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Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain

C. N. OLIVER*, P. E. STARKE-REED*, E. R. STADTMAN*, G. J. LIU†, J. M. CARNEY‡, AND R. A. FLOYD†

*Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; ‡Department of Pharmacology, University of Kentucky School of Medicine, Lexington, KY 40536; and †Molecular Toxicology Research Group, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

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ABSTRACT **Free radical-mediated oxidative damage has been implicated in tissue injury resulting from ischemia/reperfusion events. Global cortical ischemia/reperfusion injury to Mongolian gerbil brains was produced by transient occlusion of both common carotid arteries. Protein oxidation, as measured by protein carbonyl content, increased significantly during the reperfusion phase that followed 10 min of ischemia. The activity of glutamine synthetase, an enzyme known to be inactivated by metal-catalyzed oxidation reactions, decreased to 65% of control levels after 2 hr of reperfusion that followed 10 min of ischemia. We also report that the free radical spin trap *N-tert*-butyl-α-phenylnitrone [300 mg/kg (body weight)] administered 60 min before ischemia/reperfusion is initiated, partially prevents protein oxidation and protects from loss of glutamine synthetase activity. In addition, we report a *N-tert*-butyl-α-phenylnitrone-dependent nitroxide radical obtained in the lipid fraction of the ischemia/reperfusion-lesioned brains, but there was very little radical present in the comparable sham-operated control brains. These data strengthen the previous observation utilizing *in vivo*-trapping methods, that free radical flux is increased during the reperfusion phase of the ischemia-lesioned gerbil brain. The loss of glutamine synthetase would be expected to increase the levels of brain L-glutamate. Thus, the oxidative inactivation of glutamine synthetase may be a critical factor in the neurotoxicity produced after cerebral ischemia/reperfusion injury.**

It is well known from the work of McCord and his coworkers (1) that ischemia/reperfusion injury has been linked to the generation of free radicals and yet only recently have more direct methods involving free radical trapping provided clear evidence that this may be the case (2, 3). In biological systems, conditions leading to increased availability of cellular iron plus hydroxyl radical formation are most frequently implicated in free radical-mediated tissue damage (4).

It is now clear that glutamine synthetase (GS, L-glutamate ammonia lyase, E.C. 6.3.1.2) and other key metabolic enzymes are oxidatively inactivated by metal-catalyzed oxidation reactions involving the reduction of Fe(III) and the production of H_2O_2 (5–7). Considerable evidence indicates that the Fe(II) binds to a divalent cation site on the enzyme, interacts with H_2O_2, and produces an as yet unidentified activated oxygen species causing the oxidation of one or more nearby amino acid residue (5–7). For *Escherichia coli* GS, oxidative inactivation involves the oxidation of His-269 (8), Arg-344 (I. Climent and R. L. Levine, personal communication), or both, both of which overlie one of the metal binding sites of the enzyme. Peptide mapping and sequence analysis have established the site-specific location of the oxidized residues (ref. 8; I. Climent and R. L. Levine, personal communication) and x-ray crystallographic studies have confirmed their proximity to a metal binding site on the enzyme (9).

Oxidative inactivation renders GS and other enzymes highly susceptible to proteolysis by proteases such as trypsin, *Streptococcus griseus* protease (10), and subtilisin (11) as well as by a class of cytosolic proteases that selectively degrade the oxidized proteins *in vitro* and *in vivo* (12–16). We have proposed that oxidative modification of proteins is a marking step for selective proteolysis (17). Additional evidence suggests that this process is important in a variety of normal and pathological processes, such as aging (18, 19), neutrophil function (20), rheumatoid arthritis (21), age-related changes in lens (22), and oxygen toxicity (19).

