Ammonia-induced activation of p53 in cultured astrocytes: Role in cell swelling and glutamate uptake

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ARTICLE INFO

Article history:
Received 25 November 2008
Received in revised form 29 December 2008
Accepted 30 December 2008
Available online 11 February 2009

Keywords:
Ammonia toxicity
Astrocytes
Cell swelling
Glutamate transport
Hepatic encephalopathy
MAP kinase
Mitochondrial permeability transition
NF-κB
Oxidative stress
p53

ABSTRACT

Cytotoxic brain edema, due principally to astrocyte swelling, is a major neurological complication of the acute form of hepatic encephalopathy (HE) [acute liver failure, ALF], a condition likely caused by elevated levels of brain ammonia. Potential mediators of ammonia-induced astrocyte swelling include oxidative/nitrosative stress (ONS), the mitochondrial permeability transition (mPT), mitogen-activated protein kinases (MAPKs) and nuclear factor-kappaB (NF-κB), since blockade of these factors reduces the extent of astrocyte swelling. As p53, a tumor suppressor protein and transcription factor, is a downstream target of ONS and MAPKs, we examined its potential role in the mechanism of ammonia-induced astrocyte swelling. Astrocytes exposed to NH4Cl (5 mM) showed increased phosphorylation (activation) of p53 at 1 h and such phosphorylation was significantly reduced by inhibitors of MAPKs (ERK1/2, JNK and p38-MAPK), antioxidants (vitamin E, catalase, PBN, desferoxamine, MnTBAP), as well as by L-NAME, an inhibitor of nitric oxide synthase, indicating a key role of oxidative/nitrosative stress and MAPKs in the ammonia-induced activation of p53. Since p53 is known to induce the mPT and to activate NF-κB (factors leading to ONS and implicated in ammonia-induced astrocyte swelling), we examined whether inhibition of p53 activation blocked mPT induction, NF-κB activation, as well as cell swelling. Pifithrin-α (PFT), an inhibitor of p53, blocked these processes. Impairment of astrocytic glutamate uptake is another important feature of HE and hyperammonemia. We therefore examined the potential role of p53 in the ammonia-induced inhibition of glutamate uptake and found that PFT also reversed the ammonia-induced inhibition of glutamate uptake. Our results indicate that a potentially important downstream target of ammonia neurotoxicity is p53, whose activation contributes to astrocyte swelling and glutamate uptake inhibition, processes likely a consequence of ONS derived from the mPT and activation of NF-κB.

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1. Introduction

Hepatic encephalopathy (HE) is a major neurological complication in patients with severe liver disease (Lockwood, 1992; Jones and Weissenborn, 1997). HE can present acutely [acute liver failure, ALF] as a result of massive liver necrosis after viral hepatitis, acetaminophen toxicity, or exposure to other hepatotoxins. One major consequence of acute HE is cytotoxic brain edema which results in increased intracranial pressure and brain herniation. The mortality of individuals with brain edema in the setting of acute HE approaches 80–90% (Capocaccia and Angelico, 1991).

Astrocyte swelling is generally believed to be a key component of the cytotoxic brain edema associated with ALF (Norenberg, 2001; Blei, 2005). While pathogenetic factors responsible for the brain edema in acute HE are still not clear, elevated levels of brain ammonia, due to failure of the liver to adequately detoxify it, has been strongly implicated as an important etiological factor (Clemmesen et al., 1999; Albrecht and Jones, 1999; Hazell and Butterworth, 1999). Ammonia has been shown to cause astrocyte swelling in vivo (Voorhies et al., 1983; Willard-Mack et al., 1996), in brain slices (Ganz et al., 1989), as well as in astrocyte cultures (Norenberg et al., 1991; Zwingmann et al., 2000). For review, see Norenberg et al. (2007).

Another dysfunction associated with HE is a reduction of astrocytic glutamate uptake, possibly resulting in abnormal
glutamatergic neurotransmission and excitotoxicity (for review, see Butterworth, 2001). Ammonia suppresses high affinity glutamate uptake (Bender and Norenberg, 1996) and decreases levels of the glutamate–aspartate transporter (GLAST, EAAT-1) mRNA in cultured astrocytes (Zhou and Norenberg, 1999). Downregulation of the GLUT-1 (EAAT-2) glutamate transporter has also been shown in hyperammonemic rats, in rats with portacaval anastomosis (model of chronic HE), as well as in thioacetamide-induced acute liver failure (Knecht et al., 1997; Norenberg et al., 1997).

