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EFFECT OF 2-CYCLOHEXENE-1-ONE-INDUCED GLUTATHIONE DIMINUTION ON ISCHEMIA/REPERFUSION-INDUCED ALTERATIONS IN THE PHYSICAL STATE OF BRAIN SYNAPTOSOMAL MEMBRANE PROTEINS AND LIPIDS

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Abstract—Glutathione is able to protect membrane proteins from oxidative stress. In ischemia/reperfusion injury, free radicals cause synaptosomal membrane protein and lipid oxidation that is prevented by the free radical scavenger *N*-tert-butyl-α-phenylnitrone (Hall N. C. *et al.* (1995) *Neuroscience* **64,** 81–89; **69,** 591–600). We wondered if diminution of glutathione would lead to further membrane alterations. Accordingly, the effects of glutathione depletion, by intraperitoneal administration of 2-cyclohexene-1-one, on the physical state of cortical synaptosomal membrane proteins and lipids, with and without global ischemia/reperfusion, were studied *in vivo* and *in vitro* in adult and aged gerbils utilizing electron paramagnetic resonance spectrometry. 2-Cyclohexene-1-one (100 mg/kg, i.p.) was administered 30 min prior to 10-min ischemia followed by 1 or 14 h reperfusion. This glutathione reduction agent was also administered to gerbils under the same temporal schedule in the absence of ischemia and compared to untreated controls. Synaptosomal membranes were labeled with a protein-specific spin label, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl, or a lipid-specific spin probe, 5-doxylstearic acid. There were no significant changes in the physical state of the lipid portion of synaptosomal membranes when comparing ischemia reperfusion and 2-cyclohexene-1-one-treated ischemia reperfusion in either the adult or aged gerbils. However, glutathione depletion without ischemia/reperfusion caused significant changes in the physical state of the protein portion of cortical synaptosomal membranes in both the adult and aged models. Glutathione depletion, without ischemia/reperfusion, in the adult model showed a maximum change at 3 h that returned to control values by 14 h. In contrast, the aged model showed significant changes at 1 h reperfusion, which did not return to control values by 14 h reperfusion. Glutathione depletion combined with ischemia/reperfusion caused initial protein change in both adult and aged models at 1 h reperfusion, which did not return toward control values by 14 h reperfusion.

The results of this study suggest that glutathione depletion increases the severity of membrane protein damage associated with ischemia/reperfusion injury. Copyright © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: ischemia/reperfusion, glutathione, gerbil, electron paramagnetic resonance, 2-cyclohexene-1-one, spin label.

There is considerable evidence suggesting that free radicals are produced by ischemia/reperfusion injury.[7,13,17,30,31] These free radicals have been proposed to initiate neuronal damage,[18,19,22,31,39] including alterations in membrane proteins and lipids.[20–22,30,32] Oxidations of proteins and lipids are consequences of the production of these free radicals.[14,18–22] The brain is particularly sensitive to free radical insults because it contains high concentrations of easily peroxidizable polyunsaturated fatty acids[14,15] and is not particularly enriched with protective antioxidant enzymes or other antioxidant compounds.[26]

We have reported previously that transient 10-min ischemia followed by 1 h reperfusion led to

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Abbreviations: CHX, 2-cyclohexene-1-one; EDTA, ethylenediaminetetra-acetate; EGTA, ethyleneglycolbis(aminoethyl ether)tetra-acetate; EPR, electron paramagnetic resonance; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; HWHH, half-width at half-height of the low-field resonance line of the lipid-specific spin label 5-doxylstearic acid in cortical synaptosomal membranes; MAL-6, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl; M_I, magnetic quantum number describing the three resonance lines of nitroxide spin labels: M_I=+1 is the low-field line; 5-NS, 5-doxylstearic acid; W/S ratio, EPR spectral height of the M_I=+1 low-field resonance line of the protein-specific spin label MAL-6 attached to weakly immobilized sites on synaptosomal membrane proteins to that of MAL-6 attached to strongly immobilized sites on this system

significant changes in the physical state of cortical synaptosomal membrane proteins and lipids.[20] The lipid changes returned to control values after additional reperfusion, but at 12–14 h reperfusion, a second, polyamine-related increase in lipid motion was observed.[20,21] Addition of a brain-accessible free radical scavenger prior to ischemia abrogated these changes in membrane lipids and proteins.[21]