GS is a key enzyme in the regulation of amino acid metabolism as well as brain L-glutamate and γ-aminobutyric acid levels. Because ischemia/reperfusion injury is thought to be associated with the generation of free radicals and because GS is a pivotal enzyme in brain metabolism, we have investigated the possibility that GS is oxidatively inactivated in brain after ischemia/reperfusion injury. In this report we show that protein oxidation is significantly increased and GS specific activity is lost during reperfusion of ischemia-lesioned gerbil brain. There is a high correlation between these parameters as a function of reperfusion time after an ischemic insult. Finally, time-course studies indicate that oxidative modification of proteins and loss of enzyme activity is an early intracellular indicator of tissue damage in this model.

MATERIALS AND METHODS

Animals. Mature male Mongolian gerbils were obtained from Tumblebrook Farms (West Brookfield, MA). In pentobarbital-anesthetized animals, a loop of dental floss was placed around each common carotid artery and the free ends were passed through a double-lumen catheter. The catheter was fixed in the dorsum of the neck and glued in place with cyanoacrylate adhesive. Ischemia was induced by closing the carotid artery against the septum of the double-lumen catheter using the loop of dental floss. Release of tension and removal of the dental floss allowed reperfusion to occur. Ischemia/reperfusion was induced as described (3). Reperfusion was allowed for specific times before decapitation. The whole brain was removed, dissected on a cold stage, and then submerged in liquid nitrogen. The brain stem, which is not supplied by the common carotid, was used as an internal control. All tissue was frozen at −75°C until analyzed.

Preparation of Extracts. Brain cortex was minced and resuspended in 10 mM Hepes (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, and 0.6 mM $MgSO_4$.

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Abbreviations: GS, glutamine synthetase; PBN, *N-tert*-butyl-α-phenylnitrone; DNPH, 2,4-dinitrophenylhydrazine.

The buffer also contained the following protease inhibitors to prevent proteolysis of oxidized proteins during preparation of crude extracts: leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), phenylmethylsulfonyl fluoride (40 μg/ml), aprotinin (0.5 μg/ml), and 1.1 mM EDTA. The cells were disrupted by sonication using a Vibra cell sonicator (two 10-sec bursts; Sonics and Materials, Danbury, CT) at an output setting of 20–30. The insoluble cellular debris was removed by centrifugation of 1.0- to 1.5-ml samples at 100,000 × *g* for 5 min in a Beckman TL-100 refrigerated ultracentrifuge. Under these conditions there is little or no nucleic acid or lipid in the extract preparations. The clear supernatant fluid was recovered and used for determination of oxidized protein and GS specific activity.

Assays. The protein concentration of the soluble protein fraction was determined by the Pierce BCA method (23). The protein carbonyl content was determined spectrophotometrically using the 2,4-dinitrophenylhydrazine (DNPH)-labeling procedure as described (18). The protein hydrazone 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 nmol of DNPH incorporated per mg of protein calculated from an absorbtivity of 21.0 $mM^{-1} \cdot cm^{-1}$ for aliphatic hydrazones (24). GS activity was determined by the method of Rowe *et al.* (25) as modified by Miller *et al.* (26). The assays were corrected for nonspecific glutaminase activity by comparing the activity in the presence and absence of ADP and arsenate. At pH 7.5, the nonspecific glutaminase activity did not change with different conditions of ischemia/reperfusion injury. For all assays the results are reported as the mean of duplicate determinations from four to six gerbil brains for each condition. *N-tert*-Butyl-α-phenylnitrone (PBN) was made up fresh to 1.5 M in 0.9% NaCl, kept in the dark, and administered at 50 mg/kg at 1 hr prior to the ischemia treatment.

Spin Trapping. The cortex was homogenized at 4°C in 0.5% NaCl (5 g/ml) and the homogenate was then extracted with 20 vol of chloroform/methanol, 2:1 (vol/vol). The crude extract was washed thoroughly with 0.5% NaCl, 5:1 (vol/vol), and the mixture was allowed to separate into two phases by standing at 4°C for 2 hr. The organic phase was recovered and bubbled with nitrogen to concentrate the sample. In most cases the organic phase was evaporated to dryness and the sample then redissolved in chloroform. The samples were transferred to a Pasteur pipette (sealed at the capillary end) and bubbled with nitrogen for 5 min. The pipette was then placed in the sample cavity of an IBM Bruker ESP300 EPR spectrophotometer and scanned for the presence of spin adducts. The spectrometer settings were as follows: microwave power, 19.8 mW; modulation amplitude, 0.975 G; time constant, 1310.72 ms; scan range, 100 G; and scan time, 6 min. All spectra were recorded at room temperature.

