Short communication

Transfection of the plasma-type platelet-activating factor acetylhydrolase gene attenuates glutamate-induced apoptosis in cultured rat cortical neurons

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Abstract

Using an adenoviral vector, we induced overexpression of the plasma type of platelet-activating factor acetylhydrolase in cultured rat neurons. Neurons overexpressing this enzyme showed a decrease in glutamate-induced injury, mainly, apparent as decreased apoptosis. Reduction of lipid peroxidation by this enzyme and protection of mitochondrial function were demonstrated, and these may be the basis of the resistance to glutamate-induced neuronal injury that we observed. © 2000 Elsevier Science B.V. All rights reserved.

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Platelet-activating factor (PAF) acetylhydrolase (PAFAH) removes the acetyl group at the sn-2 position of PAF. Forms of mammalian PAFAH include an intracellular (tissue) type and an extracellular (plasma) type. In turn, tissue cytosol includes at least two types of tissue PAFAH, isoforms Ib and II [7]. Isoform Ib is a tertiary G protein complex-like heterotrimeric enzyme, while isoform II is a 40-kDa monomer. Plasma PAFAH is a 45-kDa monomeric enzyme in humans and a 63-kDa monomeric enzyme in guinea pigs [5, 12, 24]. Isoform II has an amino acid sequence with 41% identity to that of human plasma PAFAH [8, 9, 24]. Although human and guinea pig plasma PAFAH show some differences in physical properties such as association of lipoproteins or molecular mass, they share many similar biochemical properties [11]. Isoform II preferentially hydrolyzes oxidized phospholipids as well as PAF [8], and therefore may function as an antioxidant [15].

Plasma PAFAH also has the ability to catabolize oxidized phospholipids [11, 21, 22]. Although plasma PAFAH is secreted in vitro from various cells such as macrophages [4] and hepatocytes [23], the exact cellular source(s) of this enzyme in vivo remains unclear. Recently, Asano et al. reported that most of the PAFAH activity in human plasma originates from hematopoietic lineage cells [2].

Neurotoxicity initiated by overstimulation of glutamate receptors and subsequent depolarization of mitochondria has been suggested to contribute to oxidative stress in neuronal injury [6, 26]. Previous investigation has indicated that two types of neuronal death, apoptosis and necrosis, can result from glutamate receptor-mediated excitotoxicity [1]. Mild insults lead to transient mitochondrial depolarization, reversible energy failure, and apoptosis, while intense insults produce irreversible mitochondrial depolarization, permanent energy collapse, and ionic imbalances resulting in cellular swelling and necrosis [1]. Overexpression of isoform II in Chinese hamster ovary K1 cells has been reported to suppress tert-butylhydroperoxide (t-BuOOH)-induced apoptotic cell death, most likely by its antioxidant

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effects [15]. In the present study, we determined whether glutamate-induced neuronal death was reduced in neuronal cultures by overexpression of the plasma PAFAH gene transfected with an adenovirus vector in place of tissue-type PADAH gene and estimated whether oxidative stress due to impairment of mitochondrial function contributed to neuronal apoptosis.

We purchased 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) from Wako (Osaka, Japan). Thiobarbituric acid (TBA) reagent as well as 1,1,3,3-tetraethoxypropane, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT reagent), and Hoechst 33258 was obtained from Sigma (St. Louis, MO). A protein assay kit was obtained from Bio-Rad (Hercules, CA).

Replication-defective E1- and E3-adenoviral vectors without an inserted gene (Ad1W) or alternatively containing either guinea pig plasma PAFAH gene (AdPAFAH) [12] or β-galactosidase gene (AdLacZ) [14] under control of cytomegalovirus enhancer and a chicken β-actin promoter together constituting a CA promoter [17] were prepared as described previously [25].

Cerebral cortical neuronal cells were prepared from 17-day-old rat embryos according to a previously reported procedure [18,19]. The dissociated cells were plated in polyethylenimine-coated 96-well and 24-well culture plates and 2-well glass slides. Incubation with recombinant adenovirus (10–50 multiplicity of infection (m.o.i.)) was carried out for 6 h using 4-day-old cultures. Cells next were exposed to 1 mM glutamate for 60 min on day 7 in culture, and the medium was replaced. Cultures then were maintained for 24 h before neuronal death was assessed. PAFAH activity was assayed as described by Stafforini et al. [20].

X-gal staining was performed to demonstrate gene expression for β-galactosidase, as previously described in detail [25]. Briefly, cells on glass slides were prefixed with 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS), incubated in 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, and postfixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS, with a thorough rinse with PBS before each step.

The TBA test protocol of Lee and Csallany [13] was used with the modification that phosphoric acid was replaced with 10% trichloroacetic acid (TCA) in 1% HCl [3]. After addition of the TCA solution samples, 0.5 ml of TBA reagent (1% w/v TBA in 50 mM NaOH) was added, and the mixture was heated for 15 min at 100°C. After cooling, the chromophore was extracted into 1.5 ml butanol-1-ol, and absorbance was measured at 532 nm. Since the TBA test measures malondialdehyde (MDA), we prepared a standard plot for MDA in the TBA test system, which was linear up to 20 nmol MDA per sample [3]. The plot was used to convert absorbance values to nmol of MDA. MDA content was normalized to protein concentration.

