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The Nrf2-Inducible Antioxidant Defense in Astrocytes can be Both Up- and Down-Regulated by Activated Microglia: Involvement of p38 MAPK

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Abstract

The effects of microglia-conditioned medium (MCM) on the inducible Nrf2 system in astrocyte-rich cultures were investigated by determination of glutathione (GSH) levels, γ glutamylcysteine ligase (γ GCL) activity, the protein levels of Nrf2, Keap1, the modulatory subunit of γ GCL (γ GCL-M) and activated MAP kinases (ERK1/2, JNK and p38). Microglia were either cultured for 24 h in serum-free culture medium to achieve microglia-conditioned medium from non-activated cells (MCM₀), used as control condition, or activated with different concentrations (0.1–1,000 ng mL⁻¹) of lipopolysaccharide (LPS) to produce MCM_{0.1–1,000}. Acute exposure (24 h) to MCM₁₀₀ increased GSH, γ GCL activity, the protein levels of γ GCL-M, Nrf2, and activated JNK and ERK1/2 in astrocyte-rich cultures. In contrast, treatment with MCM₁₀ for 24 h decreased components of the Nrf2 system in parallel with activation of p38 MAPK. Stimulation of the Nrf2 system by α BHQ was partly intact after 24 h but blocked after 72 h treatment with MCM₁₀ and MCM₁₀₀. This down-regulation after 72 h correlated with activation of p38 MAPK and lack of ERK1/2 and JNK activation. The negative effects were partly reversed by an inhibitor of p38 which restored α BHQ mediated protection against oxidative stress. In conclusion, the study showed a negative effect of MCM₁₀ on the inducible anti-oxidant defense in astrocyte-rich cultures at both 24 and 72 h that correlated with activation of p38 and was partly reversed by a p38 inhibitor. A transient protective effect of MCM₁₀₀ on astrocyte-rich cultures against H₂O₂ toxicity was observed at 24 h which coincided with activation of JNK and ERK1/2.

Keywords

neuroinflammation; Nrf2; microglia; astrocytes; α BHQ

INTRODUCTION

The pathological processes in diseases such as stroke, Alzheimer's, Parkinson's diseases, and amyotrophic lateral sclerosis (ALS) are not fully characterized but both oxidative stress and inflammation are considered to be involved (for review see Vargas and Johnson, 2009). One inducible system that can be activated in response to oxidative stress is the transcription factor Nrf2 (Nuclear factor-erythroid 2-related factor 2) (Kobayashi et al., 2006). Under normal conditions, Nrf2 binds to the cytoskeleton associated protein Keap1 (Kelch-like ECH-associated protein 1) (Dhakshinamoorthy and Jaiswal, 2001; Itoh et al., 1999) which targets Nrf2 for proteasomal degradation (Cullinan et al., 2004; Sun et al., 2007). Oxidant and/or electrophilic stress dissociate Nrf2 from Keap1 (Kobayashi et al., 2006). Nrf2 can then translocate into the nucleus and interact with specific DNA-sequences, called antioxidant responsive element (ARE, also named electrophile responsive element (EpRE), (Kobayashi and Yamamoto, 2005). ARE-sites are located in promoter regions of several genes encoding antioxidant proteins such as NAD(P)H:quinone oxidoreductase (Bloom and Jaiswal, 2003; Nioi and Hayes, 2004), glutathione-S-transferase (Ho et al., 2005), hemeoxygenase-1 (Jaiswal, 2004), and the catalytic and modulatory subunits of γ -glutamyl cysteine ligase (γ GCL-C and γ GCL-M, respectively), (Solis et al., 2002).

Activation of the Nrf2 system is protective in animal models of various neurodegenerative diseases (Johnson et al., 2008). Some studies on human tissue indicate that dysfunctional induction of the Nrf2 system may be an important factor in ALS, Parkinson's and Alzheimer's diseases (Sarlette et al., 2008). In cerebral ischemia Nrf2 activation by tBHQ (*tert*-butylhydroquinone) limits the damage in the penumbra region but not in the core (Shih et al., 2005). There is less information about the Nrf2 system in neonatal hypoxia-ischemia. We have earlier showed that the level of GSH is decreased in rat brain after hypoxia-ischemia (Wallin et al., 2000) and that the reduction in GSH levels is more pronounced when the hypoxia-ischemia is preceded by inflammation induced 3 days earlier (Wang et al., 2007). Both the damage and the decrease in GSH were normalized by the anti-oxidant and GSH precursor *N*-acetylcysteine indicating that GSH synthesis was reduced by the inflammation (Wang et al., 2007). However, LPS injection 24 h before an insult *protected* against hypoxia-ischemia induced damage (Eklind et al., 2005). Thus it appears that inflammation may have dual, time related, effects on the anti-oxidant defense systems that are of importance for brain protection after hypoxia-ischemia in neonatal rats.

In this study we used separate purified microglial and astrocyte-rich primary cultures. This allowed us to study temporal effects of microglia-conditioned medium (MCM) on the Nrf2 system in astrocytes. To obtain the MCMs, microglia was cultured without or with different concentrations of LPS (0.1–1,000 ng mL⁻¹) in serum-free culture medium for 24 h to generate MCM₀ and MCM_{0.1–1,000}, respectively (Lund et al., 2006; Taylor et al., 2010). Astrocyte-rich cultures were treated with MCMs up to 72 h and the expression of different components of the Nrf2 system as well as GSH levels, γ GCL activity, and activated MAP kinases (JNK, ERK1/2, p38) were determined. We found that 24 h exposure to MCM₁₀ down-regulated whereas treatment with MCM₁₀₀ for 24 h up-regulated the components of the astroglial Nrf2 system. The up-regulation at 24 h correlated with elevated JNK and ERK activation whereas in the case of the down-regulation by MCM₁₀ at 24 h also p38 was activated. Treatment for 72 h with either MCM₁₀ or MCM₁₀₀ down-regulated the Nrf2 system by a mechanism that involved sustained p38 MAPK activation.