Chemicals. DNPH was obtained from Eastman. Leupeptin, pepstatin, aprotinin, 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. Solutions of PBN were dissolved in saline on the day of testing. The stocks were prepared in amber bottles shielded from light and used only once.

RESULTS AND DISCUSSION

Oxidative inactivation of enzymes and oxidative modification of proteins by metal-catalyzed oxidation reactions are accompanied by the generation of protein carbonyl derivatives that can react with DNPH to form protein hydrazone derivatives (28). By using this property, we determined the protein carbonyl content of the soluble fraction of crude brain cortical extract preparations from gerbils subjected to various conditions of ischemia/reperfusion. GS activity was determined in the same preparations. The results show (Fig. 1) that protein carbonyl content increased and GS activity decreased in brains of animals subjected to ischemia/reperfusion treatment compared to sham-operated controls. Protein carbonyl content increased progressively as a function of reperfusion time exhibiting a maximum 2-fold change at 2 hr. After 2 hr the levels of oxidized proteins decreased significantly but still remained slightly higher than controls even after 24 hr of reperfusion. GS activity in the same samples declined to minimum of 65% of the GS level in sham-operated control animals after 2 hr of reperfusion. In the 2- to 3-hr interval of reperfusion, there was a decrease in the protein carbonyl content that was not accompanied by an increase in GS activity. Between 3 and 24 hr of reperfusion, GS activity returned to initial levels. We also have found that administration of PBN at 300 mg/kg (body weight) 60 min prior to ischemia partially prevented the ischemia/reperfusion-mediated loss of GS activity and the increase in protein carbonyl content. Moreover, in contrast to cerebral cortex, brain stem showed no loss of GS activity or increase in protein carbonyl content during the reperfusion period (Fig. 2). Because significant changes were observed within 60 min of reperfusion (Fig. 1), it is likely that protein oxidation and loss of enzyme activity is an early intracellular indicator of tissue damage. The data in Fig. 3 show that when GS specific activity is plotted versus protein carbonyl content in the same samples, a predictable relationship exists with a correlation coefficient of −0.9048, indicating that these parameters are closely linked.

Fig. 4 shows the results of the spin-trapping experiments. The spectra were obtained on five animals, two sham-operated control animals, and three animals subjected to ischemia/reperfusion treatment. The results clearly show that more free radicals (11.6-fold higher) were trapped in the

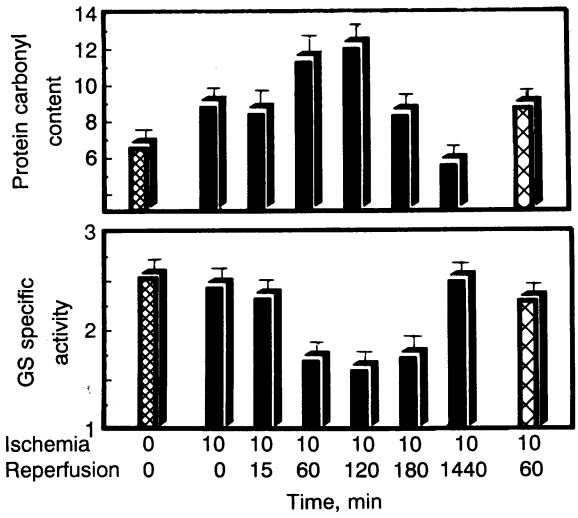

FIG. 1. Changes in protein carbonyl content and GS activity after ischemia/reperfusion injury in gerbil cortex. Soluble protein fractions were prepared from brains of gerbils subjected to various conditions of ischemia reperfusion. Protein carbonyl content (DNPH, nmol/mg of protein) and GS activity (units/mg of protein) were determined. The results are presented as the means of duplicate determinations for each experimental time point. Close-cross-hatched bars, sham-operated control; solid bars, after ischemia for 10 min, brains were reperfused for various times as indicated; loosely cross-hatched bars, PBN (300 mg/kg of body weight) was administered prior to ischemia.

