Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound \( N \)-\( \text{tert} \)-butyl-\( \alpha \)-phenylnitrone

J. M. Carney*, P. E. Starke-Reed†, C. N. Oliver‡, R. W. Landum*, M. S. Cheng*, J. F. Wu*, and R. A. Floyd§

*Department of Pharmacology, Chandler Medical Center, University of Kentucky, Lexington, KY 40536; †Department of Experimental Medicine, College of Medicine, George Washington University, Washington, DC 20037; ‡Merck & Co., Rahway, NJ 07065; and §Molecular Toxicology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

Communicated by Earl R. Stadtman, January 29, 1991

ABSTRACT Oxygen free radicals and oxidative events have been implicated as playing a role in bringing about the changes in cellular function that occur during aging. Brain readily undergoes oxidative damage, so it is important to determine if aging-induced changes in brain may be associated with oxidative events. Previously we demonstrated that brain damage caused by an ischemia/reperfusion insult involved oxidative events. In addition, pretreatment with the spin-trapping compound \( N \)-\( \text{tert} \)-butyl-\( \alpha \)-phenylnitrone (PBN) diminished the increase in oxidized protein and the loss of glutamine synthetase (GS) activity that accompanied ischemia/reperfusion injury in brain. We report here that aged gerbils had a significantly higher level of oxidized protein as assessed by carbonyl residues and decreased GS and neutral protease activities as compared to young adult gerbils. We also found that chronic treatment with the spin-trapping compound PBN caused a decrease in the level of oxidized protein and an increase in both GS and neutral protease activity in aged Mongolian gerbil brain. In contrast to aged gerbils, PBN treatment of young adult gerbils had no significant effect on brain oxidized protein content or GS activity. Male gerbils, young adults (3 months of age) and retired breeders (15–18 months of age), were treated with PBN for 14 days with twice daily dosages of 32 mg/kg. If PBN administration was ceased after 2 weeks, the significantly decreased level of oxidized protein and increased GS and neutral protease activities in old gerbils changed in a monotonous fashion back to the levels observed in aged gerbils prior to PBN administration. We also report that old gerbils make more errors than young animals and that old gerbils treated with PBN made fewer errors in a radial arm maze test for temporal and spatial memory than the untreated aged controls. These data can be interpreted to indicate that oxidation of cellular proteins may be a critical determinant of brain function. Moreover, it also implies that there is an age-related increase in vulnerability of tissue to oxidation that can be modified by free radical trapping compounds.

Oxygen free radicals have been implicated as an etiological agent in the process of aging (1). Aging in brain is of particular interest in this regard. It is becoming clear that brain is particularly vulnerable to oxidative damage. This may be because brain contains relatively high concentrations of easily peroxidizable fatty acids (2). In addition it is known that certain regions of brain are highly enriched in iron, a metal that is catalytically involved in the production of damaging oxygen free radical species (3). Also, brain is not particularly enriched with protective antioxidant enzymes or antioxidant compounds (4); however, ascorbate is found in relatively high levels in brain (5). Ascorbate may contribute to oxidative damage because if tissue organizational disruption occurs it will act in combination with iron to catalyze peroxidative damage to brain homogenate (6, 7).

Key metabolic enzymes are inactivated by oxygen free radicals produced either by mixed-function oxidation systems or by nonenzymatic methods (8). It has been demonstrated that ischemia/reperfusion injury to brain results in a tissue-dependent inactivation of a highly oxidatively sensitive enzyme, glutamine synthetase (GS), and this is also accomplished by a general increase in the level of protein oxidation (9). Ischemia/reperfusion injury in the gerbil brain is accompanied by the generation of hydroxyl free radicals as detected by salicylate hydroxylation (10). It has been shown that there is an increase in the amount of oxidized protein in cultured fibroblasts as the age of the donor increases (11). These combined observations suggest that oxidative inactivation of enzymes and the intracellular accumulation of oxidized proteins and other cellular components may play a critical role in the changes in cellular function and cell loss as aging occurs. Therefore, it is possible that reducing the rate or extent of cellular oxidation may diminish or retard the process of aging. We have conducted an initial evaluation of the effects of daily administration of a free-radical spin trapping compound, \( N \)-\( \text{tert} \)-butyl-\( \alpha \)-phenylnitrone (PBN), on cellular protein oxidation, GS and neutral protease activity, and the amount of errors committed in a radial arm maze test. Young adult and retired breeder gerbils were treated with PBN for 14 days and evaluated for changes in both biochemical and behavioral endpoints.

