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PICROTOXIN-INDUCED CONVULSIONS AND LYSOSOMAL FUNCTION IN THE RAT BRAIN

Munjal M. Acharya, Surbhi H. Khamesra* and Surendra S. Katyare***

**Department of Biochemistry, Faculty of Science, M.S. University of Baroda, Vadodara 390002, Gujarat, India.*
***** Author for correspondence***

ABSTRACT

The effect of picrotoxin-induced convulsions on lysosomal function in rat brain were evaluated by measuring the free as well as total acid phosphatase, cathepsin D, acid ribonuclease (RNAse II) and acid deoxyribonuclease (DNAse II) activities. Following picrotoxin treatment the free RNAse II activity increased whereas the total activities of practically all the other enzymes decreased. Paradoxically, the cathepsin D activity, free as well the total was completely abolished. In case of all the enzymes the ratio of Total activity / Free activity decreased indicating increased lysosomal membrane fragility which could lead to process of neurodegeneration in the epileptic animals.

KEYWORDS

Picrotoxin, Convulsions, Epilepsy, Lysosomal function, Lysosomal enzymes

INTRODUCTION

Epilepsy, a chronic seizure disorder results due to uncontrolled hypersynchronous discharges from the glutamatergic system and leads to neuronal cell loss, e.g. loss of hippocampal pyramidal cells and of granular and hilar cells in dentate gyrus and neurodegeneration (1-3). While the former is believed to be the underlying cause for the lowered seizure threshold, neurodegeneration has been shown to be associated with memory impairment in animals as well as in humans (1-3). It has also been recognized that neurodegeneration results as a consequence of excitotoxicity (2, 3). In the light of the above, it may be anticipated that in the epileptic condition the cerebral function may be affected at the cellular and/or subcellular level.

Interestingly, it has been reported that in cobalt epilepsy the acid phosphatase activity in rat brain was significantly elevated (4). By contrast, in the skin biopsy samples and in the leukocytes from epileptic patients the arylsulfatase A activity decreased (5, 6). In kainate-evoked seizures, the levels of cathepsin D mRNA in rat brain were found to be elevated (7). Enhanced cathepsins activity had also been reported in progressive myoclonus epilepsy (8,9). These reports are thus the suggestive of the possible alterations in lysosomal functions in epilepsy. However, no in depth studies have been carried out to evaluate the lysosomal function in the epileptic condition. The chronic treatment with picrotoxin (PTX) is known to induce tonic-clonic type of major convulsions in the rats (10). Using this model, we examined the cerebral lysosomal function in comparison with the untreated controls. The studies included measurement of the activities of the marker enzymes viz. acid phosphatase, cathepsin D, acid ribonuclease (RNAse II) and acid deoxyribonuclease (DNAse II). The results of these studies are summarized here.

Dr. Munjal M. Acharya
Department of Biochemistry, Faculty of Science, M.S. University of Baroda, Vadodara 390002, Gujarat, India.
Tel.: (+91-265-2795594)
E-mail: manjulacharya@yahoo.co.in

MATERIALS AND METHODS

Picrotoxin (PTX), yeast RNA, calf thymus DNA and hemoglobin were purchased from Sigma Chemical Co. U.S.A. Sodium β glycerophosphate (βGP) was from Mallinkrodt, U.S.A. All other chemicals were of analytical-reagent grade and were purchased locally.

Animals and treatment with PTX

Male albino rats of Charles-Foster strain weighing between 200-250 g were used. The animals had free access to food and water. PTX solution was prepared fresh daily in saline. The animals were injected with PTX intra-peritoneally (i. p.) at the dose of 1.5 mg/kg body weight around 9 AM for 20 consecutive days (10). In the initial stages the animals developed seizures within 20-30 min of PTX administration and the tonic-clonic convulsions were well established within 8-10 days of treatment. In the later stages of the treatment

the seizures developed within 10-20 min of PTX administration. The animals were kept in individual cages and were observed for incidences, character and intensity of epileptic manifestations. The animals were scored according to the scale described by Kubova et al. (11) as indicated below:

0 No changes

1 Uneasiness, scratching tremor, single myoclonic jerks

2 Atypical minimal seizures

3 Minimal seizures consisting of clonic convulsions involving the head and forelimb muscles and leaving righting reflexes intact

4 Major seizures without the tonic phase

5 Complete major seizures i.e. generalized tonic-clonic convulsions with loss of righting reflexes.

The animals that reached the scale of 5 were used for further studies. The mortality rate was zero. The controls received equivalent volume of saline.