MATERIALS AND METHODS

Reagents

Escherichia coli LPS (serotype O55:B5), *α*BHQ, GSH, cysteine, glutamic acid, 5-sulfosalicylic acid (5-SSA), naphthalene-2,3-dicarboxaldehyde (NDA) and hydrogen peroxide (H₂O₂) were from Sigma (Stockholm, Sweden). SB203085, U0126, SP600125 were from Cell Signaling Technology (Beverly, USA). Anti-phospho-p38, anti-phospho-SAPK/JNK, anti-phospho-ERK1/2 were from New England Biolabs (Beverly, USA). Anti-Nrf2 and anti-Keap1 antibodies were from R&D Diagnostics (Minneapolis, USA). Anti- α -tubulin, anti- γ GCL-C and anti- γ GCL-M antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse secondary antibodies were from Vector Laboratories (Burlingame, USA). Dulbecco's modified Eagle medium, poly-D-lysine; foetal bovine serum and penicillin/streptomycin solution were from Gibco/Invitrogen (Merelbeke, Belgium). Other common reagents were purchased from standard suppliers.

Primary Microglia Cultures and Preparation of Microglia-Conditioned Medium (MCM)

Primary mixed glial cultures were prepared as described previously (Correa et al., 2008). Briefly, after decapitation, forebrains of newborn Sprague-Dawley rats were dissociated mechanically, filtered through a 150- μ m nylon mesh, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse-serum and 1% penicillin/streptomycin and plated on poly-D-lysine-coated (PLD; 15 μ g mL⁻¹) 75 cm² flasks (Falcon; Le Pont de Claix, France). After 15 days in culture the flasks were shaken at 230 rpm at 37°C for 3 h to remove loosely adherent microglia. The supernatant was plated on 12-well culture plates for 2 h (5×10^4 cells mL⁻¹). After this, medium was changed to remove non-adherent cells. Cells were grown in a humidified environment containing 5% CO₂ and held at a constant temperature of 37°C.

One hour prior to LPS exposure, medium was removed from microglial cultures and replaced by fresh serum-free DMEM. Then, stimulation with LPS (0.1–1,000 ng mL⁻¹) was performed for 24 h to achieve MCM_{0.1–1,000}. In parallel, medium without addition of LPS was conditioned for 24 h to obtain non-activated microglia-conditioned medium, MCM₀. After collection, the conditioned media were sterile filtered through a 0.2- μ m filter and frozen at –20°C. Conditioned media derived from independent culture preparations were pooled before used.

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 (Hansson et al., 1984; Nodin et al., 2005). In brief, the rats were decapitated and cortices were carefully dissected. The tissue was mechanically passed through a nylon mesh (80- μ m mesh size) into culture medium consisting of DMEM supplemented with 10% heat-inactivated horse-serum and 1% penicillin/streptomycin. The cells were grown in 12- or 24-well plates at 37°C in a humidified atmosphere of 5% CO₂. Cells were used after 7–10 days in culture when near confluency was reached.

One hour before treatments, culture medium was replaced by fresh DMEM and then exposed to the different undiluted MCM for 24 or 72 h. After that time, cultures were washed with ice-cold PBS and used for Western Blot determinations, GSH content or γ GCL activity measurements.

Western Blot Analysis

After treatments, cultures were washed with ice-cold PBS and lysed in Tris-buffered saline pH 7.6 (TBS), containing 10% glycerol, 1% Nonidet P-40, EDTA 1 mM, EGTA 1 mM plus complete protease inhibitors cocktail (Roche, Stockholm, Sweden). Cell lysates were mixed with 5× Laemmli sample buffer and boiled for 5 min. Then equal amount of protein (30 µg) were resolved on 10% SDS-PAGE in a MOPS 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; Sundbyberg, Sweden) in TBS with 0.1% Tween 20 (TBST). Then, the membranes were incubated overnight at 4°C with the corresponding primary antibodies in 5% bovine serum albumin (BSA)-TBST, extensively washed with TBST solution and incubated with the correspondent secondary antibodies 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 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 exposure to different undiluted MCM the media were changed to fresh undiluted MCM after 48 h of incubation and incubation was carried out to complete the 72 h *in vitro*. 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). In addition we also used an assay that provides inversely proportional values of cytotoxicity and viability [MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega; Nacka, Sweden)] according to manufactures instructions. Data was calculated as a ratio between live cells and dead cells and plotted as percentage of the values obtained for astroglial cells cultured in MCM₀.

GSH Determination

Intracellular GSH content was assessed using the method described by Lewicki et al. (2006) with minor modifications. Briefly, cells were washed with PBS and scraped out of the dish and sonicated before an aliquot was reserved for measurement of protein content using the Bradford reagent (Bio-Rad; Hercules, USA). Proteins in the remaining cell solution were precipitated with the addition of 3.3% perchloric acid and removed by centrifugation at 14,000g for 15 min at 4°C. Next, the pH of the supernatant was adjusted with 2M NaOH 2M and 0.4 M borate buffer (pH 9.2). Derivatization was performed with a solution of NDA (1 mg mL⁻¹ in ethanol). Incubation was carried out at 20°C in darkness for 10 min. The fluorescence intensity was immediately measured in a fluorometer Spectra Max Gemini (Molecular Devices; Sunnyvale, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. GSH levels were calculated in nmol GSH/mg protein and are presented as a percentage of control conditions.

γ -Glutamate Cysteine Ligase Activity

The activity of γ GCL was measured with a fluorescence-based microtiter plate assay as described by White et al. (2003) with minor modifications. Briefly, after sonication and centrifugation, an aliquot was reserved for protein content analysis. Another aliquot was mixed with the reaction cocktail (400 mM Tris buffer, 20 mM L-glutamic acid, 2 mM EDTA, 20 mM sodium borate, and 40 mM ATP freshly added). The reaction was initiated by adding 2 mM cysteine. After incubation, reaction was stopped with 200 mM 5-sulfosalicylic acid. The amount of γ -glutamyl cysteine (γ -GC) formed was measured by NDA fluorescence. γ GCL activity was calculated in mole γ -GC/min $\times$ mg proteins and is presented as a percentage of control conditions.

