



ELSEVIER

Biochimica et Biophysica Acta 1453 (1999) 407–411



Rapid report

In vivo modulation of rodent glutathione and its role in peroxynitrite-induced neocortical synaptosomal membrane protein damage

Tanuja Koppal^a, Jennifer Drake^a, D. Allan Butterfield^{a,b,*}

^a Department of Chemistry and Center of Membrane Sciences, 409 Kinkead Hall, University of Kentucky, Lexington, KY 40506-0055, USA

^b Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA

Received 28 December 1998; accepted 1 February 1999

Abstract

Peroxynitrite, formed by the reaction between nitric oxide and superoxide, leads to the oxidation of proteins, lipids, and DNA, and nitrates thiols such as cysteine and glutathione, and amino acids like tyrosine. Previous *in vitro* studies have shown glutathione to be an efficient scavenger of peroxynitrite, protecting synaptosomal membranes from protein oxidation, the enzyme glutamine synthetase from inactivation, and preventing the death of hippocampal neurons in culture. The current study was undertaken to see if *in vivo* modulation of glutathione levels would affect brain cortical synaptosomal membrane proteins and their subsequent reaction with peroxynitrite. Glutathione levels were depleted, *in vivo*, by injecting animals with 2-cyclohexen-1-one (CHX, 100 mg/kg body weight), and levels of glutathione were enhanced by injecting animals with *N*-acetylcysteine (NAC, 200 mg/kg body weight), which gets metabolized to cysteine, a precursor of glutathione. Changes in membrane protein conformation and structure in synaptosomes subsequently isolated from these animals were examined using electron paramagnetic resonance, before and after *in vitro* addition of peroxynitrite. The animals injected with the glutathione depletant CHX showed greater damage to the membrane proteins both before and after peroxynitrite treatment, compared to the non-injected controls. The membrane proteins from animals injected with NAC were comparable to controls before peroxynitrite treatment and were partially protected against peroxynitrite-induced damage. This study showed that modulation of endogenous glutathione levels can affect the degree of peroxynitrite-induced brain membrane damage and may have potential therapeutic significance for oxidative stress-associated neurodegenerative disorders. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Peroxynitrite; Glutathione; Oxidative stress; *N*-Acetylcysteine

Peroxynitrite (ONOO⁻) is the cytotoxic product of the reaction between nitric oxide and superoxide anion, and this anion is known to lead to the oxidation of several cellular components, including proteins, lipids, and DNA, and result in the nitration

of many amino acids, mainly tyrosine [1]. Peroxynitrite is also known to inactivate key mitochondrial enzymes and affect the cellular energy status resulting in apoptosis (for a review, see [1]). The protonated form of peroxynitrite, peroxynitrous acid (ONOOH), is known to be a highly reactive oxidant species (reviewed in [2]). But whether it is the active intermediate of ONOOH (ONOOH*) with a hydroxyl radical-like reactivity or the hydroxyl radical itself that is

* Corresponding author. Fax: +1 (606) 257-5876;
E-mail: dabens@pop.uky.edu

formed by the decomposition of ONOOH that is responsible for the deleterious effects of peroxynitrite is still questionable.

Consistent with the notion of peroxynitrite-induced oxidative stress, previous studies showed that peroxynitrite caused conformational and structural changes in brain cortical synaptosomal membrane proteins and led to increased protein carbonyl levels [3]. Peroxynitrite also led to inactivation of the highly oxidation-sensitive enzyme glutamine synthetase (GS), and caused cell death in hippocampal neuronal cultures [3]. In all these *in vitro* experiments when the cellular antioxidant glutathione (GSH) was added to the system prior to the addition of peroxynitrite, significant protection was observed against the peroxynitrite-induced damage [3]. Glutathione is a tripeptide (Glu-Cys-Gly) and a known scavenger of peroxynitrite [2]. Peroxynitrite was shown to nitrate glutathione and such *S*-nitrosothiol species have been found in ALS patients [4] and in rat cerebellar extracts [5].

The goal of the current study was to assess the susceptibility of synaptosomal membrane proteins to peroxynitrite-mediated oxidative stress by modulating the endogenous levels of glutathione *in vivo* and studying the protein damage to subsequently isolated cortical synaptosomes. Intracellular levels of glutathione were reduced employing *i.p.* injections of 2-cyclohexen-1-one (CHX), while the levels of intracellular glutathione were increased using *N*-acetylcysteine (NAC).