There are a series of physiological defense mechanisms, including superoxide dismutase, glutathione peroxidase, catalase and glutathione, which protect the cell against toxic oxygen metabolites. The efficacy of these systems has been shown to diminish with ischemia/reperfusion injury.[26,27] Glutathione is a thiol-containing tripeptide, glutamylcysteinylglycine, found in high concentrations in most cells and found predominantly in its reduced form in the brain.[40] Glutathione is the principal non-protein sulfhydryl present in cells, and its presence provides cells with their reducing environment.[40] The functions of glutathione include maintenance of protein thiols and the reduced forms of other antioxidants, such as α-tocopherol and ascorbic acid, and protection of cells against oxidative damage or other types of toxicity.[34–36] Glutathione plays an important role in protection against oxidative stress and free radical damage via the intervention of enzyme scavengers such as glutathione peroxidase and superoxide dismutase, which in turn oxidize glutathione to GSSG.[4] Thus, glutathione functions not only as a substrate for glutathione peroxidase but also as a free radical scavenger in antioxidant systems.[4]

Recently, there has been significant research involving glutathione depletion and resulting effects on oxidative stress[34,37,38,41] and post-ischemic brain damage.[46] The approach has been to modify brain glutathione levels in order to assess the resistance or susceptibility to oxidative stress.[35] Glutathione levels may be decreased by administration of compounds that oxidize glutathione to GSSG, sulfhydryl reactants that alkylate glutathione, compounds that inhibit the synthesis of glutathione, and compounds bearing an electrophilic site that form conjugates with glutathione.[4,35] An example of a compound which has been proven to be an effective brain glutathione-depleting agent is 2-cyclohexene-1-one (CHX).[34,35] CHX forms a conjugate with glutathione via glutathione *S*-transferase[36] and is particularly useful in depleting brain glutathione because it can pass through the blood–brain barrier.[35]

As yet unanswered questions in this research include: (1) Does glutathione depletion itself affect the physical state of brain membrane proteins and lipids, and are these alterations a function of age? (2) Does glutathione diminution affect alterations in the physical state of synaptosomal membranes produced by free radicals associated with ischemia/reperfusion? In this report, these questions are addressed.

EXPERIMENTAL PROCEDURES

Materials

CHX (95+%), used as a glutathione-depleting agent, was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Ultra-pure sucrose, used for synaptosome isolation, was obtained from ICN Biochemicals. The protease inhibitors leupeptin, pepstatin A and aprotinin were obtained from Calbiochem. The protein-specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and the lipid bilayer-specific spin label 5-doxylstearic acid (5-NS) were obtained from Aldrich Chemical Co. All other chemicals were obtained from Sigma in the highest possible purity.

Animals

All procedures involving animals have been approved by the University of Kentucky Institutional Animal Care and Use Comittee. Male Mongolian gerbils were obtained from Tumblebrook Farms (West Brookfield, MA, U.S.A.). Adult (three months of age) and aged (15–18 months of age) male gerbils were acclimated to the University of Kentucky Medical Center facility for a minimum of seven days prior to experimentation. The animals were housed under 12-h light/dark condition and fed standard Rodent Laboratory Chow (Purina) *ad libitum* in the home cage. In each experiment, along with the particular experimental manipulations, two control gerbils were used and were given no ischemia and no CHX. This experimental design was employed to control for biological and instrumental variations. Additional control experiments (no ischemia) involved dosing of animals with 100 mg/kg (i.p.) CHX at times ranging from 1 h 40 min to 14 h 40 min prior to decapitation. Ischemia/glutathione depletion experiments involved surgery on the animals prior to experimentation, as described previously.[20,21] Briefly, in pentobarbital-anesthetized animals, a loop of dental floss was placed around each common carotid artery and the free ends passed through a double-lumen catheter, as described previously.[12] The catheter exited in the dorsum of the neck and was glued in place with cyanoacrylate adhesive. Forty-eight hours after surgery, animals were dosed with 100 mg/kg CHX 30 min prior to ischemia. Ischemia was induced for 10 min followed by reperfusion for specific times before decapitation. Upon decapitation, the whole brain was removed and dissected on a cold stage, as described previously.[12,20,21] Cortices from two to three animals were then pooled and suspended in approximately 20 ml of ice-cold isolation buffer (0.32 M sucrose containing 4 μg/ml leupeptin, 4 μg/ml pepstatin A, 5 μg/ml aprotinin, 20 μg/ml type II-S soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES at pH 7.4 and homogenized in a Wheaton 30-ml motor-driven Potter-type homogenizer with a Teflon pestle.

