Neurochemistry International 55 (2009) 143–150

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Responses of astrocyte to simultaneous glutamate and arachidonic acid treatment

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ARTICLE INFO

Article history:
Received 1 December 2008
Received in revised form 17 February 2009
Accepted 25 February 2009
Available online 5 March 2009

Keywords:
Astrocyte
Caspase-3
Bcl-2
Hsp70
Synergistic effect
AFM

ABSTRACT

After cellular injury many endogenous toxins are released from injured cells and result in secondary injury. To elucidate mechanisms of such injury many of these toxins have been studied individually. However, the data obtained is only useful for reference and does not accurately represent the multifactorial situation under pathophysiological conditions. Primary astrocytic cultures were treated individually and simultaneously with two well-studied toxins, glutamate (Glu) and arachidonic acid (AA). Both are simultaneously released from neural cells during injury. Measurements of cellular protein content, intracellular water space, lactate dehydrogenase release, and malondialdehyde formation indicated that Glu and AA act through different mechanisms. Glu + AA applied together had a synergistic effect on the levels of *Caspase-3* gene expression, and Bcl-2 and Hsp70 protein. Atomic force microscopy observed that Glu caused cell membrane roughness and nuclear swelling, while AA induced pores in the cell membrane and nuclear shrinkage. Glu + AA accelerated nuclear shrinkage and resulted in more serious cell damage. This study not only distinguishes the different responses of astrocytes to Glu and AA, but also provides a new view into the synergistic effect of these biochemicals; highlighting the need to be cautious in applying single factor experimental data to interpret complex physiological and pathological conditions in animals. Two or more factors may act not only on different targets but also on the same target synergistically.

1. Introduction

The real situation in vivo is always more complex than what can be discovered from in vitro studies. It is common to take a basic approach with single factor studies to elude a mechanism for cell injury and death. However, multiple factor studies are also useful in order to investigate any possible synergistic effects and further understand the real situation. Also, many studies of neuronal injury and death under acute and chronic conditions have been undertaken but few look at the involvement of astrocytes. Here we hypothesize that glutamate (Glu) and arachidonic acid (AA), two well-known toxic agents, can exhibit synergistic effect in cellular responses of astrocytes. In particular the areas of cell swelling and apoptosis-associated protein levels are expected to be of most interest.

The pathophysiology of ischemic injury in the brain involves a primary injury and a delayed secondary injury (Lipton, 1999). Secondary injury has been attributed to a number of mechanisms including abnormal intracellular shifts of ions such as Na^+ and Ca^{2+}, free radical associated lipid peroxidation of the cell membrane, and excitotoxic cell death (Hall and Springer, 2004; Lipton, 1999; Stiefel et al., 2005; Xu et al., 2003). Various studies have reported that high levels of toxins such as K^+, cytokines, Glu and AA, released into the extracellular space during ischemia can also contribute to neural injury. To investigate this, we attempted to examine synergistic mechanisms in injury induction by treating astrocytes with Glu and AA, individually and simultaneously.

Glutamate is the major excitatory amino acid neurotransmitter in the mammalian CNS. Over-stimulation of Glu receptors by high extracellular Glu levels can induce neural cell death by a mechanism known as excitotoxicity (Had-Aissouni et al., 2002). It is thought that an uptake system primarily involving astrocytic Glu transporters (Had-Aissouni et al., 2002) controls extracellular

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doi:10.1016/j.neuint.2009.02.016

Glu concentrations and hence oxidative toxicity to non-excitable cells, including astrocytes. Moreover, AA is actually a downstream product of Glu-triggered excitotoxicity in neurons, which may be released into the extracellular space and affect astroglial structure and function.

AA is present in the highest concentration among all polyunsaturated fatty acids in the neural cell membrane and also plays important roles in signal transduction (Haag, 2003; Spehr et al., 2002). During cerebral ischemia, AA levels can reach up to 10-fold higher concentrations than those estimated during normal physiological conditions (Lipton, 1999; Matsubara et al., 1983). Under neural damage, unesterified AA produces O_2^-, increasing oxidative stress and making astrocytes more susceptible to oxidative insult. These free radicals can then elicit a harmful cascade of reactions altering the microenvironment and the structure of proteins in mitochondrial and plasma membranes (Blomgren and Hagberg, 2006; Chan, 1996; Lipton, 1999).

Glu and AA individually induce cytotoxicity through seemingly unrelated mechanisms. Moreover, they are both released under cerebral injury and together can potentially increase the rate of cell injury and death. We previously reported that AA can inhibit astrocyte Glu uptake (Yu et al., 1986). In addition, potassium-evoked Glu release liberates AA from cortical neurons (Taylor and Hewett, 2002). Thus Glu and AA have a complex relationship in neural cells, especially in injury or ischemia.

Concentrations of Glu (1 mM) and AA (0.2 mM) on astrocytes were chosen for their known toxicity to neural cells although they are not the maximum toxicity levels observed under certain pathophysiological conditions (Lipton, 1999). A broad spectrum of methods were used for a comprehensive analysis, from traditional molecular methods, RT-PCR and Western blotting to modern methods including morphological analysis by atomic force microscopy (AFM), phase contrast microscopy and Hoechst staining. Also, the changes in cellular protein content (CPC), lactate dehydrogenase (LDH) release, intracellular water space (IWS) and malondialdehyde (MDA) formation, were measured. Apoptosis-associated proteins were examined for further information regarding a possible synergistic effect. *Bcl-2* and *Hsp70* are both strongly protective against apoptosis and free radical-mediated cell death (Giffard et al., 2008; Setroikromo et al., 2007; Soane and Fiskum, 2005). *Bad* and *Caspase-3* are also involved in apoptosis regulation as pro-apoptotic factors or executors (Miyawaki et al., 2008). Our focus is to show Glu and AA induce cytotoxicity through different pathways and iterate the importance of multiple factor studies.