Mitochondrial function was assessed by the MTT assay. Using 96-well microplates, a modified MTT assay was performed essentially as described by Musser et al. [16]. MTT reagent was dissolved at 5 mg/ml in PBS, filter-sterilized using 0.22 μm disposable filters (Milllex-GV; Millipore, Bedford, MA), and stored at 4°C. MTT stock solution (25 μl) was added to each well and incubated at 37°C for 4 h to allow mitochondria to form a colored, insoluble formazan product. After the supernatant was removed by aspiration, 100 μl of dimethylsulphoxide was added to each well to solubilize the formazan crystals. The plate was agitated to fully dissolve the crystals, and absorbance at 550 nm was measured by a model 340 ATTC microplate reader (SLT, Saltzburg, Austria).

Cells on glass slides were stained with Hoechst 33258 (10 μM) for 5 min and analyzed morphologically under a nonconfocal fluorescence microscope (Olympus BX50, Tokyo, Japan). A photomicrograph was taken immediately with color reversal film (Fujichrome Provia 400, Fuji, Tokyo, Japan). Apoptotic cells identifiable by nuclear fragmentation were counted from the color print.

Data are presented as the mean±S.D. For statistical comparisons, one-way analyses of variance followed by Fisher’s protected least-squares difference test were used for multiple comparisons. P-values <0.05 were considered to indicate significant differences.

We first examined the expression of LacZ by X-Gal staining in neurons exposed to AdLacZ at various m.o.i. for 72 h. Neurons showed deep blue coloration with intensity dependent on vector quantity from 10 to 50 m.o.i. (data not shown). The percentage of neurons with positive X-gal staining at 50 m.o.i. was 70.8±10.9% (n=4). Next, we measured PAFAH activity in neurons infected with AdPAFAH. Like LacZ, the activity of PAFAH increased with increasing m.o.i. (Fig. 1). PAFAH activity in culture

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**Fig. 1.** Platelet-activating factor acetylhydrolase (PAFAH) activity in neurons transfected with AdPAFAH (recombinant adenovirus vector containing the guinea pig plasma PAFAH gene). Values are expressed as the mean±S.D. (n=4). ***, P<0.01 and ***, P<0.001 compared with controls. ‡‡‡, P<0.001 compared with AdPAFAH at 10 m.o.i.; ‡‡, P<0.01 compared with AdPAFAH at 50 m.o.i. (Significance testing: one-way analysis of variance followed by Fisher’s protected least-squares difference test.)
medium showed no difference between media from neurons without infection (control), neurons infected with Ad1W, and neurons infected with AdPAAHA. Activity in medium was negligible compared with activity in cell suspensions (data not shown). Plasma type of PAAHA should be produced and secreted from some type of cells [2,4,23] and some enzyme activity was expected in the culture medium infected with AdPAAHA. The discrepancy may be explained by the pooling system of the enzyme in neurons. The result suggested that transfection at m.o.i. 50 for 72 h achieves sufficient cellular expression of plasma PAAHA.

Oxidation of lipids, (lipid peroxidation) was assayed with TBA. In experiments without glutamate, neurons infected with Ad1W were associated with more reaction product (MDA) than neurons without infection (control) or those infected with AdPAAHA. Infection with adenovirus then, may induce oxidation of lipids (Fig. 2) [11,15]. Although glutamate treatment resulted in increased MDA in neurons without infection (control) or infection with Ad1W, MDA content was not significantly increased by glutamate loading in neurons infected with AdPAAHA (Fig. 2). PAAHA may attenuate increased membrane lipid peroxidation resulting from both viral infection and glutamate treatment.

Extent of neuronal injury was assayed in terms of mitochondrial function in the MTT assay [16]. Neurons infected with AdPAAHA and those without infection (control) showed significantly higher amounts of MTT neuronal mitochondrial function from this insult in addition to attenuating oxidation of membrane phospholipids [11,15].
Damage to neurons from 1 mM glutamate exposure was estimated by the MTT assay 24 h after exposure to glutamate for 60 min. Significantly more MTT product was present 24 h after exposure to 1 mM glutamate for 60 min in neurons infected with AdPFAH than in those infected with Ad1W or those without infection (Fig. 3). Tissue-type, 40 kDa monomer P AFAH (isoform II) acts as an antioxidant phospholipase that protects cells from oxidative stress [15]. Plasma-type P AFAH has an amino acid sequence similar to isoform II [8,9,24] and also has the ability to catabolize oxidized phospholipids [11,21,22]. Overstimulation of glutamate receptors induces cell death by reactive oxygen intermediates that result from impairment of mitochondrial function [6,26]. The present results suggest that like tissue-type isoform II, plasma-type PAF AH can protect neurons from apoptosis by reducing oxidative stress.

Mild glutamate excitotoxic insults lead to transient mitochondrial depolarization and apoptosis, while intense insults produce irreversible mitochondrial depolarization and necrosis [1]. According to the MTT assay, impairment of mitochondrial function was relatively mild after 1 mM glutamate exposure in the present study, in accordance with our previous observation that neuronal death in culture was mainly apoptosis 24 h after exposure to 1 mM glutamate [10]. With Hoechst 33258 staining of neurons 24 h after exposure of 1 mM glutamate for 60 min, apoptotic neurons with fragmented nuclei were widespread in cultures infected with Ad1W or with no infection (control), while neurons infected with AdPFAH did not show marked apoptotic features (Figs. 4 and 5). Thus, like the tissue type, 40-kDa PAF AH monomer, plasma-type PAF AH has an antioxidant action that protects cells from death due to oxidative stress.

References