MATERIALS AND METHODS

Animals. Male Mongolian gerbils were obtained from Tumblebrook Farms (West Brookfield, MA). Adult (3 months of age) and retired (15–18 months of age) male gerbils were purchased and acclimated to the University of Kentucky Medical Center facility for a minimum of 14 days prior to experimentation. They were fed standard Rodent Laboratory Chow (Purina) ad libitum in the home cage. Gerbils were injected (i.p.) with either saline or PBN (dissolved in saline) for 14 days prior to sacrifice. Animals were sacrificed by decapitation. Brains were rapidly removed and frozen in liquid nitrogen. Samples were stored at –70°C until analyzed.

Preparation of Extracts. Each brain cortex sample was divided into two equal portions. One set of the samples was

Abbreviations: GS, glutamine synthetase; PBN, \( N \)-\( \text{tert} \)-butyl-\( \alpha \)-phenylnitrone; DNPH, 2,4-dinitrophenylhydrazine.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. §1734 solely to indicate this fact.
menced and resuspended in 10 mM Hepes buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH2PO4, and 0.6 mM MgSO4. These samples were used for the protease activity determinations. The other matching samples, used for protein oxidation determinations, were treated the same except that the buffer also included the protease inhibitors leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), apronin (0.5 μg/ml), and phenylmethylsulfonyl fluoride (40 μg/ml), as described (9), to prevent proteolysis of oxidized proteins during preparation of crude extracts. All samples were then processed as described (9).

Assays. The protein concentration of the soluble protein fraction was determined by the Pierce BCA method (12). The protein carbonyl content was determined spectrophotometrically by using the 2,4-dinitrophenyldiazine (DNPH)-labeling procedure as described (13). The protein hydrazones derivatives were sequentially extracted with 10% (wt/vol) trichloroacetic acid, treated with ethanol/ethyl acetate, 1:1 (vol/vol), and reextracted with 10% trichloroacetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride, and the difference spectrum of the sample treated with DNPH in HCl was determined versus the sample treated with HCl alone. Results are expressed as nmole of DNPH incorporated per milligram of protein calculated from an extinction coefficient of 21.0 mM−1cm−1 for aliphatic hydrazones (14). GS activity was determined by the method of Rowe et al. (15) as modified by Miller et al. (16). The assays were corrected for nonspecific glutaminase activity by comparing the activity in the presence and absence of ADP and arsenate. For all assays, the results are reported as the mean of triplicate determinations from three gerbils at each dosing interval. Alkaline protease activity was determined according to the fluorescancemethod described by Böhlen et al. (17) as modified by Rivett (18). An alkaline protease is any protease that exhibits proteolytic activity at alkaline pH. Protease assays were conducted at pH 8.0.

Behavioral Testing. Gerbils were tested for temporal and spatial memory using an eight-arm radial maze (19). Experimentally naive gerbils were tested for their efficiency in patrolling behavior. Gerbils were placed into the central (start) chamber and then allowed to explore the maze. Errors were defined as reentry of previously entered arms of the maze. Both the time to explore all eight arms of the maze and the number of errors prior to exploring all arms of the maze were recorded for each subject. The observer was blind to the treatment groups.

Chemicals. DNPH was obtained from Eastman Kodak. Leupeptin, pepstatin, apronin, and phenylmethylsulfonyl fluoride were purchased from Boehringer Mannheim. L-Glutamine and ATP were obtained from Sigma. All other reagents were of the highest available grade. PBN was obtained from Aldrich. PBN was dissolved in saline and stored in amber bottles at 4°C in the dark.

Statistics. Data are presented as the mean ± SE for protein oxidation and enzyme activity. The significance level of treatment effects and the reversal of PBN effects were determined using analysis of variance and post-hoc analysis. A P < 0.05 was considered significant.