Experimental

The animals were killed by decapitation on day 21 of PTX treatment and the brains were quickly dissected out and placed in beakers containing chilled (0-4 °C) 0.25 M sucrose. 10% (w/v) tissue homogenates were prepared in 0.25 M sucrose using a Potter-Elvehjem type glass – Teflon homogenizer. The homogenate was subjected to centrifugation at 2000 rpm for 10 min at 4 °C in a Sorvall RC 5B plus centrifuge (SS 34 rotor) and the pellet containing nuclei and unbroken cell debris was discarded. The supernatant (S_1 fraction) was used within 15 min for the measurement of the 'free' activity. For the measurement of the 'total' activity the S_1 fraction was diluted (1:5) with tris-HCl buffer (10 mM, pH 7.4) containing 0.1% Triton-X-100 and subjected to three cycles of freezing and thawing.

Enzyme assays

Acid phosphatase activity was measured in the medium (total volume 0.6 ml) containing 0.1 M sodium acetate buffer, pH 5.0 (12). After pre-incubating 0.2 ml of the enzyme at 37 °C for 1-2 min the reaction was initiated by adding βGP at the final concentration of 15 mM. The reaction was allowed to proceed for 15 min and was terminated by the addition of 1 ml of 5% (w/v) trichloroacetic acid (TCA). The tubes were kept on ice for 30 min and then centrifuged at 2000 rpm for 10 min. The amount of liberated inorganic phosphorous in the supernatant was estimated by the method of Fiske and Subba Row (13).

Cathepsin D activity was measured in the medium (total volume 0.5 ml) containing 50 mM sodium acetate buffer, pH 3.8 and 0.25 M sucrose (12,14). After pre-incubating 0.05 ml of enzyme at 37 °C the reaction was initiated by adding 0.5 mg of hemoglobin. At the end of 10 min of incubation period the reaction was terminated by the addition of 2.5 ml of 5% (w/v) TCA. The tubes were kept on ice and the contents were filtered using a Whatman filter paper No. 1. The tyrosine positive materials in the filtrate were estimated by the method

Table 1. Effects of picrotoxin treatment on the lysosomal enzyme activities.

Enzyme	Treatment	Activity		Total activity
		Free	Total	Free activity
Acid phosphatase	Control	104.0 ± 3.0	339.0 ± 11.0	3.26 ± 0.06
	Epileptic	94.0 ± 5.0	220.0 ± 15.0**	2.34 ± 0.14**
Cathepsin D	Control	17.98 ± 1.2	96.53 ± 6.9	5.37 ± 0.38
	Epileptic	ND	ND	-
RNAse II	Control	31.0 ± 1.5	320.0 ± 14.0	10.39 ± 0.31
	Epileptic	70.0 ± 4.0**	190.0 ± 11.0**	2.77 ± 0.16**
DNAse II	Control	42.0 ± 1.3	520.0 ± 51.0	12.38 ± 0.81
	Epileptic	34.0 ± 2.0*	150.0 ± 17.0**	4.41 ± 0.38**

The experimental details are as described in the text. Results are given as mean ± S.E.M. of 16 independent observations in each group. Acid phosphatase activity is expressed as nmole P_i / 10min / mg protein and the Cathepsin D activity is given as mg tyrosine positive materials / 10min / mg protein. The nucleases (RNAse II and DNAse II) activity is expressed as nmole nucleotides / 10min / mg protein.
ND, not detectable
*, $p < 0.01$ and **, $p < 0.001$ compared with the corresponding control.

as described (15).

RNAse II activity was measured in the assay medium (total volume 0.2 ml) containing 0.15 M sodium acetate buffer, pH 5.0 (12). After pre-incubating 0.05 ml of enzyme for 1-2 min at 37 °C the reaction was initiated by the addition of 300 µg of RNA. The reaction was carried out for 30 min and was terminated by the addition of 2 ml of ice-cold 10% perchloric acid (PCA) containing 2.5 mg of uranyl acetate per ml. The tubes were kept on ice for one hr and then centrifuged at 2000 rpm for 10 min. The amount of nucleotides released in the supernatant was determined by measuring the absorption at 260 nm (15).

DNAse II activity was determined in the assay medium (total volume 0.2 ml) containing 0.15 M sodium acetate buffer, pH 5.0, and 0.15 M KCl (12). After pre-incubating the enzyme (0.05 ml) at 37 °C for 1-2 min the reaction was initiated by the addition of 150 µg of DNA. The reaction was allowed to proceed for 30 min and was terminated by the addition of 2 ml of ice-cold 10% PCA. The tubes were kept on ice for one hr and then centrifuged at 2000 rpm for 10 min. The amount of nucleotides released in the supernatant was determined by recording the absorbance 260 nm (15).

