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Carnosine and taurine protect rat cerebellar granular cells from free radical damage

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Abstract

Carnosine and taurine have been suggested to protect excitable tissues against oxidative stress. We have investigated the protection of cerebellar granule cells (neurons) by these compounds against free radicals generated by kainic acid (KA), and 3-morpholinosydnonimine hydrochloride (SIN-1) treatment. Carnosine decreased free radical levels in KA and SIN-1 treated cells, and increased cell viability. The KA effect, but not that of SIN-1, was dependent on the presence of external Ca^{2+} ions. Taurine increased cell viability, but did not decrease free radical levels. These results suggest that there are multiple pathways leading to cell death, not all of which involve decreases in intracellular free radical levels, and also indicate that multiple mechanisms of cellular defense exist against oxidative stress.

Keywords: Kainic acid; 3-Morpholinosydnonimine hydrochloride; Free radicals; Carnosine; Taurine; Neuron

Oxidative stress and damage in neurons, which results from free radical attack on cellular components such as membrane lipids, proteins and DNA, have been correlated with an excess of reactive oxygen species (ROS) produced by a number of intracellular mechanisms [14]. Although antioxidants may protect cells against ROS and increase cell viability [21], in some cases, delayed cell death has no direct connection to ROS generation and antioxidants have no protective effect [6,8].

A variety of factors, including activation of glutamate receptors and NO production, are known which can stimulate ROS formation and imitate oxidative stress in neurons. Thus, Ca^{2+} ions can activate the cytoplasmic enzyme nitric oxide (NO) synthase [1], which generates either NO radicals in the presence of arginine or in its absence, superoxide anion [9]. The cytotoxic potential of NO and superoxide radicals can also be increased by formation of peroxynitrite [18], which attacks membrane phospholipids, nucleic acids and proteins [9]. In addition to the biogenic origins of these ROS, the synthetic compound 3-morpholinosydnonimine hydrochloride (SIN-1) can be used to generate NO and superoxide simultaneously and independently of cellular mechanisms [18] with these two compounds then combining to form peroxynitrite.

In the present studies, we have used fluorescence techniques [10] to examine the effects of carnosine (β-alanyl-L-histidine) and taurine (2-aminoethane sulfonic acid) on cell viability, free radical production and Ca^{2+} ion levels in neurons treated with KA or SIN-1, compounds which respectively increase intracellular ROS levels by intracellular and extracellular mechanisms. Carnosine and taurine were selected for this study because they are both found in excitable tissues where they may be involved in protec-

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tion against oxidative stress because they are hydrophilic antioxidants [3,16], and because they have different effects on enzymes of free radical metabolism such as myeloperoxidase [4,11]. Because of the possible involvement of Ca^{2+} ions in metabolic disordering, we have also investigated the relationship between intracellular Ca^{2+} ion levels and the generation of ROS by KA and SIN-1 in the presence of carnosine and taurine.

Neurons (cerebellar granule cells) were prepared from the cerebella of 10–13 day old Sprague–Dawley rat pups in accordance with our previous protocols [10]. Non-viable neurons were identified in flow cytometry by staining with propidium iodide (PI), a DNA-binding dye which is excluded from cells with intact plasma membranes. The cellular content of ROS was determined by the use of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). For experimental purposes, cell preparations were loaded with 100 μM DCFH-DA for 60–80 min and then treated with PI (10 μg/ml) for 1–2 min immediately before cell cytometry [10]. The fluorescence response of the cells was found to be constant up to 120 min, the maximal time for the experiment. In experiments with the calcium-sensitive dye, Fluo-3, cells were treated as described earlier [10] and intracellular Ca^{2+} levels were estimated by using an excitation wavelength of 488 nm and measuring the fluorescence emission intensity at 515–545 nm [20]. In various experiments, cells were treated at 37°C with up to 1 mM KA for up to 2 h [17] or 100 μM SIN-1 for 30 min, and ROS, Ca^{2+} levels and cell viability were then measured by flow cytometry.

In order to characterize the possible protective effects of the excitable tissue antioxidants carnosine and taurine, neuronal preparations were pre-treated during the restitution period at physiologically appropriate concentrations (10–25 mM for carnosine [3] and 10 mM for taurine [16]), and control samples were treated by the addition of concentrations of *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) buffer equal to those of each of the antioxidants used. After restitution, samples were diluted 10 times during subsequent manipulations; and in Table 1, the final concentrations of the compound used are listed. Experiments were performed at least twice (from neuron preparations from different animals) with triplicates of each condition in each experiment and data sets from experiments were analyzed for statistically significant differences ($P < 0.05$) by one-way ANOVA.

Table 1

The effects of carnosine and taurine on cerebellar granule cells treated with KA or SIN-1. Mean DCF fluorescence intensities obtained as arbitrary units in the experiments were normalized to values based on the control cell DCF fluorescence set to 100 units. Cell viabilities (expressed relative to the percentage (75 ± 1%) of viable cells in control samples) were calculated from PI staining of cells by flow cytometry. Data (n = 5) are expressed as the mean ± SD.

