GLUTAMATE INDUCED LESIONS TREATED BY SELECTIVE TARGETING ANTIOXIDANTS TO HIPPOCAMPAL AND CORTICAL NEURONS

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Abstract: Oxidative stress contributes significantly to neurodegenerative dis orders , and age related dysfunctions. To improve the functions of mitochondria we have produced targeted antioxidants by covalently attaching lipophilic triphenyl phosphonium cation to them. We tested targeted antioxidants e.g. glutathione and curcumin, on hippocampus and cortical regions of mice brain. Our results show that application of glutamate to brain subregions leads to the enhancement of lipid peroxidation as well as protein bound carbonyls, which was reversed by the application of antioxidants. Next, we found that the targeted synthetic derivatives of glutathione and curcumin were significantly more efficient in regulating oxidative stress as it was evident in lower lipid peroxidation and protein carbonyl content. We also found that these synthetic antioxidants get accumulated several hundred folds in the brain subregions compared to their non synthetic forms. Therefore, these results show that targeting of antioxidants could provide a effective method to reduce oxidative stress.

Key words: Triphenyl phosphonium cation; antioxidant; neurodegenerative disorders, Hippocampus.

Introduction: Oxidative stress is due to production of reactive oxygen species (ROS) which are free radicals and super oxide radicals that results in lipid per oxidation (LPO) of plasma membrane, DNA damage, etc. Malondialdehyde (MDA) is a recognized end product of such LPO [1] and is considered as a good indicator of oxidative stress. Glutathione in its reduced form (GSH) is an important antioxidant molecule that functions as a cofactor for a variety of enzymes and is involved primarily in protecting cells from oxidative damage. GSH after reacting with ROS become oxidized GSH (GSSG). Therefore, the concentrations of GSH and GSSG and their molar ratio can also be used for indicating the oxidative stress status. Abnormal calcium level is linked to abnormal neuronal firing and the abnormal propagation of action potentials [12] Such conditions are often seen during excitotoxicity. Excitotoxicity is a pathological state in which excitatory synapses are stimulated in an uncontrolled fashion. This may results from either excessive release of excitatory neurotransmitter i.e. glutamate or its inefficient clearing from the synaptic site [5]. Excitotoxicity often results in oxidative stress in the neurons which in turn leads to cell death. Hippocampus and cortical neurons are susceptible to damage caused by oxidative stress . As hippocampaus and neocortex are important brain regions involved in learning, memory and thinking, it is very important to develop therapies which would minimize such cell damage especially the oxidative damage.

Earlier studies have shown the antioxidant properties of curcumin, which protect cells by scavenging free radicals [11]. In this study, we hypothesized that if we can target these antioxidants directly to mitochondria, it would result in enhanced protective outcome in terms of lower LPO and cell damage as compared to their non-targeted forms. Here, we chemically synthesized targeted glutathione and curcumin derivatives by covalently attaching lipophilic triphenylphosphonium (TPP) cation. Our results are in agreement with our hypothesis.

Materials and methods: Normal healthy adult (3 months old) swiss albino mice (Mus musculus albinus) of both the sexes, were procured from institute of Animal Health and Veterinary Biologicals, Mhow, India. These mice were reared and maintained under an automated light/dark (12h:12h) cycle with the provision of standard chow and water available ad libitum. Twenty four animals were used (four animals per testing condition) in the present study. All animals were placed in standard size cage (size 30×20×12 cms). The bedding used for mice was sterile paddy husk, which was changed every week. All animals experimentation was performed in compliance with guidelines of ethical committee of Vikram University Ujjain MP (India).

Synthesis of targeted glutathione (TargetedGSH): Synthesis of targeted glutathione was performed as described [13]. Breifly, Reactions for the synthesis of targeted antioxidants were carried out under nitrogen atmosphere. To synthesize targeted derivative of glutathione, a solution of bromo-antioxidant (Glutathione; 2 gm, 6.50 mmol) was refluxed and evaporated the volatiles under vaccum below 100 oC. The compound obtained was hygroscopic, dissolved in methanol and precipitated after adding n-hexane. Filtered precipitate was again dissolved in methanol containing triphenylphosphine (0.80 gm, 3.00 mmol). After refluxing the solution, evaporated the volatiles under vaccum to obtain targeted derivative of glutathione.