Statistical Analysis

Results are presented as means $\pm$ standard error mean (SEM) of at least three separate experiments with different cell preparations. One or two-way ANOVA followed by the Bonferroni's post-hoc test for multiple comparison were used to determine statistical significance (95%; $P < 0.05$). If the Levene's test for homogeneity of variances was significant then the Kruskal-Wallis non-parametric test was used.

RESULTS

Effect of Microglia-Conditioned Media (MCM) on the Astroglial Nrf2 System

Culturing microglia in different concentrations of LPS (0.1–1,000 ng mL⁻¹) for 24 h to generate MCM did not cause cytotoxicity (Supp. Info. Fig. 1A). When astrocyte-rich cultures were treated with the MCMs, only MCM_{1,000} induced increased cell death after 24 h. Therefore, MCM_{1,000} was not used in further experiments. Treatment with MCM₁₀₀ for 72 h caused increased cell death and decreased viability compared to treatment with MCM₀ as measured by LDH release (see Fig. 5) and the cytotoxicity and viability assay (Supp. Info. Fig. 1). We therefore did not use MCM₁₀₀ in the mechanistic studies after 72 h treatment.

The effects of acute (24 h) and chronic (72 h) exposure of astrocyte-rich cultures to MCM on the GSH content, γ GCL activity and components of the inducible Nrf2 system are shown in Fig. 1. First, the effects of MCMs obtained with different doses of LPS (0.1–100 ng mL⁻¹; MCM_{0.1}–MCM₁₀₀) on astroglial GSH content after 24 h of exposure were evaluated (Fig. 1A). Culturing in MCM₁₀ significantly reduced the content of GSH in astrocytes whereas MCM₁₀₀ increased GSH levels. Similarly, culturing in MCM₁ and MCM₁₀ reduced γ GCL activity whereas MCM₁₀₀ increased the activity (Fig. 1B). The effects of treating astrocyte-rich cultures with MCM₁₀ and MCM₁₀₀ on Nrf2, Keap1, γ GCL-C, and γ GCL-M protein expression are shown in Fig. 1C,D. We found that MCM₁₀, which reduced the GSH content and γ GCL activity, down-regulated Nrf2 and γ GCL-M protein expression whereas Keap1 was up-regulated [the reasons for the appearance of a double band with the Keap1 antibody are unknown but this has been reported by several other groups (Lo et al., 2006; Tanigawa et al., 2007; Zhang et al., 2004)]. No change was observed for the levels of the γ GCL-C subunit. Since increased levels of Keap1 and low levels of Nrf2 suggest an increased degradation of Nrf2 by the proteasome, we evaluated the effect of the proteasome inhibitor MG-132 (100 nM). When the proteasome was inhibited, the negative effects of MCM₁₀ on Nrf2 protein expression were completely prevented (Fig. 1E,F).

Exposure of astrocyte-rich cultures to MCM₁₀ and MCM₁₀₀ for 72 h decreased the intracellular content of GSH as well as γ GCL activity (Fig. 2A,B). Similarly, the Nrf2, Keap1, γ GCL-C, and γ GCL-M protein expression were down-regulated by MCM₁₀ and MCM₁₀₀ after 72 h, whereas the levels of tubulin were unchanged (Fig. 2C,D).

To rule out that the observed effects of MCMs on the astrocyte-rich cultures were due to a direct action of LPS, we added LPS to the culture medium. We found that the high levels of LPS (100–1,000 ng mL⁻¹) down-regulated the GSH content after 24 h but did not modify γ GCL activity (Supp. Info. Fig. 2A,B). Treatment of astrocytes for 72 h with LPS did not change the levels of GSH but the γ GCL activity was increased by 100 ng mL⁻¹ of LPS (Supp. Info. Fig. 2C,D).

Effects of the Nrf2 Inducer α BHQ on Astrocytes-Rich Cultures Exposed to MCM for 24 or 72 h

The phenolic compound α BHQ increase both GSH levels and γ GCL activity in astrocytes but only marginally in neurons (Eftekharpour et al., 2000; Lavoie et al., 2009). The protective effects are due to activation of the Nrf2 system (Kensler et al., 2007; Shih et al., 2005). Here we investigated whether α BHQ increased the intracellular levels of GSH and γ GCL activity in astrocyte-rich cultures exposed for 24 and 72 h to MCMs. Co-treatment with MCM and α BHQ (20 μ M) increased the GSH content in astrocytes exposed for 24 h to MCM₀, MCM₁₀ and MCM₁₀₀ (Fig. 3A). A similar stimulatory effect of α BHQ was found for γ GCL activity in astrocytes treated with MCM₀ and MCM₁₀ (Fig. 3B). In astrocytes treated with α BHQ and MCM₁₀₀ no further increase in γ GCL activity was observed compared with the elevated level induced by MCM₁₀₀ alone (Fig. 3B). The Nrf2 and γ GCL-M protein contents were elevated to a similar degree by α BHQ in astrocyte-rich cultures exposed to MCM₀ or MCM₁₀ for 24 h (Fig. 3C,D).

When α BHQ was added after 48 h to MCM₁₀ and MCM₁₀₀, no increases in the intracellular GSH content of astrocytes was observed after 72 h (Fig. 4A). Similarly no increases in γ GCL activity, Nrf2 or γ GCL-M were observed by α BHQ in astrocytes exposed to MCM₁₀ or MCM₁₀₀ for 72 h (Fig. 4B–D). Importantly, the effect of α BHQ in astrocyte-rich cultures treated with MCM₀ for 72 h was intact (Fig. 4A–D).