2. Materials and methods

2.1. Primary culture of astrocytes

Cerebral cortical astrocytes were prepared from newborn ICR, imprinting control region to investigate mice according to Yu et al. (1986, 2003, 2007). Culture medium was changed every 3–4 days with DMEM containing 10% (v/v) fetal bovine serum (FBS, Hyclone, UT, USA) for the first 2 weeks, and 7% (v/v) FBS thereafter. The astrocyte purity was over 95%, determined by GFAP-staining. Cultures of at least 3 weeks were treated with Glu (1 mM), AA (0.2 mM) (Sigma–Aldrich, MO, USA) and Glu (1 mM) + AA (0.2 mM) in serum-free DMEM with freshly added glutamine. AA was dissolved in alcohol as vehicle that has been previously tested and found it exerts no toxicity to astrocytes (Yu et al., 1986).

2.2. CPC, LDH release, IWS and MDA formation

Cellular Protein content was determined photometrically in triplicate samples using Lowry's method (1951) according to Yu et al. (1989) and Peng et al. (2003). LDH (DD, EC 1.1.1.27) activity in cell incubation media was measured utilizing the Sigma Diagnostic Lactate Dehydrogenase Reagent (Sigma–Aldrich, MO, USA) according to Yu et al. (1989) and Jiang et al. (2002). IWS of intact astrocytes in culture was measured using 3-O-[^{14}C]-methyl-D-glucose (OMG method) (Kletzien et al., 1975) according to Yu et al. (1989). MDA formation was measured as an index of the amount of lipid peroxidation in the cell membrane and carried out according to Yu et al. (1989).

2.3. Semi-quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by TRIzol reagent according to the manufacturer's instructions (Invitrogen Corporation, CA, USA). For RT-PCR, RNA was reverse-transcribed using M-MLV Reverse-Transcriptase (Promega, WI, USA), and random primers (Invitrogen Corporation). cDNA was amplified by PCR (reagents from Promega). Forward and reverse primers and PCR conditions, respectively, are listed below. *GFAP*, 5′-AGAAGGTCCGCTTCCTGGAA-3′ and 5′-TCCAAATCCACACGAGCCA-3′ (23 cycles; annealing temperature 60 °C); *Caspase-3*, 5′-TTCAGAGGCGACTACTGCCG-3′ and 5′-CCTTCCTGTTAACGCGAGTGAG-3′ (23 cycles; annealing temperature 60 °C); *Bad*, 5′-GGGATGGAGGAGGAGCTTAG-3′ and 5′-GATCCCACCAGGACTGGATA-3′ (25 cycles; annealing temperature 60 °C); *Bcl-2*, 5′-GCCACCATGTGTCCATCTGAC-3 and 5′-GATCCAGGTGTGCAGATGCC-3′ (29 cycles; annealing temperature 61 °C); *Hsp70*, 5′-GGTTGTCACTGTCCCATTTGA-3′ and 5′-TGGTGTTTGTGGAAAGGACC-3′ (28 cycles; annealing temperature 58 °C); *GAPDH*, 5′-GGGTGGTGCCAAAAGGGTC-3′ and 5′-GGAGTTGCTGTTGAAGTCACA-3′. Primer sequences, PCR products and specific amplification conditions were according to Chen et al. (2005). *GAPDH* was used as internal control. Semi-quantification was by TotalLab software (v. 201; Nonlinear Dynamics Ltd., UK).

2.4. Western blot

Standard Western blot analysis was performed. Monoclonal mouse primary antibodies against *Bcl-2* and *Hsp70* (Santa Cruz Technology, Inc., CA, USA) were used at 1:1000, against GFAP (Sigma–Aldrich) at 1:2000 and against β-actin (Santa Cruz Technology) at 1:4000. Goat anti-mouse secondary antibodies (Santa Cruz Technology) were used at 1:1000. Quantitative results were expressed as a ratio of *Bcl-2*, *Hsp70* and GFAP to β-actin.

2.5. Atomic force microscopy (AFM)

Astrocytes after treatment for 4 h were fixed for 30 min in 2.5% glutaraldehyde in 0.9% NaCl at room temperature. Crystals of NaCl and other salts were removed by triple distilled water. A piece of culture 1 cm × 1 cm was cut with the plastic from the culture plate, immobilized by double-sided adhesive tape to the steel base and placed on the AFM scanner for imaging. AFM imaging was carried out with an SPM-9500J3 Scanning Probe Microscope (Shimadzu Corporation, Japan) in contact mode at room temperature (22–24 °C) according to Yingge et al. (2003). Surface roughness of cells was evaluated in terms of height deviation from root mean square (RMS) values (Chung et al., 2002; Yu and Ivanisevic, 2004). For each treatment, RMS values were collected for five individual cells from each batch, for a total of three batches. Five areas of 2 μm × 2 μm were randomly selected on each cell. Nuclear sizes were also measured from three different photos from each batch, for all three batches. Parameters measured by AFM were quantitatively determined by Shimadzu SPM-9500J3 analysis software. MetaMorph Software (V. 4.5 r 5, Universal Imaging Corp., U.S.) analysis software was used for measuring changes in area of nuclei stained with Hoechst 33342 (Sigma–Aldrich). Treated astrocytes were also observed by phase contrast microscopy and images captured at: 10 min, 1 and 4 h. Three fields were randomly selected from one culture and photographed. The cultures used as control and the three treatments for each experiment, were taken from the same batch and three batches were used.

