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NFκB in the Mechanism of Ammonia-Induced Astrocyte Swelling in Culture

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Abstract

Astrocyte swelling and brain edema are major neuropathological findings in the acute form of hepatic encephalopathy (fulminant hepatic failure, FHF), and substantial evidence supports the view that elevated brain ammonia level is an important etiological factor in this condition. Although the mechanism by which ammonia brings about astrocyte swelling remains to be determined, oxidative/nitrosative stress and mitogen-activated protein kinases (MAPKs) have been considered as important elements in this process. One factor known to be activated by both oxidative stress and MAPKs is nuclear factor κB (NFκB), a transcription factor that activates many genes, including inducible nitric oxide synthase (iNOS). Since the product of iNOS, nitric oxide (NO), is known to cause astrocyte swelling, we examined the potential involvement of NFκB in ammonia-induced astrocyte swelling. Western blot analysis of cultured astrocytes showed a significant increase in NFκB nuclear translocation (a measure of NFκB activation) from 12 h to 2 days after treatment with NH₄Cl (5 mM). Cultures treated with antioxidants, including superoxide dismutase, catalase and vitamin E, as well as the MAPKs inhibitors SB239063 (an inhibitor of p38-MAPK), and SP600125 (an inhibitor of c-Jun N-terminal kinase, JNK) significantly diminished NFκB activation by ammonia, supporting a role of oxidative stress and MAPKs in NFκB activation. The activation of NFκB was associated with increased iNOS protein expression and NO generation, and these changes were blocked by BAY 11-7082, an inhibitor of NFκB. Additionally, ammonia-induced astrocyte swelling was inhibited by the NFκB inhibitors BAY 11-7082 and SN-50, thereby implicating NFκB in the mechanism of astrocyte swelling. Our studies indicate that cultured astrocytes exposed to ammonia display NFκB activation, which is likely a consequence of oxidative stress and activation of MAPKs. NFκB activation appears to contribute to the mechanism of ammonia-induced astrocyte swelling, apparently through its upregulation of iNOS protein expression and the subsequent generation of nitric oxide.

Keywords

ammonia; astrocyte swelling; hepatic encephalopathy; iNOS; MAPK; NFκB; oxidative stress

INTRODUCTION

Hepatic encephalopathy (HE) is a complex neuropsychiatric syndrome resulting from severe liver failure (Jones and Weissenborn, 1997). Brain edema, increased intracranial pressure and brain herniation are the principal neurological complications in patients with the acute form of HE (fulminant hepatic failure, FHF) that occurs following viral hepatitis, acetaminophen toxicity and other acute liver disorders (Ware *et al.*, 1971; Jalan, 2005; Blei, 2005). It presents with the abrupt onset of delirium, seizures, and coma and it has an extremely poor prognosis with an 80–90% mortality rate (Capocaccia and Angelico, 1991). The only effective treatment for this condition is an emergency liver transplantation (Bismuth *et al.*, 1996).

Astroglial swelling is generally believed to represent a major component of the brain edema in FHF (Norenberg 1977; Traber *et al.* 1987; Swain *et al.* 1991). Ammonia has been strongly implicated as a major toxin in liver failure (for review, see Albrecht and Jones, 1999; Hazell and Butterworth, 1999), and ammonia has been shown to induce astrocyte swelling in *in vivo* models of hyperammonemia (Voorhies *et al.*, 1983; Willard-Mack *et al.*, 1996), brain slices (Ganz *et al.*, 1989) and astrocyte cultures (Norenberg *et al.*, 1991; Zwingman *et al.*, 2000). While the precise mechanism by which ammonia brings about such swelling remains poorly understood, recent studies have invoked oxidative/nitrosative stress and mitogen-activated protein kinases (MAPKs) in the mechanism of ammonia-induced astrocyte swelling (for review, see Norenberg *et al.*, 2007).