Peroxynitrite was synthesized using sodium azide and ozone as described earlier [6]. Before each experiment the solution containing peroxynitrite was thawed on ice and the absorbance measured at 302 nm to determine the concentration of peroxynitrite present. Male Mongolian gerbils, 3–5 months of age, were used. The animals were injected *i.p.* with 100 mg/kg body weight CHX for the glutathione depletion studies, and 200 mg/kg body weight NAC for glutathione enhancement studies. These doses were based on previous studies [7–9]. The animals given CHX were killed 1 h later, and those given NAC were killed 3 h after injection, by decapitation, and the brains quickly dissected on ice. The times chosen for the study were based on time response studies conducted (data not shown),

The isolation of synaptosomes by ultracentrifuga-

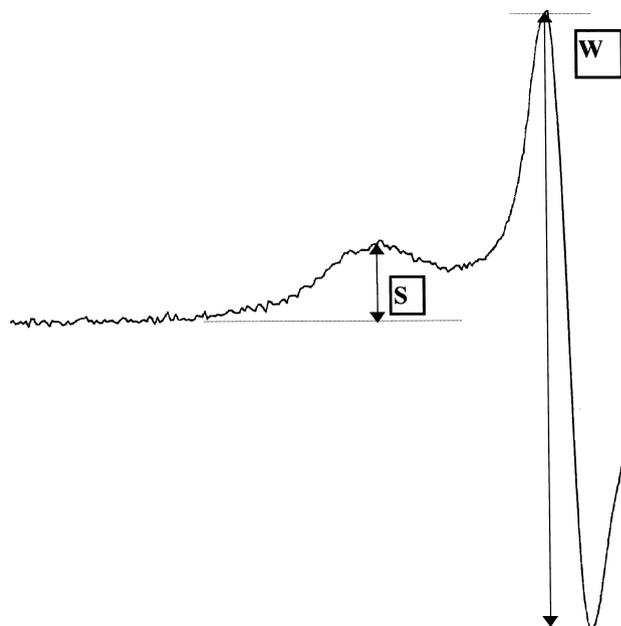


Fig. 1. Schematic of the low field region of the EPR spectrum showing the W and S components of the MAL-6-labeled synaptosomal membrane proteins.

tion of the homogenized cortices was performed as described earlier [10]. Purified synaptosomes obtained at the 1.18 M/1.10 M sucrose gradient interface were washed three times with ~30 ml lysing buffer (10 mM HEPES, 2 mM EDTA and 2 mM EGTA in deionized water, pH 7.4) containing 100 μ M diethylenetriaminepentaacetic acid (DTPA), a chelator for iron. After the three washes the synaptosome membranes were resuspended in ~1 ml lysing buffer and the protein concentration of each homogenate was adjusted to 4 mg/ml. The protein aliquots were centrifuged at 14000 rpm for 4 min. The protein pellets obtained were then treated with 250 μ M peroxynitrite, in lysing buffer, for 10 min and the washed homogenates were spin-labeled with the protein-specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and the electron paramagnetic resonance (EPR) spectra obtained by methods described previously [3,10]. The data were analyzed for statistical significance using Dunnett's test for one-way ANOVA followed by Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant for comparison between data sets.

Proteins are generally non-paramagnetic species and hence the thiol-specific spin label MAL-6 is

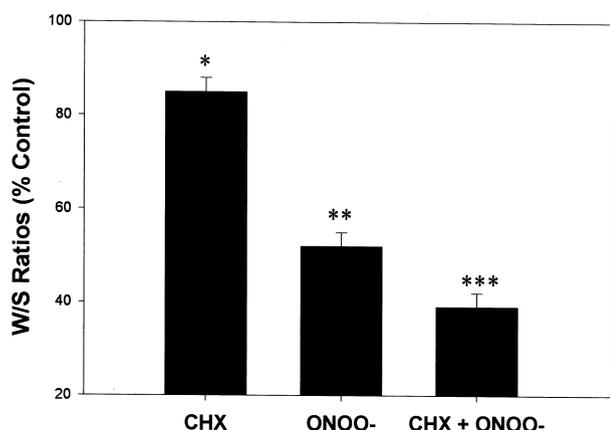


Fig. 2. Synaptosomal membranes were isolated from control animals and animals injected i.p. with CHX (100 mg/kg body weight) animals. The CHX-treated animals were decapitated 1 h after injection and synaptosomal membranes were isolated from brain cortices. W/S ratios of membrane proteins isolated from CHX-injected animals were statistically different from the W/S ratios of control animals ($*P < 0.01$). 250 μ M peroxy-nitrite added in vitro to membranes isolated from control animals showed lower W/S ratios, compared to the untreated membranes ($**P < 0.001$). Membranes isolated from CHX-treated animals and treated with peroxy-nitrite for 10 min showed an even greater decrease in W/S ratios compared to both control untreated membrane proteins ($***P < 0.001$) and control proteins treated with peroxy-nitrite ($P < 0.02$). $N = 6$ was used for each group under study.