The mechanism by which ammonia induces astrocyte swelling and glutamate uptake inhibition is not clear. However, antioxidants and L-NAME abrogated both swelling and glutamate uptake impairment (Jayakumar et al., 2006), suggesting an important role of oxidative/nitrosative stress (ONS) in the mediation of these processes. In addition, ammonia-induced ONS activates mitogen-activated protein kinases (MAPKs), and blockade of these kinases also ameliorated astrocyte swelling and glutamate transport impairment (Jayakumar et al., 2006).

One potential target of oxidative stress (OS), as well as of MAPKs, is the nuclear phosphoprotein p53. p53 is a tumor suppressor protein and transcription factor that is a key mediator of stress responses, including oxidative DNA damage, hypoxia and inflammation (Kastan et al., 1991; see Harris and Levine, 2005). For most stresses, including OS, p53 is modified through multiple post-translational events including phosphorylation and acetylation (Vogelstein et al., 2000; Harris and Levine, 2005). Once activated by phosphorylation, p53 acts as a transcription factor for many genes that contain the consensus p53-binding sites in their promoter region.

Activated p53 triggers a number of signaling pathways that may lead to cell cycle arrest, apoptosis, DNA repair (Marchenko et al., 2000; Mihara et al., 2003; Bonini et al., 2004). Activation of p53 also mediates mitochondrial dysfunction, including decreases in the mitochondrial membrane potential and reduction in complex IV activity (Bae et al., 2005; Endo et al., 2006; Sayan et al., 2006). We have shown that astrocyte cultures exposed to ammonia induces mitochondrial dysfunction, as assessed by induction of the mitochondrial permeability transition (mPT) in cultured astrocytes (Bai et al., 2001; Norenberg and Rao, 2007). The mPT is a phenomenon due to the opening of a proteinaceous pore in the inner mitochondrial membrane resulting in an increase in the permeability of this membrane and the subsequent loss of its membrane potential (ΔΨm). One consequence of the ammonia-induced mPT is the development of astrocyte swelling (Rama Rao et al., 2003), possibly through the production of free radicals (for review, see Norenberg and Rao, 2007).

Another factor activated by p53 is nuclear factor-kappa B (NF-κB). NF-κB is a ubiquitous transcription factor that is kept in an inactive form in the cytoplasm where it is bound to a member of the IκB family of inhibitory proteins (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988; Israel, 1995). IκB is modified through site-specific phosphorylation, thus liberating NF-κB and allowing it to translocate to the nucleus (Baeuerle and Baltimore, 1988). Once in the nucleus, 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). We have recently documented the activation of NF-κB when astrocyte cultures were exposed to ammonia and that preventing such activation reduced ammonia-induced astrocyte swelling (Sinke et al., 2008). Additionally, antioxidants, L-NAME and MAPK inhibitors blocked the ammonia-induced increase in nuclear NF-κB expression (Sinke et al., 2008).

In this report we demonstrate that ammonia activates p53; that such activation is due to OS and MAPKs; and that inhibition of p53 activation diminishes ammonia-induced astrocyte swelling and glutamate uptake inhibition, possibly through inhibition of the mPT and NF-κB activation.

2. Materials and methods

2.1. Materials

All chemicals used in the study were purchased from Sigma–Aldrich (St. Louis, MO) unless noted otherwise: culture media (Gibco BRL/Invitrogen); protease inhibitor cocktail (Roche Diagnostics IN, USA); chemiluminescence reagents (ECL plus, Amersham); anti-α-tubulin antibody (Oncogene, San Diego, CA); Anti-phospho-p53Ser392 (Cell Signaling Technology, Beverly, MA); anti-goat and anti-mouse HRP-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA); antioxidants: desferoxamine (DFX); manganese(III) tetrakis (4-benzoic acid)prophyrin (MnTBAP); AG Scientific, San Diego, CA). Tetramethylrhodamine ethyl ester (TMRE) and Hoechst 33258 were purchased from Molecular Probes/Invitrogen.