2.6. Statistical analysis

Statistical analysis of the data was by student's *t*-test, and one-way and two-way ANOVA performed by Prism Software (v 4.0, GraphPad Software, Inc. USA). A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Glu and AA effects on CPC, LDH release, IWS and MDA formation

Four parameters, CPC, LDH, IWS and MDA, were evaluated to study astrocyte responses to only AA, only Glu and when simultaneously applied together at the biochemical level. Glu did not change the CPC but AA decreased it significantly after 3 h (Fig. 1A). Glu + AA together also decreased CPC at 1 h and at 3 and 4 h it was reduced by 49.3% and 40.0% of the control, respectively. However, there were no significant differences between cultures treated with AA alone and with Glu + AA.

The LDH release remained unchanged in the control and Glu treated groups (Fig. 1B). However, it significantly increased in cultures treated with AA alone and with Glu + AA from 1.5 h of incubation. AA increased LDH releases 6, 16 and 22 times the control at 2, 3 and 4 h of incubation, respectively. However,

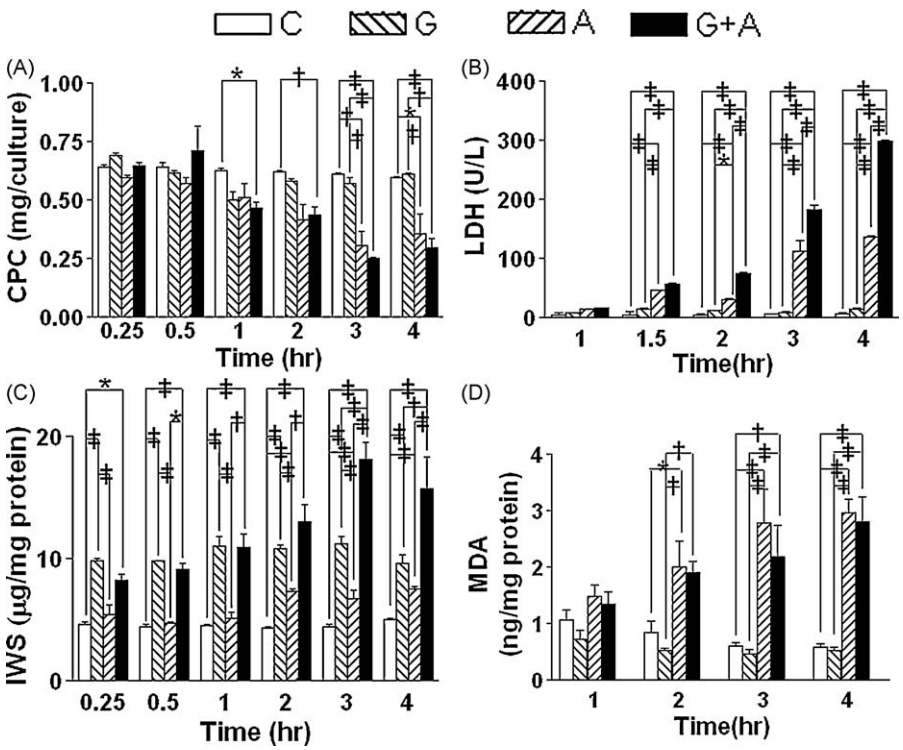

Fig. 1. Effects of Glu and AA on astrocytes on the biochemical indices. Results for untreated control and cultures treated with 1 mM Glu, 0.2 mM AA, and Glu + AA (1 mM Glu + 0.2 mM AA). For (A) cellular protein content (CPC); (B) intracellular water space (IWS); (C) lactate dehydrogenase (LDH) release; and (D), malondialdehyde (MDA) formation. Results are averaged from 3 experiments with S.E.M. as indicated by verticals bars. $^{*}p < 0.05$, $^{\dagger}p < 0.01$ and $^{\ddagger}p < 0.001$ with two-way ANOVA.

Glu + AA synergistically increased LDH release to 14, 25 and 48 times at 2, 3 and 4 h of incubation, respectively.

The synergistic effect of Glu + AA together was also observed for intracellular water space (IWS) measurements (Fig. 1C). Glu alone rapidly doubled IWS after a 15 min incubation which persisted throughout the 4 h treatment. AA also up-regulated IWS by about 50% after 2 h treatment. IWS in astrocytes treated with Glu + AA increased synergistically by a significant amount more than Glu or AA alone at 3 and 4 h. After 3 h incubation, the IWS value was over 15 μg/mg significantly more than when induced by Glu or AA alone.

Synergistic effects were not observed in MDA formation. Glu did not effect MDA formation (Fig. 1D). However, AA showed an increase in MDA formation by 1.4, 3.6 and 4.0 times of the control at 2, 3 and 4 h incubations, respectively. Glu + AA also increased MDA levels, but no significant difference from AA treatment was observed.

3.2. Glu and AA effects on mRNA levels of GFAP, apoptosis-associated genes, and Hsp70

Semi-quantitative RT-PCR quantified the mRNA levels of glial fibrillary acidic protein (*GFAP*), three apoptosis-associated genes (*Caspase-3*, *Bad*, *Bcl-2*), and *Hsp70*. The *GFAP* levels were relatively unchanged by any treatment (Fig. 2A). Glu + AA synergistically down-regulated *Caspase-3* levels in treated astrocytes by 51.2% at 12 h more than Glu or AA alone (Fig. 2B). The levels of *Bad* appeared to be decreased in presence of Glu or AA alone, and Glu + AA together showed a significantly higher inhibitory effect at 12 h of treatment (Fig. 2C). *Bcl-2* was inhibited in the presence of Glu, AA and Glu + AA (Fig. 2D); the effect was significant in the presence of Glu + AA. This synergistic effect was observed as early as after 3 h of treatment, while for Glu or AA alone inhibition was after 6 h. *Hsp70* levels were elevated at 12 h and the effect of Glu + AA was greater than single factor treatment (Fig. 2E).