Conditions	Relative mean DCF fluorescence (control = 100)	Relative cell viability (control = 100)
Control	100 ± 8	100 ± 1
+2.5 mM Carnosine	82 ± 6*	115 ± 5*
+1.0 mM Taurine	103 ± 5	98 ± 3
+KA (100 μM)	156 ± 8*	95 ± 1*
+1.0 mM Carnosine	126 ± 2*†	111 ± 3*†
+2.5 mM Carnosine	108 ± 6*†	116 ± 3*†
+1.0 mM Taurine	162 ± 11*	111 ± 4*†
+SIN-1 (100 μM)	262 ± 6*	83 ± 3*
+2.5 mM Carnosine	189 ± 17*†	103 ± 3†
+1.0 mM Taurine	283 ± 22*	88 ± 3*

*Indicates that the experimental value is significantly different from the control (with no additions) value; †indicates that the experimental value is significantly different from the respective control value with added KA or SIN-1.

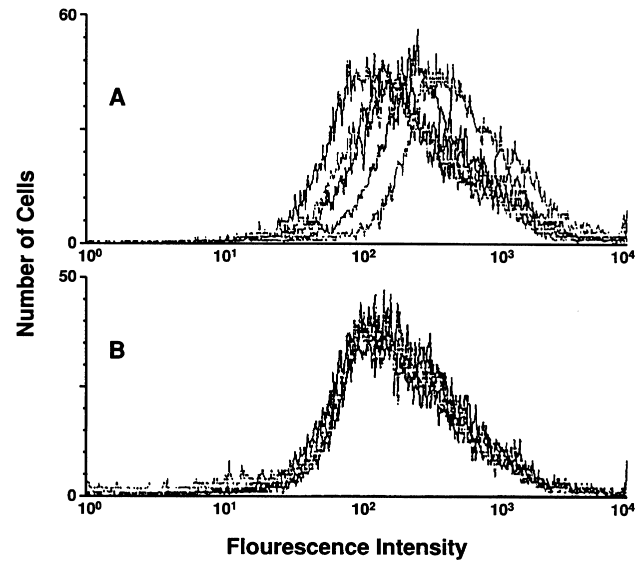

Fig. 1. The effects of different concentrations of KA on the DCF fluorescence of cerebellar granule cells. Graph A shows experiments performed in the presence of 1.2 mM Ca^{2+} ions, and graph B shows experiments performed in the absence of added Ca^{2+} ions and in the presence of 1 mM EGTA. The progressive right shifts of the DCF fluorescence peak (in arbitrary units) in graph A were achieved by the presence of 0, 100, 250, 500 and 1000 μM KA. Little shift of the DCF peak is seen in graph B where the same increasing concentrations of KA were used in the absence of Ca^{2+} ions.

Fig. 1 shows that a dose-dependent stimulation of neuronal free radical formation occurred as a result of KA exposure in the presence of 1.2 mM Ca^{2+} ions (Fig. 1A). However, as shown in Fig. 1B, free radical levels were not elevated in the presence of KA when Ca^{2+} was substituted by 1 mM ethylene glycol-bis[β-aminoethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). These observations suggest that ROS formation upon KA receptor activation is secondary to Ca^{2+} ion entry from extracellular sources.

In contrast, ROS generation following SIN-1 exposure was not reduced when calcium was absent from the incubation medium (Fig. 2), and it therefore appears that entry of Ca^{2+} ions from the external medium is not essential for the increases in ROS levels caused by SIN-1.

In order to directly measure intracellular Ca^{2+} ion con-

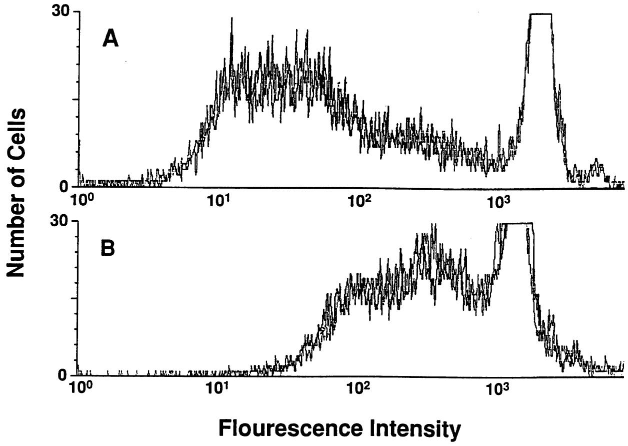

Fig. 2. The DCF fluorescence of cerebellar granule cells in the presence and absence of the free radical generator SIN-1. For each curve, the peak on the left represents DCF fluorescence and the peak on the right is PI fluorescence (fluorescence intensity in arbitrary units). Graph A shows experiments performed in the absence of SIN-1, and graph B in the presence of 100 μM SIN-1. Each graph is an overlay of analyses of cells in the presence of 1.2 mM Ca^{2+} and in the absence of Ca^{2+} ions with 1 mM EGTA present. The almost complete coincidence of the curves in each graph indicates that the position of the DCF peak in the presence and absence of SIN-1 is independent of Ca^{2+} ions.