2.2. Astrocyte cultures

Primary cultures of rat cortical astrocytes were prepared as previously described (Ducis et al., 1990). Briefly, brains of 1–2 day old rat pups were seeded on 35 mm culture dishes in DMEM containing penicillin, streptomycin and 15% fetal bovine serum (FBS) and incubated at 37 °C with 5% CO2 and 95% air. The culture media were changed twice weekly. On day 14, the cultures were treated with dibutyryl cyclic AMP (0.5 mM) to enhance cellular differentiation (Juurink and Hertz, 1985). Cultures consisted of 95–99% astrocytes as determined by glial fibrillary acidic protein (GFAP) immunohistochemistry. Cultures used in the experiments were 22–28 days old. All animal procedures followed guidelines established by National Institutes of Health Guide for the Care and use of Laboratory Animals and were approved by the local animal care committee (IACUC).

2.3. Western blots

Western blots were performed as previously described (Jayakumar et al., 2006). Briefly, astrocytes were solubilized in lysis buffer and protein levels in the supernatant were measured by the bicinchoninic acid assay (BSA, Pierce, Rockford, IL, USA). Equal amounts of protein were subjected to SDS–PAGE and electroblotted onto nitrocellulose membranes. Following blocking with nonfat dry milk, membranes were incubated with anti-phospho-p53 or anti-α-tubulin (both 1:1000) at 4 °C overnight and subsequently incubated with HRP-conjugated secondary antibodies (1:1000) for 2 h at RT. Membranes were visualized using an enhanced chemiluminescence reagent. Optical density of the bands was measured with the Chemi-Imager digital imaging system (Alpha Innotech, San Leandro, CA), and the results were quantified with the Sigma Scan Pro program (Jandel Scientific, San Jose, CA) as a proportion of the signal of a house keeping protein band (α-tubulin). Controls included omission of the primary antibodies.

To measure nuclear NF-κB expression, the nuclear extract was prepared as described below and treated with anti-nuclear (k)B or anti-α-tubulin antibodies (both 1:1000) at 4 °C overnight and subsequently incubated with HRP-conjugated secondary antibodies (1:1000) for 2 h at RT. Membranes were visualized using an enhanced chemiluminescence reagent. Optical density of the bands was measured with the Chemi-Imager digital imaging system (Alpha Innotech, San Leandro, CA) and the results were quantified with the Sigma Scan Pro program (Jandel Scientific, San Jose, CA) as a proportion of the signal of a house keeping protein band (α-tubulin). Controls included omission of the primary antibodies.

2.4. Measurement of the mitochondrial membrane potential (ΔΨm)

Changes in ΔΨm were measured with the fluorescent dye TMRE as previously described (Rao and Norenberg, 2004). Briefly, after 24 h of ammonia treatment, cells were loaded with TMRE for 20 min at a final concentration of 20 nM and examined with a Nikon Diaphot inverted fluorescence microscope (Ex 550/Em 590). At least 20 random image fields having a similar degree of cell density were selected (cells were stained with 2 μM Heoscht 33258). The selection of fields was achieved by systematically moving the microscope stage by 5 mm2 in all four directions. Images were captured with a cooled digital camera and fluorescent intensities were analyzed using the Sigma Scan Pro quantitation software. Total number of pixels were quantified on a grey scale (0–255) and the average pixel (fluorescent intensity) value in each image, containing an approximately equal number of cells, was obtained and expressed as the mean ± SEM of the total fluorescence intensity derived from a minimum of 15 random (20×) fields. The mean pixel value for TMRE was normalized to the total number of nuclei in the respective fields and the percent changes in the fluorescent intensities between control and experimental groups were calculated.

2.5. Cell volume determination

Cell volume was estimated by measuring the intracellular water space by the method of Kletzien et al. (1975), as modified by Bender and Norenberg (1998).
Briefly, 1 mM 3-O-methylglucose (3-OMG) and 0.5 μCi/ml [3H]-3-OMG were added to the culture 6 h prior to the volume assay. At the end of the incubation period, the culture medium was aspirated and an aliquot was saved for radioactivity determination. Cells were rapidly rinsed six times with ice-cold buffer containing 229 mM sucrose, 1 mM Tris–nitrile, 0.5 mM calcium nitrate and 0.1 mM phloretin (pH 7.4). Cells were harvested into 0.5 ml of 1N sodium hydroxide and mixed thoroughly by vortexing. Separate aliquots were taken for protein determination and liquid scintillation spectroscopy.