The results of all measured mRNA levels are summarized in Table 1. In summary, Glu + AA applied together synergistically down-regulated *Caspase-3 and Bad* gene levels and also slightly up-regulated *Hsp70* gene level.

3.3. Glu and AA effects on protein levels of Bcl-2, Hsp70 and GFAP

Glu treatment alone had no effect on the protein levels of Bcl-2 or Hsp70 (Fig. 3). However, AA alone significantly decreased Bcl-2 protein expression to 64.8% of control at 4 h incubation (Fig. 3A) and decreased Hsp70 levels throughout (Fig. 3B). After 2 h treatment for Bcl-2 protein levels similar results were observed for Glu + AA treatment as for Glu alone. However, after 4 h incubation with both toxins Bcl-2 had undergone a significant decrease ($p < 0.01$) and was significantly lower than with AA treatment alone, which had also decreased the protein level (Fig. 3A). The Hsp70 protein levels at 2 h treatment were similar for Glu + AA and AA. Glu alone actually showed an increase in Hsp70 levels over treatment with both toxins together ($p < 0.01$). There was a further reduction at 4 h for Glu + AA to a level lower than AA alone (Fig. 3B). Thus suggesting there is a synergistic

Table 1
Summary of gene level changes under treatments of Glu, AA and Glu + AA.

Gene examined	Treatments		
	Glu	AA	Glu + AA
GFAP	No effect	No effect	No effect
Caspase-3	No effect	No effect	↓↓↓
Bad	↓	↓	↓↓↓
Bcl-2	↓	↓	↓
Hsp70	No effect	No effect	↑

No effect represented that no detectable change was measured under treatment. Number of (↑) indicated degree of increase and number of (↓) indicated degree of decrease in mRNA under treatment.

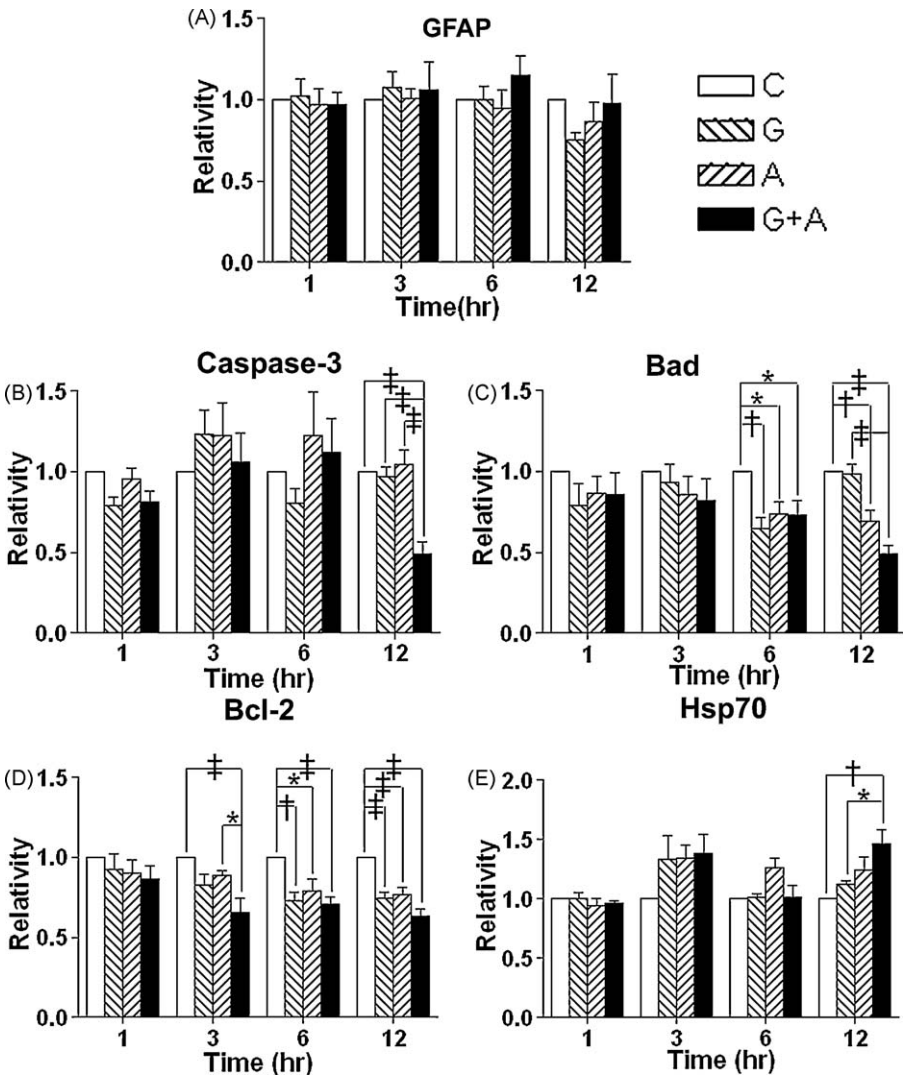

Fig. 2. Effects of Glu and AA on gene expression for GFAP, apoptosis-associated genes, and Hsp70. Including: untreated control and after treatment with 1 mM Glu, 0.2 mM AA, and Glu + AA (1 mM Glu + 0.2 mM AA). Gene levels were examined by RT-PCR for (A) GFAP; (B) Caspase-3; (C) Bad; (D) Bcl-2; and (E) Hsp70 gene expressions. GAPDH was used as internal control. Statistical analysis data represent the mean ± S.E.M. from 6 different experiments, $^{*}p < 0.05$, $^{\dagger}p < 0.01$ and $^{\ddagger}p < 0.001$ with two-way ANOVA.

effect on the levels of both proteins apparent after 4 h treatment. Interestingly, the protein levels were increased sooner than gene levels as the transcription and translation under these conditions may not be necessarily always in the order of gene expression first and protein translation later.