Synthesis of targeted curcumin (Targeted Curcumin): Synthesis of mitochondrially targeted glutathione was performed as described [13] Breifly, reactions were carried out under a nitrogen atmosphere. To synthesize targeted derivative, a solution of bromo-antioxidant (curcumin; 2 gm, 5.42 mmol) was refluxed and evaporated the volatiles under vaccum below 1000C. The compound obtained was hygroscopic, dissolved in methanol and precipitated after adding n-hexane. Filtered precipitate was again dissolved in methanol containing triphenylphosphine (0.92 gm, 3.22 mmol). After refluxing the solution, evaporated the volatiles under vaccum to obtain targeted derivative of curcumin.

***Targeting antioxidants to homogenates of brain subregions*:** In our experiments, we tested five different conditions, whereas the sixth group served as control where only buffer without the drug was given. For the first treatment, we incubated crude homogenates of brain cells (0.3 to 0.5 mg protein/0.8 ml) with glutamic acid (200 μM) to induce oxidative stress.

Comparative efficacy of targeted antioxidants (glutathione and curcumin) with their untargeted derivatives was examined after incubating 100 μmoles of these antioxidants to brain homogenates of cerebral cortex and hippocampus(0.3 - 0.5 mg protein/0.8 ml). Effects of antioxidant were observed with their targeted derivatives using the same concentrations (i.e. 100 micromoles of each antioxidant) against glutamic acid (200 μM) neurotoxicity in an incubation medium [2mM phosphoric acid (pH 7.4), 15 mM Tris- Cl (pH 7.4) and 120 mM KCl] at 300C for 10-30 minutes. Group 1 served as control. Glutamic acid (200 μM) was added to reaction mixture of experimental group 2 after adding brain cell homogenates. The rection mixture of group 3 was divided into subgroups to add targeted and untargeted derivatives of glutathione (100μM), curcumin (100 μM) in incubation medium to observe neuroprotective effect of compounds. After incubation, samples were centrifuged at 5000 rpm for 10 minutes after adding 1 mM EDTA and 10 μm ortho-toluidine to terminate the reaction. The sample was assayed for lipid peroxidation (LPO), protein carbonyls (PC) and glutathione (GSH) contents. Protein estimation was done using Lowry method using BSA as a protein standard. [8]

Assay for lipid peroxidation: 0.1 ml sample was taken and mixed with 0.05 phosphate buffer (pH 7.7). This mixture was mixed with 0.2 ml SDS, 1.5 ml Acetic acid (pH 3.5) and 1.5 ml of thiobarbituric acid. The reaction mixture was heated at 95°C for one hour. Tubes were then cooled and mixed with 1 ml of distilled water and 5 ml of n-butanol and pyridine mix (15:1 v/v). The mixture was then centrifuged at 2200x*g* for 5 minutes. The amount of MDA formed was measured using molar extinction coefficient of 1.56×10^{5}/min/cm. The results were expressed in nmol MDA/mg protein[10].

Assay for protein carbonyl (PC) content: 0.5 ml sample was mixed with 2 ml of phosphate buffer {100 mM potassium dihydrogen phosphate disodium hydrogen phosphate (pH 7.4) and 1% digitonin}. Streptomycin sulphate (10%) was added to sample to eliminate nucleic acids. The samples were then kept at room temperature for 15 minutes and centrifuged at 4000 rpm for 10 minutes. Supernatent 0.8 ml was then divided equally into two tubes. 1.6 ml DNPH (10 mM in 2 M HCl) was added to one tube and 1.6 ml, HCl (2M) was added in another tube, left the tubes for one hour at RT. The proteins precipitated by adding an equal volume of TCA to the tubes and leave for 15 minutes. Spun down the protein at 4000 rpm for 10 minutes supernatent discarded and pellets were washed with 1.5 ml of ethyl acetate and ethanol (1:1) to remove excess DNPH. This procedure was repeated three times and final protein pellets were dissolved in 2.5 ml of 100 mM NaOH and absorbance of both solution, DNPH and HCl was measured at 370 nm from which carbonyl content was calculated and the protein concentration and the protein concentration was calculated from Δ 280 of HCl samples [7].

Assay for reduced glutathione (GSH): 0.5 ml sample was mixed with 1.5 ml of metaphosphoric acid (0.1 M Ice-cold) and centrifuged at 16,000 *g* for 15 min at 0°C. Supernatent was equilibrated with 2 ml precipitating reagent and 1 ml dithiobis (2-nitrobenzoic acid (DTNB reagent) and optical density was recorded at 412 nm in a Perkin-Elmer UV-Spectrophotometer [6].