Oxidative stress (OS) and the activation of MAPKs are known to stimulate transcription factors, including nuclear factor κ B (NF κ B) (for reviews, see Bowie and O'Neill, 2000; Kyriakis and Avruch, 2001; Bubici *et al.*, 2006). NF κ B is known to activate many genes, including inducible nitric oxide synthase (iNOS), resulting in excessive nitric oxide (NO) generation (for reviews, see Xie *et al.*, 1994; Kleinert *et al.*, 2004). Previous studies have shown that the NO donors, SNAP and SIN-1, caused astrocyte swelling and that treatment of cultures with the non-specific nitric oxide synthase (NOS) inhibitor, L-NAME, significantly diminished ammonia-induced astrocyte swelling (Jayakumar *et al.*, 2006). Collectively, these studies suggest the possibility that NF κ B may represent an integral component in the mechanism of astrocyte swelling in FHF.

In this study we demonstrate that astrocytes exposed to ammonia caused activation of NF κ B, and the inhibition of such activation attenuated ammonia-induced astrocyte swelling. We also show that NF κ B activation is mediated by oxidative stress and MAPKs activation, and that the activation of NF κ B results in the upregulation of iNOS protein expression and the subsequent generation of nitric oxide (NO); the latter likely representing the proximate cause of ammonia-induced astrocyte swelling.

MATERIALS AND METHODS

Astrocyte cultures

Primary cultures of cortical astrocytes were prepared as described previously (Ducis *et al.*, 1990). Briefly, brains of 1–2 day old rat pups were seeded onto 35 mm culture dishes in DMEM containing penicillin, streptomycin, and 15% fetal bovine serum (FBS), and the culture plates were incubated at 37°C with 5% CO₂ and 95% air. Culture media was changed twice weekly. On day 10 post seeding, FBS was replaced with 10% horse serum. After 14 days, the cultures were treated with 0.5 mM dibutyryl cyclic AMP (Sigma, St. Louis, MO) to enhance cellular differentiation (Juurlink and Hertz, 1985). Cultures consisted of 95–99% astrocytes as determined by glial fibrillary acidic protein (GFAP) immunohistochemistry. All cultures used in the experiments were 25–28 days old.

Procedures followed guidelines established by the National Institute of Health *Guide for the Care and Use of Laboratory Animals*, and were approved by the local animal care committee (IACUC).

Western blots

Samples of astrocytes were analyzed for iNOS and NF κ B protein content by Western blots as described previously (Jayakumar *et al.*, 2006). In brief, equal amounts of protein were subjected to gel electrophoresis and immunoblotting. Antibodies against NF κ B-p65 (H-286) (cat# sc-7151) and iNOS (1:750 and 1:1000 dilution, respectively; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) were used to detect NF κ B and iNOS protein expression. Anti- α -tubulin antibody was obtained from Oncogene (San Diego, CA) and lamin antibody was purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA) were used at 1:1000 dilutions. Membranes were visualized using an enhanced chemiluminescence reagent. The optical density of the bands was measured with the Chemi-Imager digital imaging system (Alpha Innotech Corp, San Leandro, CA), and the results were quantified with the Multi-Analyst software (Sigma Scan Pro 5, Deerfield, IL) as a proportion of the signal of house keeping protein bands (lamin and α -tubulin, nuclear and cytosolic markers, respectively).

Preparation of nuclear extract and Western blots for NF κ B

The nuclear extract was prepared as described by Calabrese *et al.* (2005). In brief, astrocytes were washed twice with phosphate-buffered saline (PBS), then harvested in 1 ml PBS and centrifuged at 3,000 rpm for 3 min at 4°C. The cell pellet was resuspended in 200 μ l cold buffer A, consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 μ M dithiothreitol (DTT), and a complete protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet was then incubated on ice for 15 min to allow cells to swell, after which 15 μ l of 10% NP-40 was added, and the sample was vortexed thoroughly for 40 sec. An aliquot of homogenate was used for protein measurement. Equal amount of protein was then centrifuged at 3,000 rpm for 3 min at 4°C. The resulting nuclear pellet was resuspended in 30 μ l cold buffer B consisting of 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 μ M DTT, and protease inhibitors. The pellet was then incubated on ice and vortexed for 15 sec every 2 min for up to 15 min. The nuclear extract was then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant (containing the nuclear extract) was loaded on an SDS-polyacrylamide gel, and Western blot analysis with NF κ B antibody was performed as described above. The quality of the nuclear extract was analyzed by propidium iodide staining, which indicated a purity of 92–96%.