Protein levels of GFAP, a protein not involved in apoptosis, were examined after an incubation time of 4 h, when both Bcl-2 and Hsp70 had been significantly down-regulated. The GFAP protein levels were not significantly changed by Glu, AA, nor Glu + AA (data not shown).

3.4. Glu and AA effects on cell membrane

The changes in cell membrane under treatments of Glu, AA, and Glu + AA were observed under AFM for information on cell morphology. Fig. 4A shows AFM images of the cell membrane control culture of astrocytes, it appears smooth. Also, it was hard to distinguish the nucleus and cell-to-cell boundary. Astrocytes treated with Glu (Fig. 4B) showed distinct changes. The membrane became rugged and appeared sandy (Fig. 4B). The RMS, used to evaluate membrane roughness, was 5 times higher than the control (Fig. 4E). Cell swelling caused their arrangement to become regular and the cell-to-cell boundary apparent (Fig. 4B). The nucleus also appeared to be swollen and easily distinguished from other organelles. The nuclear diameter had increased to 125% of control nuclei (Fig. 4F). After 4 h AA showed no obvious membrane roughness and instead showed induced formation of many unevenly distributed pores at 4 h (Fig. 4C), mainly around the nucleus. Glu + AA treatment also caused pores to appear on the cell membrane. However, it did not cause any significant membrane roughness and measurable nuclear swelling (Fig. 4D and E). Detailed observation showed that pores were more even distributed than under AA treatment alone.

3.5. Glu and AA effects on nuclear size

Nuclear size was measured by phase contrast microscopy (Fig. 5). It is shown that Glu induced swelling of astrocytes from 10 min to 4 h after treatment. Interestingly, the nuclei of these swollen astrocytes had also swelled at 4 h Glu incubation. AA treatment led to cell swelling at 1 h but at 4 h the nuclei of these swollen astrocytes appeared to have shrunk. After 10 min, astrocytes treated with Glu + AA swelled at a faster rate than Glu treatment alone. The nuclei of most swollen astrocytes appeared to begin shrinking at 1 h and were seriously condensed at 4 h.

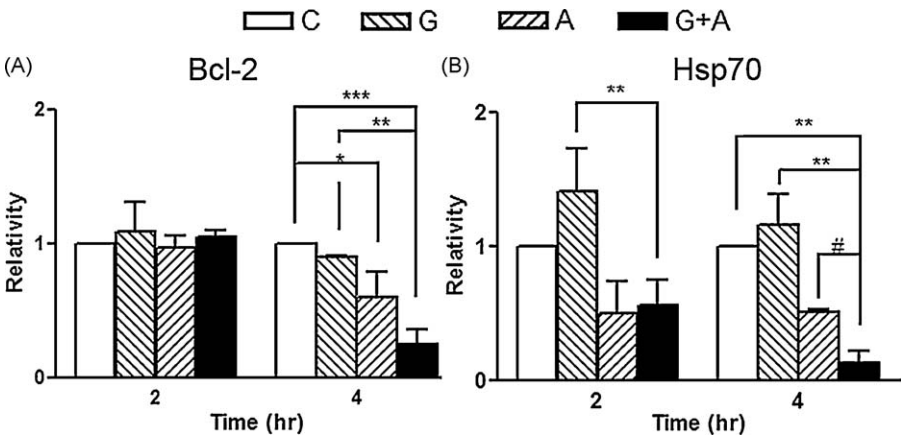

Fig. 3. Effects of Glu and AA on Bcl-2 and Hsp70 protein levels. Including untreated control and after treatment with 1 mM Glu, 0.2 mM AA and Glu + AA (1 mM Glu + 0.2 mM AA) at 2 and 4 h without serum. Protein levels of (A) Bcl-2 and (B) Hsp70 were examined. Data represent the mean ± S.E.M. from 3 independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ with two-way ANOVA. $^{\#}p < 0.05$ was the result from *t*-test analysis.