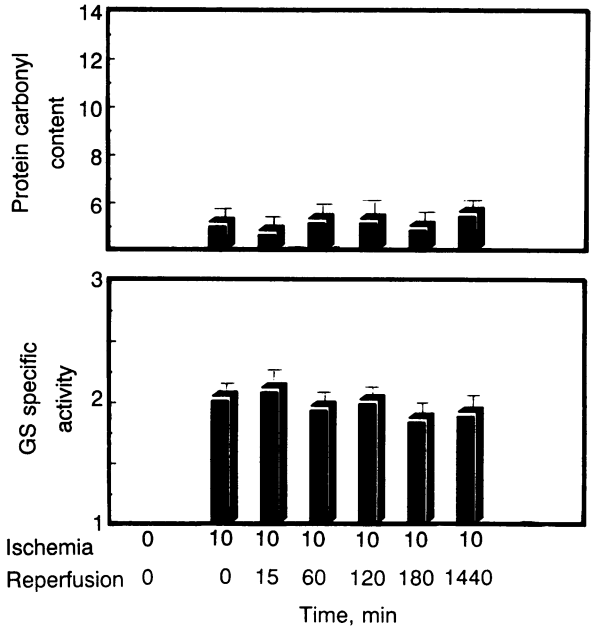

FIG. 2. Protein carbonyl and GS activity after ischemia/reperfusion injury in gerbil brain stem. Soluble protein fractions were prepared from dissected brain stem of gerbils subjected to various conditions of ischemia/reperfusion injury. Protein carbonyl content and GS activity were determined as described for Fig. 1.

ischemia/reperfusion-treated animals than in the control animals. The signals obtained were dependent upon the animals receiving the spin trap PBN. The signals obtained are triplets in contrast to the six-line spectra normally expected for spin-trapped free radicals. Attempts to spectrally separate out fine structure in the triplet signals failed. The nitrogen coupling constants (15.92 G) obtained are very similar to that of di-*tert*-butyl nitroxide, but the use of thin layer chromatography and high pressure liquid chromatography have shown that the trapped free radical(s) are not di-*tert*-butyl nitroxide. One possible explanation is that PBN spin traps one carbon-centered free radical forming a spin adduct that is oxidized to form a nitrone and then in turn traps another carbon-centered free radical to form a nitroxide. This would account for a nitroxide lacking a proton proximal to the nitroxide group.

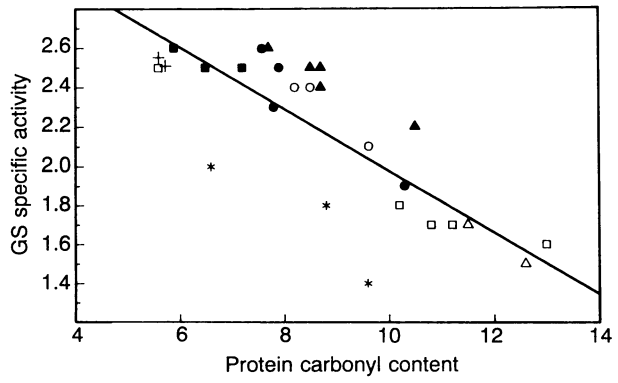

FIG. 3. Correlation of protein carbonyl content and GS activity after ischemia/reperfusion injury. Protein carbonyl content (DNPH, nmol/mg of protein) was plotted versus GS specific activity (units/mg of protein) in the same samples. The calculated correlation coefficient was determined to be $r = -0.9048$. ■, Control, 10 min of ischemia/0 min of reperfusion; ●, 10 min of ischemia/15 min of reperfusion; □, 10 min of ischemia/60 min of reperfusion; △, 10 min of ischemia/120 min of reperfusion; *, 10 min of ischemia/180 min of reperfusion; +, 10 min of ischemia/24 hr of reperfusion.