Immunofluorescence of NF κ B

Immunocytochemistry was performed on cultured astrocytes to directly visualize the translocation of the p65 subunit of NF κ B after ammonia treatment. Cultures were fixed with 4% paraformaldehyde for 30 min at RT. To prevent non-specific binding, cells were initially treated with PBST (PBS + 0.1% Triton X-100) containing 10% normal goat serum for 30 min, and then incubated overnight with a rabbit polyclonal antibody against p65 (H-286) (cat# sc-7151). Following washing with PBST, astrocytes were incubated with a goat anti-rabbit fluorescent antibody (Alexa Fluor 488, Molecular Probes, Eugene, OR, USA) for 25 min. Cultures were also stained with propidium iodide (PI; 2 μ g/ml) to determine the total number of nuclei and the results were visualized with a Nikon Diaphot inverted fluorescent microscope equipped with multivariant fluorescent filters.

Both cytoplasmic and nuclear NF κ B staining was observed in ammonia-treated as well as in control cultures, but intensity of nuclear staining was increased in ammonia-treated cells.

However, to more clearly ascertain differences in nuclear fluorescence between these two groups, the intensity threshold value of nuclei was set as low as possible in the control cultures so as to avoid any cytoplasmic staining. The same intensity threshold was then applied to the ammonia-treated cultures. The number of NF κ B-positive nuclei was counted from 5 random fields of the PI image (10x objective) using Sigma Scan Pro 5, and the results were expressed as a percent change over control. The data were analyzed using a chi-square test.

Cell volume measurement

Astrocyte cell volume (intracellular water space) was determined using 3-O-methyl-[3 H]-glucose (OMG, NEN Life sciences, Boston, MA) by the method of Kletzien *et al.* (1975), as modified for astrocyte cultures by Kimelberg (1987) and Bender and Norenberg (1998). This method is based on the principle that OMG is transported into the cell by the glucose carrier and equilibrates between the intra- and extracellular compartments. The amount of OMG accumulated in the cell is proportional to the intracellular water space or cell volume. Cultures were incubated with [3 H] OMG (1 mM containing 1 μ Ci of radioactive OMG) for 12 h, and at the end of incubation a small aliquot of medium was saved for specific activity determination. Cultures were quickly washed 5 times with ice-cold buffer containing 290 mM sucrose, 1 mM Tris-nitrate (pH 7.4), 0.5 mM calcium nitrate, and 0.1 mM phloretin. Cells were then lysed in 0.5 ml of 1N sodium hydroxide. Radioactivity was converted to intracellular water space and expressed as μ l/mg cell protein. Protein content was determined employing the bicinchoninic acid assay (Pierce, Rockford, IL).

Measurement of nitric oxide (NO) generation

NO production was analyzed in cultured astrocytes using DAF-2DA, a green fluorescence probe specific for intracellular NO (Kojima *et al.*, 1998; Fass *et al.*, 2004). Control and ammonia-treated culture plates were washed three times with serum-free culture medium and incubated with DAF-2DA (10 μ M) for 15 min at 37°C. Cultures were then washed two to three times with PBS, scraped into 500 μ l of 0.2% Triton X-100 and sonicated for 5 sec. A small aliquot of the cell extract was removed for protein estimation and 250 μ l of the extract was transferred to a 96-well microtiter plate (Fluorolite 1000, Dynex Technologies Inc, Chantilly, VA, USA). Fluorescence was measured at an excitation wavelength of 492 nm, and the emission fluorescence at 515 nm. The results were expressed as % fluorescence intensity.

Statistical Analysis

All experiments performed were repeated 4–7 times using cells derived from different batches of astrocyte cultures. The number of individual culture plates in each experimental group was 5 for astrocyte swelling, 2–4 for Western blots, and 2–3 for immunocytochemistry studies. The data of all experiments were subjected to analysis of variance followed by Tukey's *post hoc* comparisons. A value of $p < 0.05$ was considered significant. Error bars represent mean $\pm$ SEM.