An *in vitro* experiment was performed on isolated synaptosomes of aged gerbils. CHX (5.4 μl) was diluted to 10 ml in lysing buffer to give 100 μg/ml solution. This solution (250 μl) was added to 1 ml protein, at 4.0 mg/ml, to give 0.01 mg CHX/ml solution. The samples were incubated for 30 min in ice and then labeled with MAL-6 and 5-NS.

Synaptosome preparation

Synaptosomes were purified from homogenized cortices via ultracentrifugation across discontinuous sucrose gradients after the method of Ueda *et al.*,[43] adapted by Barnes,[1] and further adapted in our laboratory.[44,45] Briefly, homogenized cortices were centrifuged for 10 min at $1500 \times g$ at 4°C in a Sorvall RC-5B centrifuge. The supernatant was removed and re-centrifuged at $20{,}000 \times g$ at 4°C. The resulting pellet was resuspended in 10 ml isolation buffer and layered on top of a sucrose density gradient composed of 10 ml of 1.18 M sucrose (pH 8.5), 1.0 M sucrose (pH 8.0)

and 0.85 M sucrose (pH 8.0). Centrifugation for 2 h at 85000 × *g* at 4°C in an SW 28 rotor in a Beckman L2-65B ultracentrifuge led to isolation of nearly pure synaptosomes at the 1.18/1.0 M sucrose interface. Following isolation, synaptosomes were rinsed and suspended in lysing buffer followed by analysis of protein concentration using the method of Lowry *et al*.[33] Synaptosomal membrane suspensions from each independent pool were split into duplicates and labeled with 5-NS and MAL-6. Previous characterization of this preparation showed essentially no mitochondrial contamination.[44,45]

Spin labeling

Synaptosomal membranes were labeled with the protein-specific spin label MAL-6 as described previously.[2,3,8–11,16,20,21,23,24,45] Briefly, ice-cold MAL-6 spin label (100 μg/ml) was added to synaptosomal membranes containing 5.0 mg of protein. The resulting mixture was shaken by hand and incubated at 4°C for 16–18 h. Samples were then centrifuged and washed six times in 10–15 volumes of lysing buffer to remove excess spin label. On the last wash, the samples were brought to a total volume of ~500 μl in lysing buffer. Samples were allowed to equilibrate at room temperature for 30 min prior to electron paramagnetic resonance (EPR) analysis. All EPR spectra were obtained on a Bruker 300 EPR instrument equipped with computerized data acquisition and analysis capabilities located in a climate-controlled room of constant temperature (20 ± 1°C) and humidity. Instrumental parameters were: microwave frequency=9.78 GHz, modulation amplitude=0.32 G at 100 kHz, with a time constant of 1.28 ms. The relevant spectral parameter (EPR spectral height of the M_I=+1 low-field resonance line of the protein-specific spin label MAL-6 attached to weakly immobilized sites on synaptosomal membrane proteins to that of MAL-6 attached to strongly immobilized sites on this system: W/S ratio) was averaged for each duplicate pair of samples.