used to study the protein micro-environment by EPR. MAL-6 covalently labels sulfhydryl groups on proteins located on the protein surface or in deep pockets. The former group of -SH sites leads to spin label motion that is weakly immobilized, while reaction of MAL-6 with the latter group of -SH sites leads to spin label motion that is strongly hindered [11]. Accordingly, the EPR spectrum of MAL-6-labeled proteins in synaptosomal membranes has a weakly (W) and strongly (S) immobilized component (Fig. 1). The ratio of the signal amplitudes of these two components of the $M_I = +1$ low field region of the EPR spectrum, the W/S ratio, is extremely sensitive to alterations in the protein environment [11]. A decrease in the W/S ratio results from increased protein-protein interactions, increased protein cross-linking, and changes in protein conformation [11]. Previous models of oxidative stress studied in our laboratory, such as hydroxyl radical generation [10], hyperoxia [12], ischemia/reperfusion [7], accelerated aging [13], and amyloid- β -induced damage

[14,15], have all shown decreased W/S ratios of spin-labeled synaptosomes with increasing oxidative stress.

Confirming the results of our previous study [3] and consistent with oxidative stress, synaptosomal membranes isolated from control animals and treated with 250 μ M peroxy-nitrite for 10 min showed $\sim 50\%$ decrease in W/S ratios compared to the untreated control membranes ($P < 0.001$, Fig. 2). The membranes isolated from the CHX-injected animals showed W/S ratios lowered to about 85% compared to those isolated from control animals ($P < 0.01$, Fig. 2), confirming data previously obtained [7]. In contrast, there was essentially no difference between the W/S ratios of the NAC-injected animals (102%) and control animals, $P < 0.2$ (Fig. 3).

Synaptosomal membranes isolated from the CHX-injected animals, when treated with 250 μ M peroxy-nitrite for 10 min, showed W/S ratios lowered to 39% compared to the untreated control samples ($P < 0.001$, Fig. 2). This mean decrease in W/S ratio was statistically significant and $\sim 11\%$ lower than the mean decrease observed when the control samples were treated with peroxy-nitrite alone ($P < 0.02$), suggesting that the membrane proteins isolated from

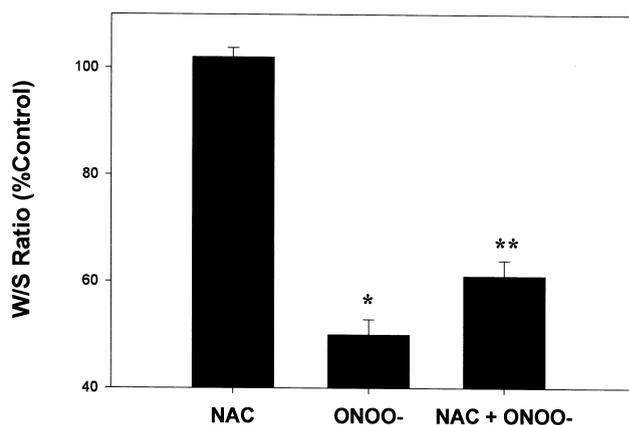


Fig. 3. Synaptosomal membranes were isolated from control animals and NAC-treated (200 mg/kg body weight) animals. Synaptosomal membranes were isolated from the animals 3 h after injection. W/S ratios of membrane proteins isolated from controls and NAC-injected animals were not statistically different ($P > 0.2$). 250 μ M peroxy-nitrite added in vitro to membranes isolated from NAC-treated animals for 10 min showed a significant decrease in W/S ratios from untreated control values ($*P < 0.001$) but was significantly higher than the values obtained with peroxy-nitrite-treated control membranes ($P < 0.01$). $N = 7$ was used for each group in the study.

CHX-injected animals were more vulnerable to damage mediated by peroxynitrite. In contrast, the decrease observed in W/S ratios of membrane proteins isolated from NAC-injected animals and treated with peroxynitrite was about 61% ($P < 0.001$), which is ~11% higher than the W/S ratios of membranes from control animals treated with peroxynitrite ($P < 0.01$, Fig. 3), implying a lesser extent of protein damage. Thus, increased glutathione levels from NAC injections offered partial protection against peroxynitrite-induced oxidative stress.