RESULTS

Ammonia activates NF κ B in cultured astrocytes

Cultured astrocytes were exposed to a pathophysiological concentration of ammonia (NH $_4$ Cl, 5 mM) (Swain *et al.*, 1992) for different time periods (1–48 h), and changes in NF κ B nuclear translocation (activation) were determined by Western blots of the nuclear fraction. Astrocytes showed no significant change in nuclear NF κ B translocation for up to 6 h following ammonia treatment. A significant increase in nuclear NF κ B translocation was

first observed at 12 h, which remained elevated at 24 and 48 h (84.2, 137.8, and 161.0%, respectively; $p < 0.05$ vs control) (Fig. 1). This increase was blocked by inhibitors of NF κ B, BAY 11-7082 (5 μ M) and SN-50 (3 μ M) (76.3 and 66.8%, respectively, as compared to control, $p < 0.05$) (Fig. 2).

NF κ B translocation to the nucleus was also assessed by immunocytochemistry by measuring the number of NF κ B positive nuclei in control and ammonia-treated astrocyte cultures. The percent of NF κ B-positive nuclei was greater in ammonia-treated cultures when compared to controls at 24 h (4-fold increase as compared to controls; $p < 0.05$) (Fig. 3).

Antioxidants attenuate NF κ B activation by ammonia

To determine whether oxidative stress is involved in the mechanism of ammonia-induced NF κ B activation, we examined the effect of various antioxidants, including superoxide dismutase (SOD; 25 units/ml), catalase (250 units/ml), α -tocopherol (vitamin E; 100 μ M), desferrioxamine (DFX) and N-tert-butyl- α -phenylnitron (PBN; 50 μ M) on NF κ B activation by ammonia. SOD, vitamin E and catalase all significantly diminished ammonia-induced NF κ B activation (69.7, 59.8 and 55.0% respectively, $p < 0.05$), whereas DFX and PBN attenuated the ammonia-induced NF κ B activation, but this decrease was not statistically significant (Fig. 4).

MAPK inhibitors attenuate NF κ B activation by ammonia

Ammonia-induced OS was recently shown to activate MAPKs in cultured astrocytes (Jayakumar *et al.*, 2006). Since MAPKs are known to activate NF κ B, we examined whether inhibition of MAPK activation could diminish the ammonia-induced activation of NF κ B. Concentrations of inhibitors that prevented ammonia-induced MAPK phosphorylation (Jayakumar *et al.*, 2006) were chosen for the present studies. Astrocyte cultures were treated with 10 μ M U0126 (1, 4-diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio) butadiene), an inhibitor of MEK, the upstream kinase of ERK1/2; 10 μ M SB239063 (an inhibitor of p38-MAPK) and 1 μ M SP600125 (an inhibitor of JNK), with and without ammonia. Cultures treated with SB239063 and SP600125 significantly diminished ammonia-induced NF κ B activation (at 24 h by 79.5% and 46.6%, respectively) (Fig. 5). U0126 showed no significant inhibitory effect suggesting a lack of involvement of ERK1/2 in the ammonia-induced NF κ B activation (Fig. 5). Cultures treated with drug alone had no effect on NF κ B activation.

NF κ B inhibitors diminish ammonia-induced astrocyte swelling

To investigate the role of NF κ B in ammonia-induced astrocyte swelling, we examined the effect of NF κ B inhibitors on cell swelling. Astrocytes were treated with different concentrations of NF κ B inhibitors BAY 11-7082 and SN-50, as mentioned above, with or without ammonia for 24 h. Ammonia significantly increased astrocyte swelling by 47.2% ($p < 0.05$, compared to control). Treatment with BAY 11-7082 (at 5 μ M) and SN-50 (at 3.5 μ M) significantly diminished ammonia-induced astrocyte swelling by 85.2 and 78.6%, respectively ($p < 0.05$) (Fig. 6).