The ratio of Total activity / Free activity is taken as the index of lysosomal membrane integrity (12,14,15). Protein estimation was done according to the method of Lowry et al. with bovine serum albumin used as the standard (16). Statistical evaluation of the data was done by Students' t-test.

RESULTS

The present studies were initiated to examine if the cerebral lysosomal functions are affected in epileptic condition. As can be noted from data in Table 1 in the control group the free and total activities of the four enzymes and the ratio of Total activity / Free activity was in the expected range (12,14). The latter values were in the range of 3.3 to 12.4 depending on the enzyme under consideration.

As is evident, PTX treatment resulted in 2.3 fold increase in the free RNAse II activity, while the free DNAse II activity decreased by 20%. The total acid phosphatase, RNAse II and DNAse II activities decreased from 35 to 71%, with maximum effect being seen for DNAse II activity. The ratios of Total activity / Free activity were significantly lower suggesting that there is loss of lysosomal membrane integrity, which makes it more permeable following PTX treatment. The cathepsin D activity, both free and total was completely abolished.

Since the effect on cathepsin D was so strikingly different, we decided to find out if the observed changes occurred as a consequence of prolonged exposure to PTX or were the effects of PTX *per se*. To ascertain this possibility, the S_1 fraction was pre-incubated with PTX (1 to 1000 µM) prior to estimating the free and total cathepsin D activities. It was observed that the free and total activities were unaffected by added PTX thereby ruling out the direct effect of PTX on cathepsin D activity (data not shown).

DISCUSSION

The process of neurodegeneration in epilepsy as a consequence of excitotoxicity (2, 3) can result in the accumulation of the cell debris. One may hence anticipate that the lysosomal enzymes in general and cathepsin D in particular may play a role in the removal of the debris. Neurodegeneration and accumulation of lipofuchsin pigments in brains of cathepsin D deficient (CD-/-) mice has been reported (17). However, the mechanism of clearance of cell debris remains unclear.

Deficiency of lysosomal enzymes leading to pathological conditions has been reported by several investigators (5,6,17-23). The cathepsin D deficient (CD-/-) mice manifest seizures and become blind near the terminal stage i.e. around postnatal day 26 (17). Mutation in ovine cathepsin D gene causes congenital lysosomal storage disease with profound neurodegeneration (22). Earlier, Hetman et al. reported increased cathepsin D mRNA and increased cathepsin D immunoreactivity in the rat brain 3 days after kainate treatment (7). In contrast to this, we found that the cathepsin D activity was completely abolished after PTX treatment. However, it may be pointed out here that in their experiment Hetman et al. did not measure the enzyme activity; their estimates were restricted only to the measurement of mRNA and immunoreactivity as determined by histochemistry (7). It is possible that in their experiments the authors detected the cathepsin D peptide which had the immunoreactive epitope but not enzyme activity. In other words, despite the increased mRNA levels, either the enzymatically active protein was not synthesized and/or a truncated cathepsin D protein was synthesized. Complete loss of cathepsin D activity as we observe here possibly suggest that cathepsin D may not play any role in clearing of cell debris which originate due to excitotoxicity. Interestingly, reports reveal that cathepsins B, L and S activities are found to be enhanced in progressive myoclonus epilepsy (8,9).

Also, of interest to note in this context is the reported presence of proteases other than cathepsin D e.g. cathepsin A and calcium activated neutral protease (CANP) in the brain (23). These proteases act on neurofilament proteins in humans and mouse brain (23). Also, the elevation of cathepsin A and carboxypeptidase in neuropathological conditions has been reported (23). Neurodegeneration in epilepsy due to excitotoxicity is believed to result in increased

intracellular concentration of Ca^{2+}. This could possibly bring about the activation of CANPs. Taken together the results would thus suggest that these proteases (23) along with other cathepsins (8,9) rather than cathepsin D might play an important role in clearing the cell debris. However, this possibility needs to be verified by more direct experiments.

In the present studies, lysosomal dysfunction is noteworthy as reflected in terms of decreased ratios of Total activity / Free activity for acid phosphatase, DNAse II and RNAse II (Table 1). It may also be noted that the free RNAse II activity in the brain increased by 1.3 fold (Table1). Earlier we have noted similar increase in free RNAse II activity in paracetamol-induced hepatotoxicity (12). The increased free nuclease activities can result in indiscriminate degradation of nucleic acids thereby hampering the metabolic activity ultimately leading to necrosis or cell death.

In conclusion, the results of present studies indicate that the lysosomal dysfunction was manifested especially in terms of increased membrane permeability following prolonged exposure to PTX. This in turn could possibly lead to process of neurodegeneration.

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