This study showed that injecting the animals with CHX caused a decrease in W/S ratios of MAL-6 covalently bound to cortical synaptosomal membrane proteins isolated from such animals. A previous study also showed significant changes in the physical state of membrane proteins after administration of CHX alone and CHX followed by ischemia/reperfusion in gerbils [7]. This result thus suggested that diminution of glutathione levels alone leads to increased oxidative stress. In contrast, animals injected with NAC showed no change in W/S ratios of membrane proteins compared to control animals, which implied no change in protein interactions and conformation or oxidative stress status.

CHX and NAC are well documented to be glutathione-depleting and glutathione-enhancing agents, respectively [7–9,16,17]. Studies have shown CHX to be very effective in lowering the amounts of reduced glutathione (GSH) because of its ability to cross the blood-brain barrier and conjugate with glutathione present in the brain [7,18,19]. On the other hand, NAC acts as a glutathione agonist by metabolizing to form cysteine, a precursor for the biosynthesis of glutathione. NAC is also known to scavenge radicals like hydroxyl radicals, and superoxide and react with reactive oxygen species such as H_2O_2 and HOCl [20]. Glutathione is one of the most effective non-enzymatic, cytosolic antioxidants and is present in high concentrations in the cell (millimolar range). Glutathione mainly exists in the reduced form (GSH), which is converted to its oxidized form (GSSG) to maintain the reduced state of protein thiols. Thus, the ratio of GSSG/GSH is an important marker of oxidative stress. The role of GSH in oxidative stress-mediated neuronal death in neurodegenerative diseases is discussed in detail in the review by Bains et al. [21]. Glutathione scavenges oxygen

radicals like hydroxyl radicals and singlet oxygen, and also acts as a substrate for the antioxidant enzyme glutathione peroxidase; the latter helps convert hydrogen peroxide to water. Glutathione peroxidase is also known to reduce peroxynitrite [22].

This study showed that *in vivo* depletion of glutathione by systemic injection of CHX alone can lower W/S ratios of membrane proteins. If glutathione levels are low the thiol groups on membrane proteins can be oxidized to form disulfide bonds that are not easily reduced back to the free sulfhydryl groups. Disulfide bonds can lead to increased protein cross-linking, thereby decreasing protein segmental motion, increasing steric hinderance, and lowering the W/S ratios of MAL-6-labeled membranes. *In vitro* addition of peroxynitrite to the glutathione-depleted membranes can further exacerbate these effects, since both the current investigation and our earlier study [3] showed that 250 μM peroxynitrite by itself can increase protein-protein interactions, following oxidative stress. Also, with lowered glutathione levels peroxynitrite cannot be scavenged efficiently.

Administering NAC alone to the animals protected against autooxidation of the membrane proteins, and treatment of synaptosomal membranes isolated from NAC-treated animals with peroxynitrite caused a significant increase in the W/S ratios compared to the peroxynitrite-treated control animals. However, the protection offered was only partial. These results showed that administering NAC intraperitoneally was not the most effective route for delivering the drug. NAC delivered by some other route could conceivably metabolize faster to cysteine, the precursor for the synthesis of glutathione.

The current study reiterated that glutathione is an extremely important cellular antioxidant that can work against peroxynitrite-induced damage to membrane proteins. Parkinson's disease patients known to have low glutathione and glutathione peroxidase levels are already being put on glutathione therapy [23,24]. There is increased evidence for oxidative stress in Alzheimer's disease brain [14,25], such as protein oxidation, assessed by EPR and other means [26–28]. Also, amyloid- β peptide is known to stimulate iNOS, which can lead to increased peroxynitrite formation [29]. Hence, the current results are consistent with the concept that increasing endogenous levels of glutathione in Alzheimer's disease patients con-

ceivably could become a promising therapeutic strategy.

This work was supported in part by grants from NIH (AG-05119, AG-10836). We are grateful to Dr. Dibakar Bhattacharyya, Department of Chemical and Materials Engineering, University of Kentucky, for the use of his ozonator for the synthesis of peroxynitrite.