A separate aliquot of each of the synaptosomal membrane preparations was labeled with the lipid-specific spin label 5-NS as described previously.[8,20,21] The lipid-specific spin label was dissolved in chloroform to a concentration of 0.2 μM. In a test tube, 80 μl of this solution was evaporated under nitrogen gas, resulting in a thin layer of label on the test tube wall. The synaptosomal membranes were adjusted to a protein concentration of 4.0 mg/ml with lysing buffer and 400 μl added to each spin label tube. Duplicate labeling was performed for each sample. The samples were incubated at room temperature for 30 min prior to spectral acquisition. The instrumental parameters were the same as above. The relevant spectral parameter (half-width at half-height of the low-field resonance line of the lipid-specific spin label 5-NS in cortical synaptosomal membranes: HWHH) was averaged for each duplicate pair.

Data analysis

Data were analysed by appropriate Student *t*-tests, two-way ANOVA and *post hoc* analyses. A value of $P<0.05$ was considered statistically significant.

RESULTS

Changes in the physical state of cortical synatosomal membrane proteins

In the present study, the protein-specific spin label MAL-6 was used to monitor structural alterations in synaptosomal cytoskeletal and transmembrane proteins to which it specifically binds.[8,45] MAL-6 is thought to react primarily with two distinct populations of spin label binding sites on proteins, one which restricts the isotropic motion of the spin label (the strongly immobilized or S sites) and one which does not significantly restrict the motion of the spin label (the weakly immobilized or W sites). As shown in numerous publications from our laboratory (see, for example, Refs 3, 8–11, 16, 20, 21, 23, 24, 45 and 47), the resulting EPR spectrum is an overlap of the W and S populations. The relevant parameter for monitoring protein conformational changes is the W/S ratio. We and others have shown that perturbations in the normal interactions of cytoskeletal proteins cause changes in the W/S ratio.[2,3,8–11,16,20,21,23,24,45,47] Our laboratory has shown that, *in vitro* (via iron/H_2O_2, menadione, lipopolysaccharide or β-amyloid)[3,11,23,42] and *in vivo* (via ischemia/reperfusion[20,21]), hyperoxia[24,25] oxidation of synaptosomal membranes decreases the W/S ratio. It was our hypothesis that the oxidatively induced decrease in the W/S ratio was due to changes in the physical state of membrane proteins associated with increased protein–protein interactions, for example by S–S cross-linking and/or formation of carbonyl groups, which might change the secondary or tertiary protein structure of membrane proteins and thus alter the motion of the MAL-6 spin label.

We used the protein-specific spin label MAL-6 to investigate the potential membrane alterations due to glutathione depletion and glutathione depletion coupled with ischemia/reperfusion. A comparison of basal W/S values of MAL-6 in synaptosomal membranes from adult and aged animals indicated lower W/S values for aged animals, confirming our earlier report.[24] Table 1 shows the effects of glutathione depletion and 10-min transient ischemia/reperfusion following glutathione depletion, in comparison to the effects of 10-min transient ischemia/reperfusion, on the W/S ratio of adult gerbil synaptosomes. The maximum effect of CHX administration on the W/S ratio in the adult model was seen at 3 h after injection, with the W/S ratio returning toward, but still significantly lower than, control values in the adult gerbil by 14 h reperfusion. CHX administration 30 min prior to ischemia/ reperfusion caused a decrease in the W/S ratio in adult gerbils at 1 h reperfusion, which was significantly greater than with ischemia/reperfusion alone. This effect remained constant through the 14-h point in the adult model, in contrast to the ischemia/reperfusion model, which partially recovered by 14 h reperfusion. At both 1 and 14 h reperfusion, the W/S ratio change was significantly larger in glutathione-depleted adult gerbils than with ischemia alone ($P<0.03$).

Changes in the W/S ratio in the aged model are shown in Table 2. CHX administration to aged gerbils caused a significant decrease in the W/S ratio at 1 h ($P<0.0001$), which, in contrast to that in adult animals (Table 1), did not return to control values by the 14-h reperfusion time-point. Glutathione depletion coupled with ischemia/reperfusion caused a decrease in W/S ratio at 1 h reperfusion which was not significantly different from the change observed

Table 1. Effects of glutathione depletion and ischemia/reperfusion on the W/S ratio of MAL-6 spin-labeled proteins in cortical synaptosomal membranes from adult gerbils