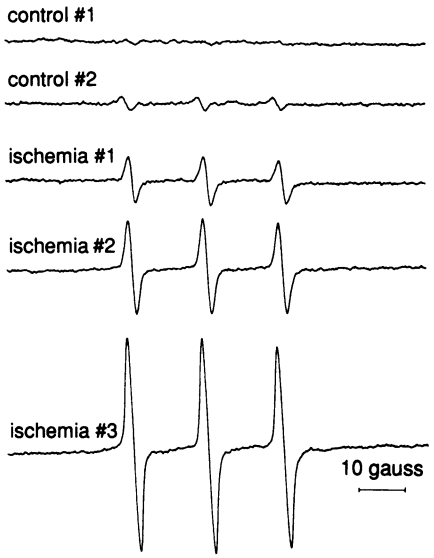

FIG. 4. EPR spectra of the lipid extract of the cortical homogenates from two sham-operated control and three ischemia/reperfusion-treated animals, which had received the spin-trap PBN prior to treatment.

It is noteworthy that these results closely parallel the time course of 2,5-dihydroxybenzoic acid formation in salicylate-pretreated gerbils. The formation of 2,5-dihydroxybenzoic acid increased in gerbil brains as a function of increased duration of ischemia/reperfusion period (3). These data are also consistent with previous studies (J.M.C., unpublished observations) that demonstrated that the rate of brain homogenate peroxidation at 37°C increased with increasing time of reperfusion after an ischemic insult. In the absence of reperfusion the rate of peroxidation was less than control homogenates.

The data clearly show that pretreatment of animals with PBN partially prevented protein oxidation and loss of GS activity. We have demonstrated (J.M.C., unpublished observations) that PBN pretreatment at 300 mg/kg (body weight) at 1 hr prior to ischemia (15 min) followed by reperfusion for 24 hr significantly reduced lethality. Thus, these data suggest that oxygen free radicals are generated in response to various conditions of ischemia/reperfusion in brain and that free radical-mediated reactions lead to protein oxidation and oxidative inactivation of enzymes during the initial reperfusion period.

After 2 hr of reperfusion, there is a decrease of protein carbonyl content without a concomitant recovery of GS activity. These results suggest that oxidized proteins including GS may be selectively degraded. Similar results were obtained in hepatocytes from rats subjected to 100% normobaric oxygen in the interval between 48 and 54 hr of exposure (18). In this case, a decrease in the level of oxidized protein coincided with the induction or activation of proteases that selectively degrade the oxidized proteins (19). In the ischemia/reperfusion experiments, there is an increase in GS activity to control levels when reperfusion is extended for 24 hr suggesting that recovery occurs in this time period and may be associated with new enzyme synthesis.

The data presented here indicate that intracellular protein oxidation and loss of GS activity are closely linked. These changes may be early intracellular indicators of tissue damage resulting from reperfusion after an ischemic insult. These results do not exclude the possibility that damage to other macromolecules also occurs. However, the data suggest that oxidative inactivation of enzymes could lead to disruption of cellular metabolism and seriously impair the capacity of the cell to repair this damage. GS is not only a key metabolic

enzyme in amino acid metabolism but it also has a pivotal role with glutamate decarboxylase and glutaminase in the regulation of L-glutamate and γ-aminobutyric acid levels in brain. In ischemia/reperfusion injury, the oxidative inactivation of GS could have serious metabolic consequences with respect to a variety of metabolic pathways but the loss of glutamate decarboxylase even in the absence of altered glutaminase activity could lead to the accumulation of L-glutamate with resulting glutamate neurotoxicity. Thus, L-glutamate-mediated neurotoxicity could occur without any damage after ischemia to glutaminergic neurons.

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