2.6. Measurement of glutamate uptake

L-Glutamate uptake was performed as described by Drejer et al. (1982) and Bender et al. (1989). Briefly, uptake experiments were initiated by adding 50 μM of unlabeled D-aspartate and 0.2 μCi of D-[3H] aspartate (a nonmetabolizable analog of L-[3H]glutamic acid) to the media for 1 min. The uptake reaction was terminated by washing cells rapidly three times in ice-cold DMEM. Cells were then harvested into 500 μl of 1N NaOH solution and mixed thoroughly by vortexing. Separate aliquots were taken for protein determination and liquid scintillation spectroscopy. Uptake rates were calculated from the measured radioactivity of the cells and the specific activity of the media, and the results were expressed as nmol/min/mg protein.

2.7. Statistical analysis

All experiments were repeated four to seven times using cells derived from different batches of astrocyte cultures. The number of individual culture plates in each experimental group was five for astrocyte swelling, two to four for Western blots. The data of all experiments were subjected to ANOVA followed by Tukey’s post hoc comparisons. A value of \( p < 0.05 \) was considered significant. Error bars represent mean ± SEM.

3. Results

3.1. Ammonia increases phosphorylation (activation) of p53 in astrocytes

Cultured astrocytes were treated once with a pathophysiological concentration of ammonia (NH4Cl 5 mM) (such value is found in brains of animals with acute liver failure, Swain et al., 1992). This single treatment with ammonia is followed by a rapid decline in ammonia concentration in the medium to undetectable levels by 30–60 min (unpublished observations). Levels of phospho-p53 (p-p53Ser392) were determined by Western blots from 1 to 48 h after ammonia treatment. A significant increase in p-p53 was observed at 1 h (51%; \( p < 0.05 \) vs. control) which remained elevated at that level for 6 h (Fig. 1A). At 12 h p-p53 levels further increased with peak phosphorylation occurring at 24 h (111% increase; \( p < 0.05 \) vs. control). A small decrease was detected at 48 h although this level was still higher than that observed at 1–6 h.

3.2. Ammonia-induced phosphorylation of p53 is attenuated by antioxidants and L-NAME

Cultures were treated with NH4Cl (5 mM) for 1 h in the presence or absence of individual antioxidants. The antioxidants chosen were previously shown to reduce ammonia-induced swelling (Jayakumar et al., 2006). The ammonia-induced increase in p-p53 levels was significantly blocked by vitamin E (100 μM), a blocker of lipid peroxidation; catalase (250 U/ml), a hydrogen peroxide decomposer; PBN (500 μM), a spin trapping free radical scavenger; DFX (20 μM), an iron chelator; and MnTBAP (10 μM) a cell permeable superoxide dismutase mimetic.

Another free radical that is increased after ammonia treatment in cultured astrocytes is nitric oxide (Sinke et al., 2008). Cultures exposed to L-NAME (250 μM), an inhibitor of nitric oxide synthase, also significantly blocked the increase in p-p53 by ammonia. Overall, antioxidants, as well as L-NAME, reduced p53 phosphorylation to below control levels (Fig. 2A). Cultures treated with antioxidants or L-NAME alone had no effect on p53 phosphorylation (data not shown).

3.3. MAPK inhibitors attenuate ammonia-induced p53 phosphorylation in cultured astrocytes

Since MAPKs are known to phosphorylate p53 (Wu, 2004), we examined whether MAPK inhibitors block the phosphorylation of p53 by ammonia. Addition of ammonia to cell cultures for 1 h with MAPK inhibitors, including SB 239063 (10 μM; p38-MAPK inhibitor), SP 600125 (1 μM; JNK inhibitor) and UO (U0126; 10 μM; an inhibitor of MEK1/2, the upstream kinase that activates ERK1/2) showed a significant reduction in p53 phosphorylation (72% 63% and 56%, respectively, \( p < 0.05 \) vs. NH4Cl) (Fig. 3). MAPK inhibitors alone had no effect on p53 phosphorylation (data not shown).

Fig. 1. (A) Representative Western blots showing the time course of p53 phosphorylation at Ser392 (C-terminus) following ammonia treatment of cultured astrocytes showing a significant increase in phospho-p53 (p-p53) as compared to control (Con). (B) Quantification of ammonia-induced phosphorylation of p53. p-p53 levels were normalized against α-tubulin. \( p < 0.05 \) vs. Con (Control). Data are means from 4 to 8 experiments. Error bars represent mean ± SEM.

