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Responses of astrocyte to simultaneous glutamate and arachidonic acid treatment

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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.

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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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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,

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