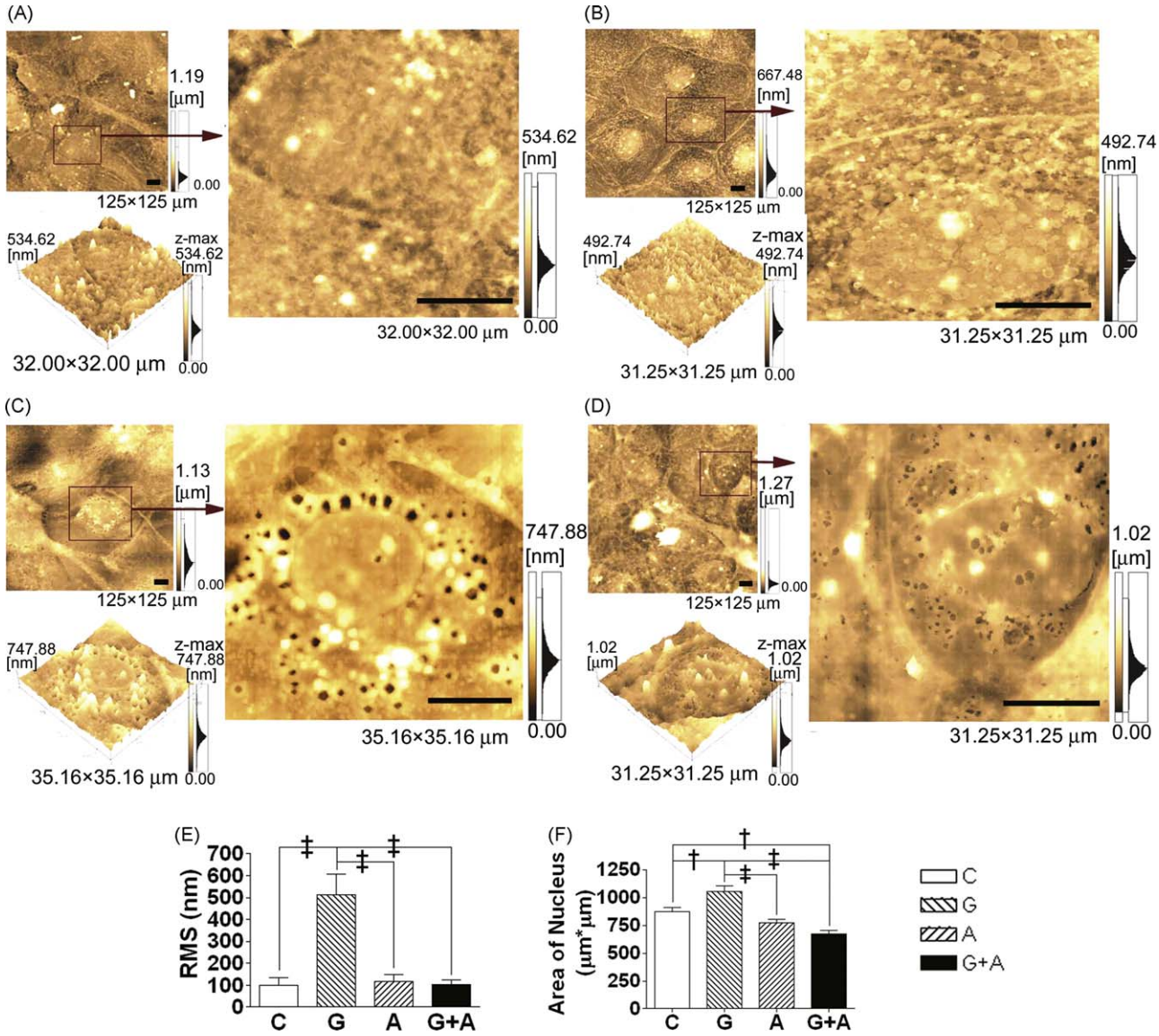

Fig. 4. Atomic force microscopy (AFM) analysis of cell membrane. (A) untreated control, and 4 h treatment with (B) 1 mM Glu; (C) 0.2 mM AA; and (D) Glu + AA (1 mM Glu + 0.2 mM AA). The height of the object is indicated as brightness of color the upper left and the right panels, i.e., the brighter the color, the higher the object. The right panels were a higher magnification of the box in the upper left panels. The lower left panels are the three-dimensional image of the upper left panel. (E) Root mean square (RMS) in nanometers, and (F) statistical analysis of the nuclear area with values measured from the AFM images. $n = 30$, $^{\ddagger}p < 0.001$ with one-way ANOVA. Bar = 10 μm.