Fig. 2. Antioxidants as well as the nitric oxide synthase inhibitor L-NAME blocked the phosphorylation of p53 induced by ammonia in cultured astrocytes. (A) Representative Western blots showing a significant decline in phospho-p53 (p-p53) compared to the NH4Cl-treated group at 1 h post-ammonia treatment. L-NAME (N-nitro-L-arginine methyl ester), Vit E (vitamin E); Cat (Catalase); PBN (N-tert-butyl-α-phenylnitrone); DFX, (desferoxamine), MnTBAP (manganese (III) tetrakis (4-benzoic acid) porphyrin). (B) Quantification of Western blots. p-p53 levels were normalized against α-tubulin. \( p < 0.05 \) vs. con (Control), \( p < 0.05 \) vs. NH4Cl. Data are means from 4 to 8 experiments. Error bars represent mean ± SEM.
Since p53 is known to activate NF-kB involved in the mediation of ammonia-induced astrocyte swelling (Norenberg et al., 2007; Sinke et al., 2008), we examined the effect of PFT on ammonia-induced glutamate uptake inhibition. Cultures were initially treated with 10 μM PFT for 3 days and the rate of glutamate uptake was performed at 1 min. PFT alone at this dose (10 μM) resulted in a significant reduction in glutamate uptake (data not shown). This may have been due to a toxic effect of PFT when used for longer periods of time (3 days). We therefore pretreated (20 min) cultures with 1 μM PFT, with or without ammonia for 3 days and the rate of glutamate uptake was performed at 1 min. Astrocytes exposed to ammonia significantly reduced D-aspartate uptake by 49.2% compared with controls (p < 0.05). This inhibition was significantly reversed by treatment with PFT (52.1%, p < 0.05 vs. NH4Cl) (Fig. 7).

### 4. Discussion

Previous studies have shown that astrocyte cultures exposed to ammonia result in the production of free radicals, including nitric oxide (Murthy et al., 2001; Sinke et al., 2008), as well as in the activation of MAPKs (Schliess et al., 2002; Jayakumar et al., 2006; Bodega et al., 2007). The present study demonstrates that astrocyte cultures exposed to ammonia phosphorylates (activates) the transcription factor p53, and such activation was significantly blocked by antioxidants, L-NAME, and MAPK inhibitors, implicating oxidative/nitrosative stress (ONS) and MAPKs in its activation. Inhibition of p53 activation by PFT blocked the ammonia-induced mPT and activation of NF-kB, suggesting the involvement of p53 in the induction of these processes. Further, the ability of PFT to block the ammonia-induced astrocyte swelling and to reverse the glutamate uptake inhibition, suggests that p53 participates in the evolution of these astrocyte abnormalities. Recently, Kosenko et al., 2007, reported increased brain cytoplasmic p53 level after acute ammonia intoxication in rats.

Cytotoxic brain edema, due primarily to astrocyte swelling by ammonia, is a major complication of ALF (Norenberg, 2001; Blei, 2005). While the mechanisms underlying ammonia-induced astrocyte swelling are not completely understood, oxidative stress, MAPKs, NF-kB, and the mPT have all been implicated as important mediators of such swelling (Norenberg et al., 2007; Sinke et al., 2008). The present study demonstrating a significant reduction of ammonia-induced astrocyte swelling by PFT, in conjunction with the involvement of ONS and MAPKs in the activation of p53, strongly suggests that p53 activation represents an important intermediate step by which ONS and MAPKs contribute to astrocyte swelling. Along with our earlier reports showing that inhibition of the mPT and NF-kB by CsA and BAY 11-7082, respectively, blocks astrocyte swelling (Rama Rao et al., 2003; Sinke et al., 2008), our findings suggest that p53, by activating the mPT and NF-kB, plays a major role in the mediation of ammonia-induced astrocyte swelling.

p53 is a tumor suppressor protein and a transcription factor that is activated by a variety of stimuli, including DNA damage and oncogene stimulation (Harris and Levine, 2005). The well-known function of p53 is its ability to regulate the transcription of target genes involved in growth arrest and apoptosis in response to DNA damage. While its role in tumor suppression is well known, p53...
has also been recognized to play a role in cell death following CNS ischemia (Chopp et al., 1992; Leker et al., 2004; Endo et al., 2006), traumatic brain injury (Napieralski et al., 1999; see Raghupathi, 2004 for review) and Huntington’s disease (Bae et al., 2005), as well to contribute to the brain edema following subarachnoid hemorrhage (Yan et al., 2008).