Inhibition of NF κ B reduces ammonia-induced iNOS protein expression and NO generation

We have recently shown that inhibition of NOS by L-NAME diminished astrocyte swelling in cultures following ammonia treatment (Jayakumar *et al.*, 2006). Since NF κ B is a major transcriptional factor responsible for iNOS gene induction, we determined whether NF κ B-mediated iNOS protein expression and subsequent increase in NOS activity and NO generation might explain the mechanism by which NF κ B activation causes astrocyte swelling. Astrocyte cultures exposed to ammonia (5 mM) for 12 and 24 h showed an

increase in iNOS protein expression (Fig. 7A), in agreement with a previous report by Schliess *et al.* (2002). Ammonia also increased NO production (Fig. 7B). These changes were significantly attenuated by the NF κ B inhibitor BAY 11-7082 (5 μ M).

Inhibition of iNOS prevent ammonia-induced astrocyte swelling

To investigate the role of iNOS in ammonia-induced astrocyte swelling, we examined the effect of the specific iNOS inhibitor, aminoguanidine, on cell swelling. Astrocytes were treated with different concentrations of aminoguanidine (0.1, 0.3, 0.5 and 1 mM) with or without ammonia for 24 h. Ammonia significantly increased astrocyte swelling by 40.2% ($p < 0.05$, compared to control). Treatment with 500 μ M aminoguanidine partially prevented ammonia-induced astrocyte swelling, while 1 mM completely blocked it (Fig. 8).

DISCUSSION

This study demonstrates that astrocyte cultures exposed to ammonia increases NF κ B nuclear translocation (a measure of NF κ B activation) as shown by Western blots and immunocytochemical detection of its translocation to the nucleus. These findings are consistent with studies by Schliess *et al.* (2002), who showed I κ B degradation following ammonia treatment in cultured astrocytes. NF κ B activation was significantly diminished by antioxidants and by inhibitors of MAPKs, indicating a role of oxidative stress and MAPKs in the ammonia-induced activation of NF κ B. Blockade of NF κ B activation reduced the extent of ammonia-induced astrocyte swelling. Such blockade was associated with a reduction in the ammonia-induced increase in iNOS protein expression and NO production. These studies suggest that oxidative stress and related MAPKs contribute to the ammonia-induced activation of NF κ B, and that such activation leads to iNOS upregulation and increased NO formation; the latter likely represents a major factor in the mechanism of ammonia-induced astrocyte swelling.

NF κ B is an important element in the biological response to stress (Bowie and O'Neill, 2000). NF κ B is kept in an inactive form in the cytoplasm where it is bound to a member of the I κ B family of inhibitory proteins (Baeuerle and Baltimore, 1988; Israel, 1995). Normally, NF κ B is activated by physiological as well as pathological stimuli. Upon cell activation, the I κ B inhibitors are modified through site-specific phosphorylation which targets them for subsequent ubiquitination and proteolytic degradation by the proteasome. Removal of the inhibitor unmasks the nuclear localization signals on subunits of NF κ B allowing it to translocate to the nucleus where it binds to target DNA elements and activates the transcription of many genes, especially those involved in immune responses, inflammation and cell proliferation (for review, see Baldwin, 1996).

Oxidative stress (OS) was previously shown to be involved in ammonia-induced astrocyte swelling (Jayakumar *et al.*, 2006). While the mechanism by which oxidative stress induces swelling remains to be elucidated, OS is known to activate NF κ B (Bowie and O'Neill, 2000), and such activation was blocked by antioxidants (Li *et al.*, 2006; Caccamo *et al.*, 2005; Suzuki, *et al.*, 1992; Schreck *et al.*, 1992; Ho *et al.*, 1999). Consistent with these results, our study shows that the ammonia-induced oxidative stress may be responsible for the activation of NF κ B, as various antioxidants were able to inhibit this activation.

One mechanism by which ammonia-induced oxidative stress may lead to NF κ B activation is through the stimulation of intracellular signaling kinases, in particular, the mitogen-activated protein kinases (MAPKs) (Kyriakis and Avruch, 2001; Bubici *et al.*, 2006). Ammonia has been shown to activate MAPKs in cultured astrocytes (Schliess *et al.*, 2002; Jayakumar *et al.*, 2006) and such activation was blocked by antioxidants (Jayakumar *et al.*, 2006). The stimulation of MAPKs was likely responsible for the NF κ B activation produced by

ammonia, since MAPK inhibitors SB239063 (p38-MAPK inhibitor) and SP600125 (JNK inhibitor) significantly diminished it.