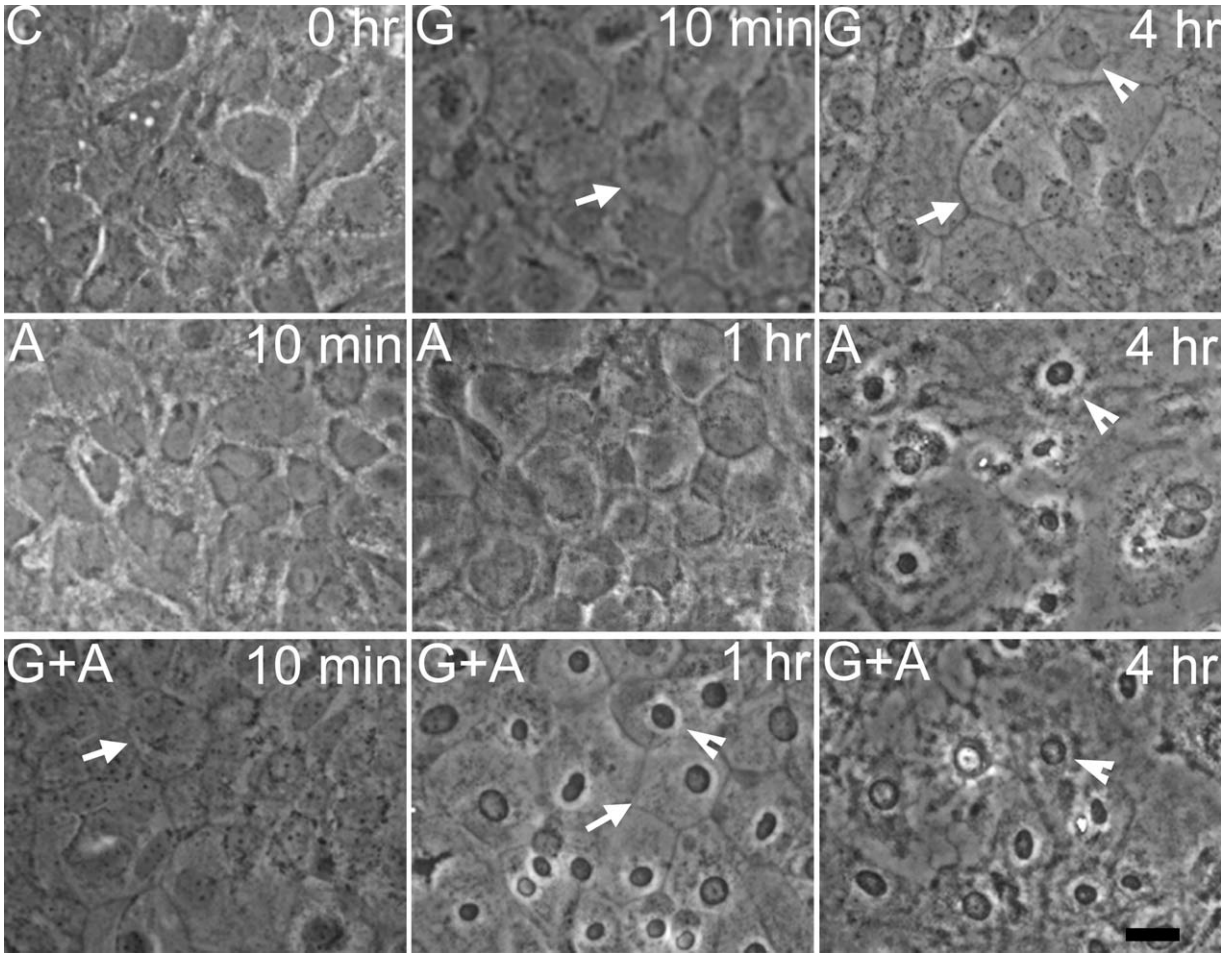

Fig. 5. Phase contrast microscopy showing Glu and AA effects on nuclear size. For untreated control, and cells treated with 1 mM Glu, 0.2 mM AA, and Glu + AA (1 mM Glu + 0.2 mM AA) for 10 min, 1 and 4 h. Arrows indicated swelling cells, and arrow-heads, nuclei. Bar = 40 μm.

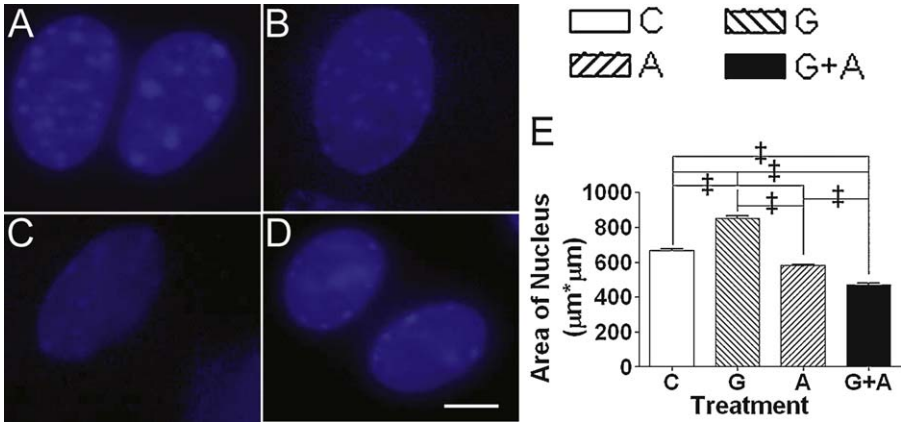

Fig. 6. Hoechst staining photos showing nuclear changes. For (A) untreated control, and treatment with (B) 1 mM Glu, (C) 0.2 mM AA, and (D) Glu + AA (1 mM Glu + 0.2 mM AA). (E) Statistical analysis of nuclear areas of Hoechst stained nuclei images. Five fields of each culture were randomly selected from a total of 3 batches; the total number of nuclei counted for each treatment was 500, $^{\ddagger}p < 0.001$ with one-way ANOVA. Bar = 10 μm.

These changes were further examined and confirmed by measuring the area of nuclei stained with Hoechst (Fig. 6A–D). From statistical analysis (Fig. 6E), Glu increased in nuclear area by 28.2% ($p < 0.001$) compared with the control. Adding Glu + AA caused the area of nuclei to shrink by 20.7% ($p < 0.001$) in the 4 h incubation. This was considerably more than under AA treatment alone (Fig. 6E).

4. Discussion

High extracellular concentrations of Glu are known to be neurotoxic. The toxic effect appears in both acute and chronic forms of neuropathology (ischemia, stroke, trauma, etc.). It was previously demonstrated by Had-Aissouni et al. (2002) that elevating extracellular Glu levels could induce astrocytic death.

Glu can induce oxidative toxicity and affect every cell type of the CNS, including astrocytes (Had-Aissouni et al., 2002; Yang et al., 2008). The result from Glu treatment was shown to cause rapid cell swelling confirmed by IWS, phase contrast and atomic force microscopy. However, this might have been partially due to Na^+ and Ca^{2+} influx along with the excitation of Glu receptors and transporters (Arundine and Tymianski, 2004; von Engelhardt et al., 2007). Glu transporters on the cell membrane may exist in a state of partial activation (Schubert and Piasecki, 2001) and allow small molecules, such as ions or water, to enter along with Glu causing cell swelling and changing the cell arrangement to a more regular 'honeycomb'. Glu in the cytoplasm might then act on Glu-specific NMDA receptors (Izumi et al., 2003) or water channels (unpublished data) on the nuclear envelope and induce further nuclear swelling. This Glu-induced nuclear swelling is important and requires further investigation. AFM showed direct evidence of Glu-induced cell membrane roughness and even spine formation that might be related to cytoskeletal rearrangements (Nowakowski et al., 2001; Weissmuller et al., 2000).

Polyunsaturated fatty acids are key components for increasing the fluidity of neural cell membranes but they can also release free radicals (Farooqui and Horrocks, 1998, 2004; Haag, 2003). High concentrations of AA induce severe cell damage (Higuchi et al., 2007; Taylor and Hewett, 2002) and can also enhance the NMDA current, induce cell swelling and cause cell death (Basselin et al., 2006; Herbert et al., 2005; Taylor and Hewett, 2002). It is shown here that a high AA concentration decreases astrocytic cellular protein content (CPC), and increases intracellular water space (IWS), LDH release and MDA formation. Cell swelling observations confirmed the IWS result. AA can cause injury indirectly by metabolite and free radical formation, resulting in lipid peroxidation. This is supported by the increase in MDA formation. LDH release reflected a loss of membrane integrity possibly through AA acting as a detergent (Friberg and Wieloch, 2002). The presence of pores on the cell membrane, observed under AFM, is the first clear evidence that unesterified AA can injure astrocytic cell membranes, probably by dehydrolysis. Therefore materials, water, electrolytes, cytokines, Ca^{2+} and AA in the medium, can freely enter the cell body leading to a high AA concentration inside the cell, where its presence as unesterified AA signals apoptosis (Cao et al., 2000; Penzo et al., 2004; Scorrano et al., 2001). Moreover, it was shown that AA induced nuclear shrinkage in astrocytes and not cellular swelling, completely different to Glu, which induces nuclear swelling.