There is evidence that some of the deleterious effects of p53 may be a consequence of mitochondrial dysfunction (Bae et al., 2005; Sayan et al., 2006). A direct action of p53 on mitochondria has been reported in astrocyte cultures following severe oxidative stress (Bonini et al., 2004). We previously demonstrated that one of the deleterious effects of ammonia in cultured astrocytes includes the induction of the mPT (Bai et al., 2001). Our findings are consistent with this view as PFT blocked the ammonia-induced depolarization of the ΔΨm, suggesting the involvement of p53 in the induction of the mPT by ammonia.

Another potential downstream target of p53 is NF-κB (Ryan et al., 2000). NF-κB is a ubiquitous transcription factor that activates the transcription of many genes, especially those involved in immune responses, inflammation and cell proliferation (for review, see Baldwin, 1996). p53 is known to activate NF-κB (Ryan et al., 2000) and such activation was shown to be mediated through stimulation of the serine/threonine kinase ribosomal S6 kinase 1 (RSK1), which phosphorylates the p65 subunit of NF-κB (Bohuslav et al., 2004). We recently documented that ammonia activates NF-κB in cultured astrocytes (Sinke et al., 2008). The
The cell type employed is not known. Whether such a difference is a consequence of the models used or following traumatic brain injury in mice (Plesnila et al., 2007). Indicate that the mPT and NF-κB activation in cultured astrocytes results in ONS (Kowaltowski et al., 2001; Bowie and O’Neill, 2000). Likewise, we have observed that the ammonia-induced mPT and NF-κB activation in cultured astrocytes results in ONS (Sinke et al., 2000a,b; Jayakumar et al., 2006). While mechanisms responsible for the inhibition of glutamate uptake in astrocytes by ammonia are not completely understood, various reports have implicated ONS in such inhibition (Volterra et al., 1994a,b; Chen et al., 2000a,b; Jayakumar et al., 2006), the swelling resulting from NF-κB activation and the mPT is in all likelihood a consequence of ONS.

How ONS leads to cell swelling is unclear. However, we recently showed that astrocyte cultures exposed to ammonia resulted in oxidation/nitration of the Na+-K+-Cl cotransporter-1 (NKCC1), an ion transporter involved in cell volume regulation, and that such oxidation/nitration of NKCC resulted in its activation that led to astrocyte swelling (Jayakumar et al., 2008). One may speculate that membrane proteins that are critically involved in the regulation of cell volume may become modified by oxidation/nitration, and that such protein modifications may lead to their dysfunction and cell swelling.

Another important aspect of HE and hyperammonemia is an alteration in glutamatergic neurotransmission (Butterworth, 2001). Cultured astrocytes exposed to a pathophysiological concentration of ammonia suppressed high-affinity uptake of glutamate (Bender and Norenberg, 1996) and decreased GLAST mRNA levels in rat brain with acute liver failure (Knecht et al., 1997). Additionally, down-regulation of the glutamate transporter GLT-1 (EAAT-2) has been shown in hyperammonemic rats, in rats with portacaval anastomosis (model of chronic HE), as well as in thioacetamide-induced acute liver failure (Knecht et al., 1997; Norenberg et al., 1997).

In summary, our results demonstrate that astrocyte cultures exposed to ammonia activate p53, and that such activation was
mediated through ONS and MAPKs. Activation of p53 resulted in induction of the mPT and NF-κB activation; the latter likely responsible for astrocyte swelling. Blockade of p53 activation suppressed astrocyte swelling and glutamate uptake inhibition. A schematic representation of pathways involved in ammonia-induced cell swelling and glutamate uptake inhibition is shown in Fig. 8. Targeting p53 may represent useful therapeutic strategy for the treatment of HE and other hyperammonemic conditions.

Acknowledgements

We thank Alina Fernandez-Revueltas for the preparation of cell cultures. This work was supported by the Merit Review Grant from Department of Veterans Affairs and NIH Grant DK063311. ARJ is supported by the American Association for the Study of Liver Disease/American Liver Foundation Grant.

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