Oxidative Stress Response of Astrocyte-Rich Cultures Exposed to MCMs

The effects of 24 h vs. 72 h exposure to MCMs were also evaluated on the resistance to oxidative stress. Treatment with MCM₀, MCM₁₀ and MCM₁₀₀ for 24 h resulted in a similar low leakage of LDH (see Fig. 5). Likewise the viability and cytotoxicity assay showed no difference comparing MCM₀, MCM₁₀, and MCM₁₀₀ after 24 h (Supp. Info. Fig. 1). When astrocyte-rich cultures were treated for 24 h with MCMs and challenged with 250 μ M H₂O₂ for three hours only, the cells cultured with MCM₁₀ showed an elevated cytotoxicity but were protected by the treatment with MCM₁₀₀ for 24 h (see Fig. 5). This corresponds well with the decreased and increased levels of GSH and γ GCL activity in cells treated with MCM₁₀ and MCM₁₀₀ for 24 h, respectively (see Fig. 1). When the treatment time was increased to 72 h cells were more sensitive. Thus, treatment with MCM₁₀₀ elevated LDH leakage and decreased the viability/cytotoxicity value compared to treatment with MCM₀ and MCM₁₀ (Fig. 5 and Supp. Info. Fig. 1). Increased cell death by H₂O₂ was also observed for astrocyte-rich cultures exposed for 72 h to MCM₁₀ and MCM₁₀₀ compared with treatment in MCM₀ (Fig. 5B). Again this correlated well with decreased levels of GSH and γ GCL activity (see Fig. 2).

p38 MAPK Signaling is Involved in Down-Regulation of Nrf2 and γ GCL-M Protein Levels

To elucidate the signaling pathways involved in the differential effects of MCMs on Nrf2 and γ GCL-M protein expression in the astrocyte-rich cultures, we investigated the pattern of phosphorylation of ERK1/2, JNK, and p38 MAPK after exposure to MCM₀, MCM₁₀, and MCM₁₀₀. In Fig. 6A, the time course (0–120 min) of ERK1/2 phosphorylation shows a similar pattern of activation in astrocytes exposed to MCM₁₀ and MCM₁₀₀ (albeit a higher phosphorylation for MCM₁₀₀ at 15 min). MCM₀ induced a small activation of ERK1/2 at 15

min but levels returned rapidly back to basal levels (Fig. 6A). Next, we analyzed the temporal pattern of ERK1/2 activation after 24 h treatment. Figure 6B shows the phosphorylation pattern of ERK1/2 after 3, 6, 12, 18, and 24 h after exposure to MCM₀, MCM₁₀, and MCM₁₀₀ respectively. MCM₀ induced an increase in ERK1/2 phosphorylation that peaked at 3-h post-exposure and then returned to basal levels whereas MCM₁₀ and MCM₁₀₀ induced a sustained activation of this signaling pathway (Fig. 6B). We also investigated the activation pattern of ERK1/2 after 24, 48, and 72 h treatments. Culturing for 72 h in MCM₀ did not change the level of ERK1/2 phosphorylation whereas treatment with MCM₁₀ and MCM₁₀₀ induced the activation of ERK1/2 which returned to basal levels at 72 h (Fig. 6C). The MEK inhibitor, U0126 (10 μ M) was used to reduce ERK1/2 phosphorylation. This inhibitor caused further down-regulation of Nrf2 and γ GCL-M protein expression compared with 24 h treatment with MCM₁₀ alone (Fig. 6D,E). Culturing astrocytes in MCM₀ with the MEK inhibitor for 24 h did not change the level of Nrf2 but decreased that of γ GCL-M (Fig. 6D,E). Determination of viability and cytotoxicity after treatment with MCM₀ and U0126 for 24 h did not reveal any adverse effects of this blocker (data not shown).

As shown in Fig. 7A, the time course analysis (0–120 min) showed that treatment with MCM₁₀₀ strongly stimulated JNK phosphorylation with a maximum level at 30 min. Treatment of astrocytes with MCM₁₀ resulted in a similar temporal but lower activation pattern compared with MCM₁₀₀. Culturing astrocyte-rich cultures in MCM₀ induced an increase in JNK phosphorylation at 15 min post-stimulation but levels returned rapidly to basal levels (Fig. 7A). The phosphorylation pattern of JNK after 3, 6, 12, 18, and 24 h after exposure to MCM₀, MCM₁₀, and MCM₁₀₀ revealed that MCM₁₀ and MCM₁₀₀ induced a sustained activation of JNK up to 24 h (Fig. 7B). In contrast MCM₀ induced a peak of JNK phosphorylation at 3 h which thereafter returned to basal levels (Fig. 7B). Treatment for longer time with MCM₁₀ and MCM₁₀₀ showed that peak levels were reached at 24 h and then returned to basal levels at 72 h (Fig. 7C). Adding the JNK inhibitor, SP600125 (10 μ M), further potentiated the negative effects of MCM₁₀ on the protein levels of Nrf2 and γ GCL-M (Fig. 7D,E). The inhibitor did not change the expression of Nrf2 and γ GCL-M in astrocyte-rich cultures treated with MCM₀ for 24 h. These observations suggest that activation of ERK1/2 and JNK, in the absence of sustained p38 MAPK activation (see below), protect the cells by limiting the down-regulation of Nrf2 and γ GCL-M.

Phosphorylated p38 MAPK in astrocyte-rich cultures treated with MCM₁₀ for 0–120 min peaked at 15 min and then returned to basal levels after 45 min. Treatment with MCM₀ increased p38 MAPK phosphorylation with a maximum at 45–60 min which remained high at 120 min. The effect of MCM₁₀₀ was an increased phosphorylation of p38 MAPK at 15 min which remained at the same level after 60 min (Fig. 8A). In Figure 8B the phosphorylation patterns of p38 MAPK after 3, 6, 12, 18, and 24 h of exposure to MCM₀, MCM₁₀, and MCM₁₀₀ respectively are shown. Levels remained unchanged with MCM₀ whereas the phosphorylation induced by MCM₁₀ continued to increase and remained high at 24-h post-exposure (Fig. 8B). MCM₁₀₀ induced a transient activation of p38 MAPK and its phosphorylation levels returned to basal after 12 h (Fig. 8B).