While ammonia was shown to activate all three MAPKs in cultured astrocytes, the activation of NF κ B was effectively prevented by inhibitors of JNK and p38-MAPK, but not by an inhibitor of ERK. Such differential effects of MAPK inhibitors have been reported by us and others (Xia *et al.*, 1995; Mori *et al.*, 2002; Xie *et al.*, 2004; Jayakumar *et al.*, 2006). These findings may be a reflection of the well known specificity of the MAPK system, in that ERK1/2 is generally activated by growth factors, whereas p38-MAPK and JNK are generally activated by stress (Davis, 1994; Cobb and Goldsmith, 1995).

One possible mechanism by which NF κ B leads to astrocyte swelling may be through the upregulation of iNOS leading to the generation of NO, an agent known to cause astrocyte swelling (Jayakumar *et al.*, 2006). Increased brain NO production was shown in portacaval-shunted rats given ammonia infusions (Master *et al.*, 1999). Treatment of brain slices with the non-specific NOS inhibitor, L-NAME, prevented tissue swelling induced by ammonia (Zielinska *et al.*, 2003). L-NAME also markedly diminished ammonia-induced cell swelling in cultured astrocytes (Jayakumar *et al.*, 2006). iNOS activity has been shown to be elevated in experimental models of hepatic encephalopathy (HE) (Rao *et al.*, 1997). The present study shows elevated iNOS protein expression in cultured astrocytes after ammonia treatment, in agreement with a report by Schliess *et al.*, 2002. Additionally, we found that inhibition of NF κ B with BAY 11-7082 blocked the elevation in iNOS protein expression and NO generation, and that treatment of astrocyte cultures with the specific iNOS inhibitor, aminoguanidine, completely blocked ammonia-induced cell swelling (Figure 8). Collectively, these studies suggest that ammonia-induced NF κ B activation and subsequent induction of iNOS protein expression and NO generation represent important signaling pathways in the mechanism of ammonia-induced cell swelling in cultured astrocytes.

While, NF κ B activation is generally considered as a major transcriptional factor responsible for iNOS induction, iNOS protein expression and subsequent increase in NO generation has also been shown to activate NF κ B (Janssen-Heininger *et al.*, 1999; Simpson and Morris, 1999; von Knethen *et al.*, 1999). Such activation of NF κ B by NO may result in a positive feedback system (Nam *et al.*, 2004). A similar positive feedback system may also occur with ammonia-induced free radical production (Rama Rao *et al.*, 2003). ROS is known to activate NF κ B which can lead to increases in intracellular ROS (for review, see Bowie and O'Neill, 2000).

The mechanism by which NO leads to cell swelling is not known. Recent studies have shown increased protein tyrosine nitration in ammonia-treated astrocyte cultures (Schliess *et al.*, 2002; Häussinger and Schliess, 2005). One may therefore speculate that some membrane proteins that are critically involved in cell volume regulation may become impaired due to nitrosylation which might result in cell swelling.

The findings of the present study highlighting an important role of NF κ B in astrocyte swelling, may be relevant to an evolving role for inflammation and cytokines in FHF-associated brain edema (Detry *et al.*, 2006; O'Beirne *et al.*, 2006; Wright *et al.*, 2007). As cytokines are well known to contribute to NF κ B activation (Stancovski and Baltimore, 1997; Verma and Stevenson, 1997), it is conceivable that ammonia, by activating NF κ B, may sensitize astrocytes to the effects of cytokines and endotoxins, thereby resulting in enhanced astrocyte swelling and brain edema in the setting of FHF.

In summary, our study demonstrates that ammonia activates NF κ B in cultured astrocytes and that such activation is a consequence of an ammonia-induced activation of MAPK and oxidative stress. NF κ B activation was associated with astrocyte swelling, likely through the

induction of iNOS gene expression and subsequent NO production. These findings help define the molecular basis for cell swelling following ammonia exposure, and identifies potential targets for novel therapies in the treatment of the brain edema associated with fulminant hepatic failure.