centration after application of KA and SIN-1, experiments were performed using the Ca^{2+}-sensitive fluorophore, Fluo-3. When neurons were incubated in solutions containing 1.2 mM Ca^{2+}, application of KA resulted in a significant increase in Fluo-3 fluorescence to 138 ± 4% of the control value (100 ± 3%), whereas SIN-1 caused a significant decrease (down to 86 ± 3%) of the Fluo-3 fluorescence level of control cells.

Table 1 shows that the larger increase in ROS caused by SIN-1 also resulted in a larger decrease in cell viability in comparison to control and KA-treated cells. Such decreases in cell viability caused by KA and SIN-1 have previously been ascribed to delayed necrotic and apoptotic cell death, respectively [9,17] and are believed to be the result of increased free radical production and lipid peroxidation in nerve tissue and cells in the presence of these compounds [13,19].

The above Table 1 also shows that when carnosine was used to pre-treat neurons before stimulation of ROS generation by KA or SIN-1, DCF fluorescence was suppressed in each case in comparison to control preparations containing no carnosine, and that significant increases in cell viability occurred in the presence of carnosine. Taurine was also found to increase cell viability, but this compound did not affect DCF fluorescence in comparison to preparations treated only with KA or SIN-1.

The data obtained from the experiments on Ca^{2+} ion effects on intracellular ROS formation caused by KA or SIN-1 provide further support for the model of stimulation of glutamate receptors on the neuronal membrane by excitotoxins such as KA, which cause an increased cytoplasmic Ca^{2+} ion concentration and then trigger intracellular NO production and subsequent oxidative damage by ROS [1]. Calcium involvement in elevated ROS formation had previously been demonstrated by electrophysiological approaches [15], but the experiments performed in these studies, showing that the effect of KA is dependent on external Ca^{2+} ions, are the first direct biochemical demonstration of this requirement. Furthermore, the data in Fig. 2 demonstrate that direct production of intracellular NO from SIN-1 bypasses the Ca^{2+} ion requirement for NO production which would be needed in the case of excitotoxin signaling via glutamate receptors.

The studies with Fluo-3 show that, even in the presence of external Ca^{2+} ions, SIN-1 caused a significant decrease in intracellular Ca^{2+}-ion levels. It is possible that this could be the result of oxidative damage to the cell membrane caused by free radicals generated by SIN-1, with intracellular damage (see Table 1) being caused by the penetration of SIN-1 or its degradation products into the cytoplasm and nucleus. Current studies in our laboratories are designed to elucidate the details of the mechanisms by which SIN-1 can increase intracellular ROS levels, decrease intracellular Ca^{2+} levels and decrease cell viability in a Ca^{2+}-independent manner.

The protective effects of carnosine in decreasing ROS generation and increasing cell viability (Table 1) are in agreement with previous studies on the protective effect of carnosine on individual neurons in ischemic brain slices [3] and confirm that carnosine is an antioxidant which can enhance cell viability. Such effects by carnosine have been related to its protection of cell structure from oxidative damage [7] by peroxynitrite and ROS [9]. The data on carnosine protection also show that this compound is effective in preventing decreases in cell viability in the presence of both KA and SIN-1, and suggest that carnosine may be able to protect against necrotic and apoptotic pathways to cell death, as KA treatment stimulates apoptosis [17] whereas SIN-1 treatment causes necrosis [9] under the conditions used in our studies.

In contrast to carnosine, taurine did not significantly affect ROS levels in neurons in the presence or absence of KA or SIN-1. Although taurine also did not change cell viability in the absence of KA and SIN-1 and in the presence of SIN-1, it did cause a significant increase in cell viability in cells exposed to KA, in agreement with other studies which have reported attenuation of cell death by this compound [2]. It therefore appears that although both compounds can prevent damage to cell lipids, DNA and proteins [3,11,16], they may have different effects on ROS levels and cell viability as a result of their different effects on cell membrane stability and intracellular enzyme activities [4,11].

These studies with carnosine and taurine also add to the body of evidence which shows the absence of a consistent inverse correlation between intracellular ROS levels and cell viability in neurons, and indicate that ROS generation may not be the only cause of cell death [8]. Such previous studies have shown that ROS generation is not necessarily associated with neuronal cell death [2], and that neurons cannot always be protected against oxidative stress by antioxidants [5,6,12] or by inhibition of ROS-generating reactions [5]. Further studies will be needed to identify the differences between the processes of attenuation of neuronal cell death which are either correlated with an increase in ROS or are independent of ROS levels.

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