References

- [1] J.S. Beckman, *Chem. Res. Toxicol.* 9 (1996) 836–844.
- [2] W.A. Pryor, G.L. Squadrito, *Am. J. Physiol.* 268 (1995) L699–722.
- [3] T. Koppal, J. Drake, S. Yatin, B. Jordan, S. Varadarajan, L. Bettenhausen, D.A. Butterfield, *J. Neurochem.* 72 (1999) 310–317.
- [4] R. White, T. Brock, L. Chang, J. Crapo, P. Briscoe, D. Ku, W. Bradley, S. Gianturco, J. Gore, B.A. Freeman, M.M. Tarpey, *Proc. Natl. Acad. Sci. USA* 91 (1994) 1044–1048.
- [5] I. Kluge, U. Gutteck-Amsler, M. Zollinger, K. Quang Do, *J. Neurochem.* 69 (1997) 2599–2607.
- [6] W.A. Pryor, R. Cueto, X. Jin, W.H. Koppenol, M. Ngu-Schwemlein, G.L. Squadrito, P.L. Uppu, R.M. Uppu, *Free Radical Biol. Med.* 18 (1995) 75–83.
- [7] N.C. Hall, J.M. Carney, O.J. Plante, M. Cheng, D.A. Butterfield, *Neuroscience* 77 (1997) 283–290.
- [8] R. Testa, M. Ghia, F. Mattioli, S. Borzone, S. Cagliaris, E. Mereto, E. Giannini, D. Risso, *Fund. Clin. Pharmacol.* 12 (1998) 220–224.
- [9] X.D. Wang, X.M. Deng, P. Haraldsen, R. Andersson, I. Ihse, *Scand. J. Gastroenterol.* 30 (1995) 1129–1136.
- [10] K. Hensley, N. Hall, W. Shaw, J.M. Carney, D.A. Butterfield, *Free Radical Biol. Med.* 17 (1994) 321–331.
- [11] D.A. Butterfield, *Biol. Magn. Reson.* 4 (1982) 1–78.
- [12] B.J. Howard, S. Yatin, K. Hensley, K.L. Allen, J.P. Kelly, J.M. Carney, D.A. Butterfield, *J. Neurochem.* 67 (1996) 2045–2050.
- [13] D.A. Butterfield, B.J. Howard, S. Yatin, K.L. Allen, J.M. Carney, *Proc. Natl. Acad. Sci. USA* 94 (1997) 674–678.
- [14] D.A. Butterfield, *Chem. Res. Toxicol.* 10 (1997) 495–506.
- [15] R. Subramaniam, T. Koppal, M. Green, S. Yatin, J. Drake, D.A. Butterfield, *Neurochem. Res.* 23 (1998) 1403–1410.
- [16] G. Benzi, O. Pastoris, A. Gorini, F. Marzatico, R.F. Villa, D. Curti, *Neurobiol. Aging* 12 (1991) 227–231.
- [17] K. Pahan, F.G. Sheikh, A.M.S. Namboodiri, I. Singh, *Free Radical Biol. Med.* 24 (1998) 39–48.
- [18] J. Martensson, A. Jain, E. Stole, W. Frayer, P. Auld, Meister, *Proc. Natl. Acad. Sci. USA* 88 (1991) 9360–9364.
- [19] T. Masukawa, M. Sai, Y. Tochino, *Life Sci.* 44 (1989) 417–424.
- [20] O.I. Aruoma, B. Halliwell, B.M. Hoey, J. Butler, *Free Radical Biol. Med.* 6 (1989) 593–597.
- [21] J.S. Bains, C.A. Shaw, *Brain Res. Rev.* 25 (1997) 335–358.
- [22] R. Radi, *Chem. Res. Toxicol.* 11 (1998) 70–721.
- [23] T.L. Perry, D.V. Godin, S. Hansen, *Neurosci. Lett.* 33 (1982) 305–310.
- [24] S.J. Kish, C. Morito, O. Hornykiewicz, *Neurosci. Lett.* 58 (1985) 343–346.
- [25] W.R. Markesbery, *Free Radical Biol. Med.* 23 (1997) 134–147.
- [26] K. Hensley, N. Hall, R. Subramaniam, P. Cole, M. Harris, M. Akesenov, M. Aksenova, P. Gabbita, J.F. Wu, J.M. Carney, M. Lovell, W.R. Markesbery, D.A. Butterfield, *J. Neurochem.* 65 (1995) 2146–2156.
- [27] C.D. Smith, J.M. Carney, P.E. Starke-Reed, C.N. Oliver, E.R. Stadtman, R.A. Floyd, W.R. Markesbery, *Proc. Natl. Acad. Sci. USA* 88 (1991) 10540–10543.
- [28] M.A. Smith, G. Perry, P.L. Richey, L.M. Sayre, V.E. Anderson, M.F. Beal, M. Kowall, *Nature* 382 (1996) 120–121.
- [29] K.T. Akama, C. Albanese, R.G. Pestell, L. Van Eldik, *Proc. Natl. Acad. Sci. USA* 95 (1998) 5795–5800.