We also investigated the activation pattern of p38 MAPK after 24, 48, and 72 h exposure to MCMs. Culturing primary astrocyte-rich cultures in MCM₀ for 72 h did not modify p38 MAPK phosphorylation. In the case of MCM₁₀ and MCM₁₀₀ both treatments induced activation of p38 MAPK, reaching similar high levels at 72-h post-exposure (Fig. 8C). However, one notable difference between treatment with MCM₁₀ and MCM₁₀₀ was that MCM₁₀, but not MCM₁₀₀, increased p38 phosphorylation at 24 h. These differential effects on p38 MAPK activation, which correlated well with the negative effects of MCM₁₀ and positive effect of MCM₁₀₀ on the Nrf2 system at 24 h of treatment, lead us to further

investigate the role of this signaling pathway by using the p38 MAPK inhibitor, SB203580 (20 μ M). In primary astrocyte-rich cultures exposed to MCM₁₀ for 24 h (Fig. 8D,E) or 72 h (Fig. 8F,G) inhibition of p38 partly or fully restored the negative effects of MCM₁₀ on the expression of Nrf2 and γ GCL-M (Fig. 8D,F). The viability and cytotoxicity was not different comparing treatment of the astrocytes for 72 h in MCM₀ with or without SB203580 (data not shown).

Inhibition of p38 MAPK Activation Induced by MCM₁₀ Partly Restores the Inducibility of the Nrf2 System

Since p38 MAPK activation appeared to down-regulate Nrf2 and γ GCL-M, we investigated whether inhibition of p38 also could restore the inducibility of the Nrf2 system. Again, SB203580 was used to inhibit p38 activation for 48 h, after which α BHQ (20 μ M) was added to the cell culture medium and Nrf2 and γ GCL-M protein expression was analyzed 24 h later. As shown in Fig. 9A, the inhibition of p38 partly restored the inducibility of the Nrf2 system after 72 h of exposure to MCM₁₀, confirming a role of this signaling pathway as a negative modulator of Nrf2 system in astrocyte-rich cultures exposed to MCM for 72 h (Fig. 9B). In accordance, inhibition of p38 and treatment with α BHQ partly protected astrocytes treated with MCM₀ and MCM₁₀ from cell death induced by H₂O₂ oxidative stress (Fig. 9C).

DISCUSSION

Our results show that activated microglia can stimulate or reduce the GSH-related anti-oxidant defense in cultured astrocytes. The effects on the astroglia were dependent on the time of treatment and on the concentration of LPS used to activate the microglia. Thus, treatment of primary astrocyte-rich cultures with MCM₁₀ decreased the antioxidant defense while MCM₁₀₀ increased GSH level and elevated γ GCL activity after 24 h. Importantly, the stimulatory effects of MCM₁₀₀ cannot be explained by a direct effect of LPS on astrocytes as the level of GSH was decreased and the γ GCL activity unchanged after 24 h treatment with 100 ng mL⁻¹ of LPS in MCM₀. Likewise the negative effects of MCM₁₀ and MCM₁₀₀ after 72 h were not due to a direct effect of LPS as 1-100 ng mL⁻¹ had no negative effects on GSH levels and γ GCL activity in primary astrocyte-rich cultures after 72 h. It is also important to note that the viability of microglia was unaltered when cultured with LPS (0.1–100 ng mL⁻¹) for 24 h. The viability of the astrocyte-rich cultures incubated in MCM₀₋₁₀₀ for 24 h were similar but increasing the culture time to 72 h revealed that cultures treated with MCM₁₀₀ were less viable compared to treatment with MCM₀ and MCM₁₀ for 72 h. Therefore, in studies on mechanisms after treatment for 72 h we focused on treatment with MCM₁₀.

Earlier studies have shown a preconditioning protective effect of LPS after 24 h on hypoxia-ischemia induced neurodegeneration (Eklind et al., 2005). Our data indicate that activation of the Nrf2 system by activated microglia could be involved as the expression of Nrf2, the modulatory subunit of γ GCL, the levels of GSH, and the activity of γ GCL were upregulated in cultures after treatment with MCM₁₀₀ for 24 h. Despite this, oxidative stress (H₂O₂) induced similar cytotoxicity in astrocytes treated with MCM₀ and MCM₁₀₀ for 24 h. This is most likely due to that toxicity of H₂O₂ partly is exerted via effects on the extracellular surface of the cells where an increase in cellular γ GCL and GSH have little effect (even if GSH is exported it will be highly diluted in the culture medium).

In our experiments treatment of astrocyte-rich cultures with MCM₁₀ for 72 h reduced all measured parameters of the GSH anti-oxidant system without any effects on the viability compared to treatment with MCM₀. This is in contrast to the studies of Röhl et al. (2008) who showed that astrocytes treated for 5 days with medium from microglia activated with 20 ng LPS/ml increased defense enzymes such as SOD2, peroxiredoxins, glutathione-S-

transferase among others. The reason for the discrepancy is not fully clear but Röhl et al. used a serum containing astroglia-conditioned medium to generate MCM, whereas in our case, we used serum-free culture media. Presumably the effect of LPS on microglia may be different in these media which could explain the partly diverging results. Another possibility is that the serum may bind cytokines and LPS which could result in a different bioavailability and free concentration of cytokines in the MCM. It is very likely that our experimental set-up represents a severe inflammatory challenge with likely higher levels of cytokines compared with the study by Röhl et al. The paradoxical decrease in Nrf2 and γ GCL-M in a situation where Nrf2 is anticipated to be activated to counteract the effects of activated microglia fit well with the finding *in vivo* that prolonged inflammation in the kidney causes a dramatic drop in Nrf2 and Nrf2-mediated transcription (Kim and Vaziri, 2010).

In our earlier study *in vivo* (Wang et al., 2007) we demonstrated that elevated brain damage after hypoxia-ischemia 3 days following systemic LPS injection correlated with a GSH deficit. Here we show that the GSH anti-oxidant system (Nrf2, GSH, and γ GCL activity) in astrocytes, which supports neuronal defense systems (Dringen et al., 1999; Shih et al., 2005), is decreased by activated microglia after 3 days. Thus, our hypothesis is that the potentiated toxicity *in vivo* 3 days following LPS injection could be caused by activated microglia and a non-supportive astroglial population. In this respect it is interesting to note that prenatal injections of LPS lead to long lasting decreased GSH and γ GCL protein levels (Zhu et al., 2007).