Statistical Analysis: All data expressed as mean ± SE. Statistical comparisons were made relative to the appropriate control groups by student’s 't' test and analysis of variance. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results: The incubation of homogenates of brain subregions with glutamic acid caused a significant ($P<0.05$) increase in protein carbonyl content in hippocampus and cerebral cortex in comparison to control. The present study has examined comparative effects of targeted derivatives of glutathione, curcumin and their plain derivatives in attenuation of glutamic acid induced increased lipid peroxidation. Results showed that compound targeted curcumin decreased LPO activity more efficiently than targeted GSH. The result showed reduction in the oxidative damage in hippocampus and cerebral cortex marked by decline in lipid peroxidation. Tables 1and 4 .

Incubation of hippocampus and cerebral cortex with glutamic acid (200 μM) caused a significant ($P<0.05$) increase in protein carbonyl content in comparison to control. The present study has examined comparative effects of targeted antioxidants in attenuation of glutamic acid induced increased protein carbonyl content.Protein carbonyl content showed significant ($P<0.05$) decline in hippocampus and cerebral cortex incubated with targeted derivatives of glutathione, curcumin in comparison to their plain derivatives as against, glutamate induced brain cells. However, targeted derivative of both the compound glutathione and curcumin was found to be more effective in reducing the protein carbonyl content in brain subregions (Tables 2, 5).

GSH provides a major defense against Reactive oxygen species (ROS) induced cell damage. GSH is utilized so as to serve the antioxidative protective function. Incubation with glutamic acid caused a considerable decline in the GSH content in hippocampus and cerebral cortex. Significant ($P<0.05$) reduction in GSH seems to enhance the risk of oxidative stress. Under these circumstances the compounds which scavenge ROS could be beneficial. In this study targeted antioxidants have been shown to scavenge O_2 marked by an increase in GSH content. However, comparative effects showed that targeted derivative antioxidants are more effective; suggesting higher scavenging activity of targeted compounds. Thus present study has examined the effects of mitochondrially targeted antioxidants in ameliorating the toxic effects of glutamic acid. (Tables 3, 6).

Table :1 Lipid peroxidation (LPO; nmoles MDA mg-1 protein) in cerebral cortex of swiss albino mice after incubation with glutamate (200μM) and targeted and untargeted derivatives of antioxidants (100μM).

Treatments	Nmoles MDA / mg protein
Control	0.010 ± 0.002
Glutamate	0.283 ± 0.007 *
Glutamate + GSH	0.150 ± 0.005
Glutamate + Targeted GSH	0.024 ± 0.002 #
Glutamate + Curcumin	0.069 ± 0.003 #
Glutamate + Targeted curcumin	0.014 ± 0.001 #

Data expressed in terms of mean ± S.E. * p<0.05, compared to control.
# p< 0.05, Compared to glutamate treated group.

Table :2 Protein carbonyl content (PC ; nmoles mg-1 protein) in cerebral cortex of swiss albino mice after incubation with glutamate (200μm) and targeted and untargeted derivatives of antioxidants (100μm)

Treatments	Protein carbonyl nmoles/mg protein
Control	4.92 ± 0.02
Glutamate	15.42 ± 0.09 *
Glutamate + GSH	14.39 ± 0.03 *
Glutamate + TargetedGSH	7.20 ± 0.02 #
Glutamate + Curcumin	9.68 ± 0.06 #
Glutamate + Targeted Curcumin	5.29 ± 0.03 #

Data expressed in terms of mean ± S.E. * p<0.05, Compared to control.
# p< 0.05, Compared to glutamate treated group.

Table :3 Reduced glutathione (GSH ; mg gm-1 wet wt.) in cerebral cortex of swiss albino mice after incubation with glutamate (200μM) and targeted and untargeted derivatives of antioxidants (100μM).

Treatments	**GSH mg/gm wet wt.**
Control	0.050 ± 0.006
Glutamate	0.025 ± 0.002 *
Glutamate + GSH	0.030 ± 0.003
Glutamate + TargetedGSH	0.035 ± 0.003 #
Glutamate + Curcumin	0.035 ± 0.004 #
Glutamate + Targeted curcumin	0.037 ± 0.005 #

Data expressed in terms of mean ± S.E. * $p<0.05$, Compared to control.
# $p< 0.05$, Compared to glutamate treated group.

Table :4 Lipid peroxidation (LPO ; nmoles MDA mg-1 protein) in hippocampus of swiss albino mice after incubation with glutamate (200μm) and targeted and untargeted derivatives of antioxidants (100μm).

Treatments	**Nmoles MDA / mg protein**
Control	0.892 ± 0.005
Glutamate	17.11 ± 0.020 *
Glutamate + GSH	8.220 ± 0.012 #
Glutamate + Targeted GSH	3.120 ± 0.009 #
Glutamate + Curcumin	1.950 ± 0.003 #
Glutamate + Targeted curcumin	1.040 ± 0.005 #

Data expressed in terms of mean ± S.E. * $p<0.05$, compared to control.
# $p< 0.05$, Compared to glutamate treated group.