RESULTS AND DISCUSSION

Oxidative damage to proteins is accompanied by an increase in the number of carbonyl residues on the protein, which react with DNPH to form stable hydrazones derivatives (14). Fig. 1 shows that protein carbonyl content of brain from retired breeders was significantly higher (185% of control) than that of young adult animals. In addition, there was a significant decrease in GS activity (65% of control) in retired breeders, compared to young adults. Similarly, alkaline protease activity was markedly reduced (33% of control) in retired breeders. Neutral proteases function to degrade oxidized proteins in cells (20). The observation of an age-related decrease in protease activity is consistent with the observed increase in oxidized cytosolic protein. It is possible that the increase in intracellular oxidized protein is due to concomitant oxidative inactivation of the neutral proteases. A decrease in protease activity would allow an accumulation of other oxidized proteins.

Chronic treatment with PBN, at 32 mg/kg (which is the threshold dose for protection against ischemia/reperfusion brain injury), administered twice daily for 14 days, resulted in a decrease in the level of oxidized cytosolic proteins and an increase in GS activity in retired breeder gerbils, com-

![Fig. 1. Age-related changes of the protein carbonyl content, GS activity, and neutral protease activity in brain of Mongolian gerbils. The 100% level indicates the values observed in young male adult gerbils, and the values presented in the histogram represent values from male retired breeder gerbils. Each bar represents the mean of three to six individual brain samples.](image1)

![Fig. 2. Effects of daily treatment of PBN on protein carbonyl content and GS activity of brain from male retired breeder gerbils. The asterisk and dashed line for both protein carbonyl and GS activity represent the control (c) value for retired breeder gerbils given saline for 14 days. They were age matched to the retired breeders treated with PBN. Young adult gerbil brains had a carbonyl content of 4.3 nmol/mg of protein and a GS activity of 2.35 units/mg of protein. Samples were taken at various times after starting the PBN treatment. Each bar represents the mean ± SE for three brains. PBN was given at 32 mg/kg twice daily.](image2)
pared to young adult gerbils (Fig. 2). The demonstration of a decrease in protein oxidation is consistent with the hypothesis that PBN interferes with oxidative events involved in the formation of oxidized protein, which is higher as animal age increases, thus implicating an increase in oxidative stress with age. The origin of this oxidative stress has yet to be defined. However, the possibility exists that there is a change in the metabolism of oxygen or changes in the compartmentalization of iron. In contrast to the changes observed in retired breeders, chronic treatment of young adult gerbils with PBN did not result in a significant change in either protein oxidation or GS activity. These results can be interpreted to suggest that the equilibrium between oxidative events that mediate oxidative damage to proteins and the catalytic removal by neutral proteases is such that in younger animals PBN does not interfere, but in older animals the equilibrium is altered such that the level of oxidized proteins is decreased. We do not know the exact mechanism(s) of PBN action in this regard, but it is possible, since it does spin trap (i.e., quench free radicals), that it reduces the flux of free radicals, which mediates protein oxidation. Another interpretation is that there is a compartmentalized process of posttranslational oxidation of proteins that is undergoing differently control as aging occurs. This idea is supported by the observation that the change in protein oxidation that occurs in aging can be decreased to the level of young adult gerbils, but not further. When PBN administration ceased, both the amount of oxidized protein and enzyme activities increased in a monotonous fashion back to the levels observed prior to the start of PBN dosing (Fig. 3). The data clearly infer that the PBN effect is reversible. The reversal of protein oxidation levels after cessation of PBN administration suggests that the original equilibrium is reestablished in a gradual time-dependent fashion and/or that the differentially compartmentalized oxidized protein level is increased back to normal levels.

Chronic administration of PBN in retired breeders resulted in a decrease in the number of errors in patrolling the radial arm maze (Fig. 4), compared to those in saline treated (age-matched) controls. Improvement in short-term memory using this testing procedure has been demonstrated for compounds that enhance cholinergic function (21). The data reported have shown that PBN decreases the level of intracellular oxidized protein. This effect of PBN supports the hypothesis that central nervous system dysfunction in aging and related conditions is in part due to the accumulation of oxidized proteins and other oxidation products. Further, the results of the present series of studies suggest that reduction in the level of intracellular oxidized protein may result in cognitive improvement.

Spin-trapping compounds have been used to identify subcellular sites of radical production (22). The demonstration of inherent differences in the oxidation of protein in young adult and retired breeders offers the possibility of utilizing spin-trapping compounds to identify the subcellular origin of free radical production in aging. To date, to our knowledge there has not been a similar demonstration of the origin of free radical production in aging.