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Figure 1. Increased nuclear NFκB was identified in cultured astrocytes treated with 5 mM NH₄Cl at 12–48 h. A. Western blots of nuclear fraction; B. Quantification of Western blots. No significant increase in nuclear NFκB was detected at 1, 3 and 6 h (data not shown). NFκB content was normalized against lamin. ANOVA, $n=5$. * $p<0.05$ vs. control.

Figure 2.

NFκB inhibitors diminish ammonia-induced translocation of NFκB to nuclei in cultured astrocytes as detected by immunoblots. **A.** Cultures exposed to 5 mM NH₄Cl by 1 day significantly increased NFκB translocation, while co-treatment with BAY 11-7082 (BAY, 5 μM) and SN50 (3.5 μM) significantly diminished such translocation. **B.** Quantification of ammonia-induced NFκB content after treatment with BAY 11-7082. NFκB content was normalized against lamin. ANOVA, *n*=5. **p*<0.05 vs. control; † *p*<0.05 vs. NH₄Cl.

Figure 3. Immunocytochemical staining for NFκB in ammonia-treated cultured astrocytes. A. Control. B. After 24 h of ammonia treatment. C. Quantification of immunocytochemical staining of ammonia-treated cultured astrocytes. Ammonia progressively increased the percentage of NFκB-positive nuclei. $n=4$. * $p<0.05$ vs. control.

Figure 4.

Antioxidants attenuate the ammonia-induced NFκB nuclear translocation. A. Cultured astrocytes exposed to 5 mM NH₄Cl significantly increased nuclear NFκB content at 24 h. Co-treatment with SOD (25 units/ml), vitamin E (100 μM) and catalase (250 units/ml) significantly diminished ammonia-induced NFκB translocation, while desferioxamine (DFX) and PBN showed minimal effect. B. Quantification of ammonia-induced NFκB translocation after treatment with antioxidants. NFκB content was normalized against lamin. ANOVA, *n*=4. **p*<0.05 vs. control; †*p*<0.05 vs. NH₄Cl.

Figure 5.

MAPK inhibitors prevent ammonia-induced NFκB translocation. A. Western blots reveal a slight, but not significant, reduction in NFκB activation at 1 day when cultures were exposed to the ERK1/2 inhibitor UO126 (10 μM). However, a significant inhibition in NFκB activation was observed when cultures were co-treated with inhibitors of p38-MAPK, SB239063 (10 μM) and JNK, SP600125 (1 μM). B. Quantification of ammonia-induced NFκB translocation after treatment with MAPK inhibitors. NFκB content was normalized against lamin. ANOVA, *n*=4. **p*<0.05 vs. control; †*p*<0.05 vs. NH₄Cl. SB, SB239063; UO, UO126; SP, SP600125.

Figure 6.

NF κ B inhibitors prevent ammonia-induced astrocyte swelling. Cultured astrocytes exposed to 5 mM NH₄Cl significantly increased cell swelling (47.19% by 24 h). Co-treatment with BAY 11-7082 (5 μ M) and SN50 (3.5 μ M) significantly diminished the extent of astrocyte swelling. ANOVA, $n=7$. * $p<0.05$ vs. control; † $p<0.05$ vs. NH₄Cl.

Figure 7.

NF κ B inhibitors prevent ammonia-induced iNOS protein expression and NO generation. A. Cultured astrocytes exposed to 5 mM NH_4Cl for 24 h increased iNOS protein expression and co-treatment with BAY 11-7082 (5 μM) significantly diminished this effect. B. Quantification of ammonia-induced iNOS protein expression. C. Cultures exposed to 5 mM NH_4Cl for 24 h increased nitric oxide generation, and such effect was significantly diminished by co-treatment with BAY 11-7082 (5 μM). iNOS expression was normalized against α -tubulin ANOVA, $n=5$. * $p<0.05$ vs. control; † $p<0.05$ vs. NH_4Cl .

Figure 8.

Inhibition of iNOS prevents ammonia-induced astrocyte swelling. Cultured astrocytes exposed to 5 mM NH₄Cl significantly increased cell swelling (40.2% by 24 h). Co-treatment with the iNOS inhibitor aminoguanidine (AGN) blocked such swelling. ANOVA, $n=3$.

* $p<0.05$ vs. control; † $p<0.05$ vs. NH₄Cl.