Table :5 Protein carbonyl content (PC ; nmoles PC mg-1 protein) in hippocampus of swiss albino mice after incubation with glutamate (200μm) and targeted and untargeted derivatives of antioxidants (100μm)

Treatments	**Protein carbonyl nmoles/mg protein**
Control	20.48 ± 2.41
Glutamate	43.62 ± 3.09 *
Glutamate + GSH	40.01 ± 2.88 *
Glutamate + Targeted GSH	32.20 ± 2.73 #
Glutamate + Curcumin	30.24 ± 2.59 #
Glutamate + Targeted curcumin	25.12 ± 2.42 #

Data expressed in terms of mean ± S.E. * $p<0.05$, compared to control.
# $p< 0.05$, Compared to glutamate treated group.

Table :6 Reduced glutathione (GSH ; mg gm-1 wet wt.) in hippocampus of swiss albino mice after incubation with glutamate (200μm) and targeted and untargeted derivatives of antioxidants (100μm).	
Treatments	**GSH mg/gm wet wt.**
Control	0.047 ± 0.004
Glutamate	0.018 ± 0.001 *
Glutamate + GSH	0.020 ± 0.003 #
Glutamate + Targeted GSH	0.030 ± 0.003 #
Glutamate + Curcumin	0.024 ± 0.002 #
Glutamate + Targeted curcumin	0.032 ± 0.003 #

Data expressed in terms of mean ± S.E. * $p<0.05$, compared to control.
# $p< 0.05$, Compared to glutamate treated group.

Discussion: In the present study, cerebral cortex and hippocampus were selected to evaluate the effect of targeted antioxidant therapy on attenuation of free radical induced oxidative damage. Progressive increase in the steady state level of molecular oxidative damage has been postulated to be a major causal factor in senesence-associated loss of cellular functional capacity. The hippocampus and cortical region are known to be vulnerable to a variety of insults, including age associated disease, cerebrovascular insufficiency and environmental toxins which results in impairment of cognition, memory or motor function. These regions of the brain also undergo significant morphological damage as a function of normal aging, and they contain subsystem which is thought to be critical determinant of age related cognitive and motor dys function[1].

Lipid peroxidation is an oxidative process in which molecules undergo a series of chemical alterations initiated by free radicals and oxygen. In this process, molecules pass through a series of complex multistage reactions. For lipid peroxidation to occur, activation of lipid molecules and pro-oxidants are both necessary. Lipid peroxidation may occur under two different settings (i) Initiation by metal oxygen interaction and (ii) Initiation by a hydroxyl radical derived from superoxide on H_2O_2 through metal catalysis. Lipid peroxidation is one of the best known mani festation of oxidative cell injury. Increase in the protein carbonyl group in different brain parts have been reported in motor cortex of sporadic ALS patients [2], in the substantia nigra[3,7], the region regarded as the most vulnerable for oxidative damage in Aging and age related diseases [9,14], the region contributed to memory function. The results of the present study show the oxidative stress caused by glutamate in hippocampus and cerebral cortex associated with an increased level of protein oxidative damage, as indicated by increase in the concentration of protein carbonyls and loss of protein sulphydryl. Both, hippocampus and cerebral cortex exhibited significant increase in protein carbonyl content. The absolute increase in carbonyl suggests that these regions of brain may be most vulnerable to putative free radical induced oxidations of proteins. The increase in LPO and PC and decrease in GSH content observed in the present study of such magnitude are likely to have marked deleterious effects on the functional potential of the tissues.

On the basis of these data and available literature, the present study hypothesizes that ROS created by glutamic acid is an important inducer of oxidizing atmosphere in the hippocampal and cortical neurons, characterized by an increased lipid peroxidation and protein carbonyl and depletion of reduced glutathione. The results showed effectiveness of ROS scavenger, GSH in ameliorating the toxic effects of ROS and managing the neurons to resume normal reducing atmosphere. The targeted antioxidants and their plain derivatives incubated with hippocampus and cerebral cortex attenuated the ROS induced damage in vitro, both LPO and PC were declined while, GSH was elevated. This shows that antioxidants were effective in reducing oxidative burden in hippocampal and cortical neurons. These and other studies using antioxidants or radical scavengers to attenuate biochemical deficits reveal that free-radicals and oxidative stress play a major role in pathogenesis of neurodegenerative diseases.

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