Previous studies have reported that MAPKs may play a role in the induction and regulation of the Nrf2 system in the brain (reviewed by Clark and Simon, 2009). Several MAPKs (ERK1/2, JNK, and p38 MAPK) have been implicated in modulating Nrf2 with partly contradictory findings (reviewed by Owuor and Kong, 2002). Overall the studies indicate that MAPK are involved in the regulation of the Nrf2 system in a way that is both tissue and cell specific (Sykietis and Bohmann, 2010). To address possible molecular mechanisms involved in MCM effects on the astroglial system, we analyzed the pattern of expression of activated (phosphorylated) ERK1/2, JNK, and p38 MAPK. Inhibition of ERK1/2 and JNK signaling pathways further down-regulated Nrf2 and γ GCL-M expression which suggest that these MAPKs are positively involved in the regulation of Nrf2 system. Interestingly, we found that in the cases where the Nrf2 system was down-regulated, as in the 24 h exposure to MCM₁₀ or 72 h exposure to MCM₁₀ and MCM₁₀₀, p38 MAPK was phosphorylated. Furthermore, a blocker of p38 activation partly restored the levels of Nrf2 and γ GCL-M, reduced oxidative stress-induced toxicity, and partly restored the inducibility of the Nrf2 system. The mechanism by which activated p38 MAPK cause this down-regulation can, at least partly, be related to a decreased level of Nrf2 in the nucleus. This has been shown in monocytic cells by genetic deletion and pharmacological inhibition of p38 MAPK (Naidu et al., 2009). Since ARE-sequences are present in the Nrf2, Keap1 and γ GCL-M genes, a decreased nuclear Nrf2 would lead to lower transcription and protein levels of these genes in an auto-regulatory loop (Lee et al., 2007). Similar effects of prolonged inflammation with decreased levels of Nrf2 have been shown after treatment of a human monocyte/macrophage cell line with cigarette smoke condensate (Goven et al., 2009) and in chronic renal failure (Kim and Vaziri, 2010). In contrast to our results which showed decreased levels of Keap1 after 72 h, no decrease but instead an increase in Keap1 was observed in these studies also after longer treatments (Goven et al., 2009; Kim and Vaziri, 2010). These apparent contradictory results may be related to the degree of decreased Nrf2. After 72 h of treatment the levels of Nrf2 was hardly detectable in our case and Keap1 was similarly severely depressed but when Nrf2 was moderately decreased Keap1 was increased. This agrees with the findings that Nrf2 protein depletion using siRNA decrease Keap1 (Lee et al., 2007).

From a functional point of view, depletion of Keap1 could be one way to try to secure an inducible oxidative stress response when Nrf2 is low.

Few studies so far have addressed Nrf2 function in human neurodegenerative diseases. However, a reduced number of astrocytes positive for Nrf2 in hippocampus of patients suffering from Alzheimer's disease have been demonstrated (Ramsey et al., 2007). Our results suggest that activated microglia in Alzheimer's disease could cause this change. If neuroinflammation *in vivo* indeed down-regulate Nrf2 the brain will be more vulnerable in analogy with brains of animals where Nrf2 is genetically "knocked-out" (Innamorato et al., 2008). Our finding that an inhibitor of p38 MAPK phosphorylation partly restored the decreased Nrf2 levels and the stimulatory effects of α BHQ indicate that blockage of p38 MAPK activation may be neuroprotective in degenerative diseases. Indeed, p38 MAPK is activated in ALS, Alzheimer's, Parkinson's diseases, and hypoxia-ischemia induced brain damage (Bendotti et al., 2005; Hee et al., 2002; Naidu et al., 2009). The factor(s) behind the phosphorylation of p38 MAPK is unknown but could be mediated by microglial proinflammatory cytokines as TNF α activate this signalling pathway in endothelial cells (Chen et al., 2009). Thus sustained p38 MAPK activation can down-regulate and even block the biological Nrf2-response to a challenge, an effect that can be of pathophysiological importance in the above mentioned diseases.

In conclusion, our results show that activated microglial-derived factors can up- or down-regulate the expression of components in the inducible antioxidant defense system in primary astrocyte-rich cultures in a dose- and time-dependent fashion. The up-regulation after 24-h exposure to MCM₁₀₀ correlated with elevated JNK and ERK1/2 activation whereas in the case of the down-regulation by MCM₁₀ after 24 h also p38 MAPK was activated. Treatment for 72 h with either MCM₁₀ or MCM₁₀₀ down-regulated the Nrf2 system by a mechanism that involved sustained p38 MAPK activation. Studies are in progress to determine the microglial mediators that modulate the neuroprotective Nrf2-inducible antioxidant system in astrocyte-rich cultures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Additional Supporting Information may be found in the online version of this article.

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Abbreviations

ALS	amyotrophic lateral sclerosis
ERK1/2	extracellular regulated kinase
γGCL	gamma-glutamylcysteine ligase
γGCL-C	gamma-glutamylcysteine ligase catalytic subunit
γGCL-M	gamma-glutamylcysteine ligase modulatory subunit
GSH	glutathione

JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
MCM	microglia-conditioned medium
MEK	mitogen-activated protein kinase
Nrf2	factor-erythroid 2-related factor 2
tBHQ	tert-butylhydroquinone

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Fig. 1.