AA can inhibit Glu uptake in astrocytes (Yu et al., 1986) mainly by inhibition of EAAT1, the most abundant subtype of Glu transporter (Fairman et al., 1998; Zerangue et al., 1995). It is also thought to enhance Glu concentrations in the synaptic cleft by increasing Glu release (Taylor and Hewett, 2002). Therefore AA may be capable of increasing Glu accumulation outside the cells and accelerating injury. Glu acting on ionotropic and metabotropic glutamate receptors can cause AA to be liberated from cell membranes through PLA_2 (Arundine and Tymianski, 2004). Hence, indicating that AA and Glu may exacerbate each other's effects after injury or ischemia leading to synergistically increasing the injury.

The results of all the biochemical indices show that the most cell injury occurred under treatment of the two reagents concurrently. Glu + AA synergistically and significantly increased IWS and LDH release, and resulted in more pores in the center of the cell body than either Glu or AA treatment alone. High concentrations of either Glu or AA are known to result in oxidative stress in neural cells (Schubert and Piasecki, 2001; Xu et al., 2003). In turn this increases free radical production and the likelihood of pore formation. The possibility of toxins, including AA and Glu entering the cells is thus increased. However, there is an accelerated nuclear shrinkage under Glu + AA treatment while increasing the cytoplasmic swelling that warrants further study.

The measurements of mRNA levels showed that some apoptosis-associated genes are both involved in responses to either toxin alone. However, when Glu + AA are applied the synergistic effect significantly modulated *Bcl-2* and *Hsp70* levels while significantly down-regulating *Caspase-3* levels, evident at 12 h treatment. Thus a synergistic effect was observed for Glu + AA on the levels of some apoptosis-associated genes. The measurement of protein contents indicated that Glu has little effect on protein translation and degradation processes. However, AA treatment alone significantly decreased *Bcl-2* and *Hsp70* protein levels after 4 h treatment. This is consistent with the induction of apoptosis. Another synergistic effect of Glu + AA treatment was realized as these protein levels were significantly decreased compared to AA alone and control. The decrease in *Bad* gene expression after AA treatment indicated that its action in apoptosis might be through changes in protein levels or by activation through dephosphorylation. mRNA levels of the apoptosis executor, *Caspase-3*, were also unchanged by AA, which may regulate *Caspase-3* activity in other ways (Cao et al., 2000).

The protein family *14-3-3* is a highly conserved acidic protein family involved in many processes including cell cycle regulation, responses to stress and apoptosis, and serving as adapters, activators, and repressors (Chen et al., 2003, 2005; Chen and Yu, 2002; Ferl et al., 2002). In preliminary gene expression experiments, not reported here, synergistically high levels of *14-3-3 Zeta* were detected that may have had a role in protecting cells and delaying apoptosis. This would be consistent with the synergistic down-regulation of *Caspase-3* levels, the apoptosis executor. Moreover, swelling observed by Glu treatment was indicated by an increase in the *Eta* isoform under Glu that supports a role in swelling regulation (Simard and Nedergaard, 2004). However a synergistically up-regulation of the gene levels under Glu + AA suggested the *Eta* expression may be dependent on cytoplasmic rather than nuclear swelling (data not shown). Further studies including the protein levels are warranted. They are reported here to give an indication of the potential importance of this family in astrocytes responses in multiple factor investigations.

These synergistic effects on the timing and nature of changes demonstrate an increased level of complexity studying the toxic effects on a real system (Table 1). Moreover the differences between Glu and AA single factor should be mentioned to reiterate the fact they induce cytotoxicity be two different pathways. The biochemical indices revealed many differences to suggest that this was the case. Interestingly, there was little difference in the gene level experiment for Glu or AA treatments alone. The only significant differences were *Caspase-3* at 6 h and *Bad* at 12 h. However, in the protein levels of Bcl-2 and Hsp70 the difference between the single factor treatments became clear again (Fig. 3). Thus, suggesting that AA treatment alone induces cytotoxicity through down-regulation of these proteins and hence injury by a different pathway to Glu. Moreover, this was reinforced by the differences in morphology (Fig. 5) and by the fact Glu treatment alone increase nuclear size (Fig. 6).

This comprehensive study of two well-known toxins released during cerebral injury revealed certain synergistic effects which may in turn increase damage caused by injury. Single toxin investigations are highly useful for eluding the mechanism but the real condition is always more complex. Facing us is system biology and not just molecular, or even cellular, biology. Importantly in drug discovery finding out about any synergistic effects increases our understanding. Drug cocktails aimed at multiple targets show promising abilities but might have unexpected side-effects because we are unable to predict the synergistic effects of two or more factors acting together. Multiple toxin studies can give a

more meaningful indication of the real situation and should become more common in the future.

Acknowledgements

We would like to thank Stewart Dods for his suggestion and contribution in the writing of this manuscript. This work was supported by the National Natural Science Foundation of China (Grant No.: 30270426; 30470543; 30670644; 30870818); the Beijing Natural Science Foundation (Grant No.: 7032026; 7051004; 7091004); and the National High Technology Research and Development Program of China (863 Program, Grant No.: 2006AA02Z452).

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