(A) Astrocyte-rich cultures were exposed for 24 h to microglial-conditioned medium (MCM) obtained by stimulating microglial cells with different LPS concentrations (0.1, 1, 10, and 100 ng mL⁻¹) for 24 h. Treatment of astrocytes with MCM₁₀ for 24 h decreased GSH whereas MCM₁₀₀ increased GSH levels. The results shown are the mean ± SEM of three independent experiments performed by triplicate and expressed as percentage of MCM₀. Statistics: **P* < 0.05 vs. MCM₀; ***P* < 0.01 vs. MCM₀. (B) Astrocyte cultures exposed for 24 h to MCM₁ and MCM₁₀ had decreased γGCL activity whereas MCM₁₀₀ increased γGCL activity. The results shown are the mean ± SEM of three independent experiments performed by triplicate and expressed as percentage of MCM₀. Statistics: **P* < 0.05 vs. MCM₀; ***P* < 0.01 vs. MCM₀. (C) MCM₁₀ induced a decrease in astroglial Nrf2 and the modulatory subunit of γGCL (γGCL-M) protein levels and increased the expression of Keap1. MCM₁₀₀ induced an increase in Nrf2 and γGCL-M protein levels and decreased the expression of Keap1. No changes were observed in the catalytic subunit of γGCL (γGCL-C) with any treatment. A representative experiment of four independent experiments is shown. (D) Densitometric analysis of Nrf2, Keap1, γGCL-C, and γGCL-M protein expression in astrocytes treated for 24 h with MCM₀, MCM₁₀, and MCM₁₀₀. Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: **P* < 0.05 vs. MCM₀; ***P* < 0.01 vs. MCM₀; #*P* < 0.05 vs. MCM₀; ##*P* < 0.01 vs. MCM₀. (E) The proteasome inhibitor MG-132 (100 nM) reversed the effects of MCM₁₀ on Nrf2 protein expression. A representative experiment of three independent experiments is shown. (F) Densitometric analysis of Nrf2 and Keap1 protein expression on astrocytes treated with MCM₁₀ in the presence or absence of the proteasome inhibitor MG-132 (100 nM). Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: ***P* < 0.01 vs. control; ****P* < 0.005 vs. control; #*P* < 0.05 vs. control; ##*P* < 0.01 vs. MCM₁₀; +*P* < 0.01 vs. MCM₁₀.

Fig. 2.

Astrocyte-rich cultures exposed for 72 h to MCM₁₀ and MCM₁₀₀ showed decreased GSH levels (**A**) and γGCL activity (**B**) compared to treatment with MCM₀. The results are the mean ± SEM of four independent experiments performed by triplicate and expressed as percentage of MCM₀. Statistics: **P* < 0.05 vs. MCM₀; ***P* < 0.01 vs. MCM₀. (**C**) Both MCM₁₀ and MCM₁₀₀ induced a decrease in the expression of Nrf2, Keap1, γGCL-M, and γGCL-C protein levels in astrocytes after 72 h of exposure. A representative experiment of four independent experiments is shown. (**D**) Densitometric analysis of Nrf2, Keap1, γGCL-C, and γGCL-M protein expression in astrocytes treated for 72 h with MCM₀, MCM₁₀, and MCM₁₀₀. Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: **P* < 0.05 vs. MCM₀; ***P* < 0.01 vs. MCM₀; ****P* < 0.005 vs. MCM₀.

Fig. 3.

(A) tBHQ (20 μM) treatment increased the GSH levels in astrocyte-rich cultures exposed for 24 h to MCM₀, MCM₁₀, or MCM₁₀₀. Mean ± SEM of four independent experiments performed in triplicate and expressed as percentage of MCM₀. Statistics: * $P < 0.05$ vs. MCM₀; ## $P < 0.01$ vs. MCM₁₀; + $P < 0.05$ vs. MCM₁₀₀. (B) tBHQ (20 μM) treatment increased the γGCL activity in astrocytes exposed for 24 h to MCM₀, MCM₁₀, and MCM₁₀₀. The results shown are the mean ± SEM of four independent experiments performed by triplicate and expressed as percentage of MCM₀. Statistics: ** $P < 0.01$ vs. MCM₀; ## $P < 0.01$ vs. MCM₁₀. (C) tBHQ (20 μM) treatment increased both Nrf2 and γGCL-M protein levels in astrocytes exposed for 24 h to MCM₁₀. A representative experiment of four independent experiments is shown. (D) Densitometric analysis of Nrf2 and γGCL-M protein expression in astroglial cultures treated for 24 h with MCM₁₀ in the presence or absence of tBHQ (20 μM). Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: * $P < 0.05$ vs. MCM₀; ** $P < 0.01$ vs. MCM₀; ## $P < 0.01$ vs. MCM₁₀; ### $P < 0.005$ vs. MCM₁₀; ++ $P < 0.01$ vs. MCM₀; +++ $P < 0.005$ vs. MCM₀.

Fig. 4. tBHQ (20 μM) treatment did not increase GSH levels (**A**) or γGCL activity (**B**) in astrocyte-rich cultures exposed for 72 h to MCM₁₀ and MCM₁₀₀. tBHQ was added after 48 h of exposure to MCMs and GSH levels and γGCL activity were measured 24 h later. The results shown are the mean ± SEM of four independent experiments performed by triplicate and expressed as percentage of MCM₀. Statistics: * $P < 0.05$ vs. MCM₀; ** $P < 0.01$ vs. MCM₀; ## $P < 0.01$ vs. MCM₀; ### $P < 0.005$ vs. MCM₀. (**C**) tBHQ (20 μM) treatment increased both Nrf2 and γGCL-M protein levels in cultures treated with MCM₀ for 72 h but not in astrocytes exposed for 72 h to MCM₁₀. A representative experiment of four independent experiments is shown. (**D**) Densitometric analysis of Nrf2 and γGCL-M protein expression on astrocyte-rich cultures treated for 72 h with MCM₀ and MCM₁₀ in the presence or absence of tBHQ (20 μM). Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: ** $P < 0.01$ vs. MCM₀; # $P < 0.05$ vs. MCM₀; ## $P < 0.01$ vs. MCM₀.

Fig. 5.

(A) Cell death in response to MCM exposure for 24 and 72 h was analyzed by LDH released into the medium. The results shown are the mean $\pm$ SEM of four independent experiments performed by triplicate. Statistics: * $P < 0.05$ vs. MCM₀. (B) Exposure to hydrogen peroxide (250 μ M) for 3 h induced increased cell death in cultures exposed for 24 h to MCM₁₀ and in astrocytes exposed for 72 h to MCM₁₀ and MCM₁₀₀ (all comparisons to treatment with MCM₀). The results shown are the mean $\pm$ SEM of four independent experiments performed by triplicate. Statistics: * $P < 0.05$ vs. MCM₀, ** $P < 0.01$ vs. MCM₀; # $P < 0.01$ vs. MCM₁₀.

Fig. 6.

(A) Patterns of time dependent phosphorylation of ERK1/2 in astrocyte-rich cultures exposed to MCMs for 0–120 min. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: $**P < 0.01$ vs. MCM₀; $\#P < 0.05$ vs. MCM₁₀. (B) Patterns of time dependent phosphorylation of ERK1/2 in astrocyte cultures exposed to MCMs for 0–24 h. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: $**P < 0.01$ vs. MCM₀; $***P < 0.005$ vs. MCM₀. (C) Patterns of time dependent phosphorylation of ERK1/2 in astrocytes exposed to MCMs for 0 to 72 h. The results shown are the mean $\pm$ SEM of four independent experiments performed by triplicate. Statistics: $***P < 0.005$ vs. MCM₀; $+++P < 0.005$ vs. MCM₁₀₀. (D) The inhibition of ERK1/2 phosphorylation with the MEK inhibitor U0126 (10 μ M) resulted in a further down-regulation of the Nrf2 and γ GCL-M protein expression in astroglial cultures exposed for 24 h to MCM₁₀. (E) Densitometric analysis of Nrf2 and γ GCL-M protein expression on astrocyte cultures treated for 24 h with MCM₁₀ in the presence or absence of U0126 (10 μ M). Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: $*P < 0.05$ vs. MCM₀; $**P < 0.01$ vs. MCM₀; $\#P < 0.05$ vs. MCM₁₀; $+P < 0.05$ vs. MCM₀.

Fig. 7.

(A) Patterns of time dependent phosphorylation of JNK in astrocyte-rich cultures exposed to MCMs for 0–120 min. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: * P < 0.05 vs. MCM₀; ** P < 0.01 vs. MCM₀; **** P < 0.001 vs. MCM₀; # P < 0.05 vs. MCM₁₀; ## P < 0.01 vs. MCM₁₀. (B) Patterns of time dependent phosphorylation of JNK in astrocyte-rich cultures exposed to MCMs for 0–24 h. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: §§ P < 0.01 vs. MCM₁₀ and MCM₁₀₀; * P < 0.05 vs. MCM₀; ** P < 0.01 vs. MCM₀. (C) Patterns of time-dependent phosphorylation of JNK in astroglia-rich cultures exposed to MCMs for 0–72 h. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: * P < 0.05 vs. MCM₀; ** P < 0.01 vs. MCM₀; + P < 0.05 vs. MCM₁₀. (D) The inhibition of JNK activation with SP600125 (10 μ M) resulted in a lower protein expression of Nrf2 and γGCL-M in astrocyte-rich cultures exposed for 24 h to MCM₁₀. (E) Densitometric analysis of Nrf2 and γGCL-M protein expression of astrocyte-rich cultures treated for 24 h with MCM₁₀ in the presence or absence of SP600125 (10 μ M). Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: ** P < 0.01 vs. MCM₀; # P < 0.05 vs. MCM₁₀; ## P < 0.01 vs. MCM₁₀.

Fig. 8.

(A) Patterns of time dependent phosphorylation of p38 MAPK in astrocyte-rich cultures exposed to MCMs for 0–120 min. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: * P < 0.05 vs. MCM₀; *** P < 0.005 vs. MCM₀; + P < 0.05 vs. MCM₁₀₀; ++ P < 0.01 vs. MCM₁₀₀. (B) Patterns of time-dependent phosphorylation of p38 MAPK in astrocytes exposed to MCMs for 0–24 h. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: ** P < 0.01 vs. MCM₀; # P < 0.05 vs. MCM₁₀; ## P < 0.01 vs. MCM₁₀; ++ P < 0.01 vs. MCM₁₀₀. (C) Patterns of time-dependent phosphorylation of p38 in cultures exposed to MCMs for 0–72 h. The results shown are the mean $\pm$ SEM of four independent experiments. Statistics: *** P < 0.005 vs. MCM₀; +++ P < 0.005 vs. MCM₁₀₀. (D) Inhibition of p38 MAPK activation with the specific inhibitor SB203580 (20 μM) in astrocytes exposed for 24 h to MCM₁₀ resulted in a partial restoration of Nrf2 and γGCL-M protein levels. A representative experiment of four independent experiments is shown. (E) Densitometric analysis of Nrf2 and γGCL-M protein expression on astrocytes treated for 24 h with MCM₁₀ in the presence or absence of SB203580 (20 μM). Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: ** P < 0.01 vs. MCM₀; # P < 0.05 vs. MCM₁₀; ## P < 0.01 vs. MCM₁₀.

Fig. 9.

(A) Inhibition of p38 MAPK activation with SB203580 (20 μ M) restored and potentiated the induction of the Nrf2 system in astrocyte-rich cultures exposed for 72 h to MCM₁₀. A representative experiment of four independent experiments is shown. (B) Densitometric analysis of Nrf2 and γ GCL-M protein expression in astrocytes treated for 72 h with MCM₀ or MCM₁₀ in the presence or absence of SB203580 (20 μ M) and tBHQ (20 μ M). Data are plotted as ratio of the protein of interest/tubulin obtained in each condition. Statistics: * P < 0.05 vs. MCM₀; ** P < 0.01 vs. MCM₀; # P < 0.05 vs. MCM₁₀; ΔP < 0.05 vs. MCM₁₀+SB203580; $\Delta\Delta P$ < 0.01 vs. MCM₁₀+SB203580. (C) Inhibition of p38 with SB203580 (20 μ M) decreased oxidative stress (250 μ M H₂O₂ for 3h) induced cell death. Cell death was further reduced when cells were previously treated with tBHQ (20 μ M). The results shown are the mean $\pm$ SEM of four independent experiments performed by triplicate. Statistics: *** P < 0.005 vs. untreated MCM₀; ## P < 0.01 vs. H₂O₂-treated MCM₀; ### P < 0.005 vs. H₂O₂-treated MCM₀; ++++ P < 0.001 vs. MCM₁₀; § P < 0.05 vs. H₂O₂-treated MCM₁₀; §§ P < 0.01 vs. H₂O₂-treated MCM₁₀; ΔP < 0.05 vs. untreated MCM₁₀.