% change in W/S ratio* Time of reperfusion (h) System	1 h	3 h	14 h
Ischemia/reperfusion	−8.2 ± 2.3	−5.4 ± 2.1	−5.7 ± 2.6
CHX administration	−10.1 ± 2.2	−18.4 ± 1.5§	−1.9 ± 1.5†
CHX administration followed by ischemia/reperfusion	−12 ± 2.0†	−16.1 ± 1.5§	−12 ± 2.3‡‖

*Mean ± S.D. of the percentage change in the W/S ratio of MAL-6 covalently bound to cortical synaptosomal membrane proteins relative to untreated controls. All nine W/S percentage changes are significantly different from those of non-treated controls (maximum P value <0.05); n=4–6.
†P<0.05 compared to ischemia/reperfusion alone.
‡P<0.003 compared to ischemia/reperfusion alone.
§P<0.001 compared to ischemia/reperfusion alone.
‖P<0.001 compared to CHX administration alone.

Table 2. Effects of glutathione depletion and ischemia/reperfusion on the W/S ratio of MAL-6 spin-labeled proteins in cortical synaptosomal membranes from aged gerbils

% change in W/S ratio* Time of reperfusion (h) System	1 h	3 h	14 h
Ischemia/reperfusion	−11.3 ± 1.5	−9.7 ± 2.5	−7.6 ± 2.2
CHX administration	−8.9 ± 3.1	−14.1 ± 3.3†	−8.0 ± 1.8
CHX administration followed by ischemia/reperfusion	−12.1 ± 1.7	−12.5 ± 3.8	−12.8 ± 3.2†‡

*Mean ± S.D. of the percentage change in the W/S ratio of MAL-6 covalently bound to cortical synaptosomal membrane proteins relative to untreated controls. All nine W/S percentage changes are significantly different from those of non-treated controls (maximum P value <0.005); n=4–6.
†P<0.03 compared to ischemia/reperfusion alone.
‡P<0.05 compared to CHX administration alone.

with ischemia/reperfusion alone (Table 2). However, at 14 h reperfusion, the W/S ratio change observed with glutathione depletion coupled with ischemia/reperfusion was significantly greater than that with ischemia/reperfusion alone (P<0.03) and had not returned toward control values at all when compared to the 1-h reperfusion time-point. In both the adult and aged models, glutathione diminution combined with ischemia/reperfusion caused a significant decrease in the W/S ratio at the 14-h reperfusion time-point when compared to ischemia reperfusion alone. It is interesting to note that, although adult gerbils were able to recover from glutathione depletion by 14 h while aged animals were not, the effect of glutathione depletion coupled with ischemia/reperfusion was the same in both models. This result is consistent with the notion of non-recoverable oxidized membrane proteins in ischemia/reperfusion injury when the normal cytoplasmic reducing environment has been significantly diminished.

Changes in the physical state of cortical synaptosomal membrane lipids

In order to determine if changes in the physical state of the cortical synaptosomal bilayer lipids occurred after ischemia/reperfusion, the lipid-specific spin label 5-NS was used. It is conceptualized that 5-NS intercalates into the lipid bilayer with its hydrophobic acyl chain embedded deep in the bilayer and its polar head group oriented near the hydrophilic surface of the bilayer.[8,9] The EPR-active nitroxide group is covalently bound to the acyl chain of the probe, which undergoes rapid anisotropic motion inside the bilayer. Therefore, the motion of the nitroxide group reflects the intramembrane motion in the adjacent segment of the molecule, and thus 5-NS is a useful probe for monitoring changes in the local environment of the hydrophobic portions of lipids near the bilayer surface.[8,9]

The relevant parameter for monitoring the motional changes of 5-NS is the HWHH of the low-field (M_I=+1) line of the 5-NS spectrum. Low-field line broadening is a result of decreased lipid order and increased lipid motion and is analogous to chemical exchange phenomena.[8,9] Thus, in a highly fluid membrane, the nitroxide would be able to rapidly switch between two extremes in orientation, causing an increase in uncertainty of a particular orientation at a given time, which leads to an increase in line width of the low-field line (M_I=+1).[8,9] The technique of lipid-specific spin labeling has been widely used by our laboratory and others to study the

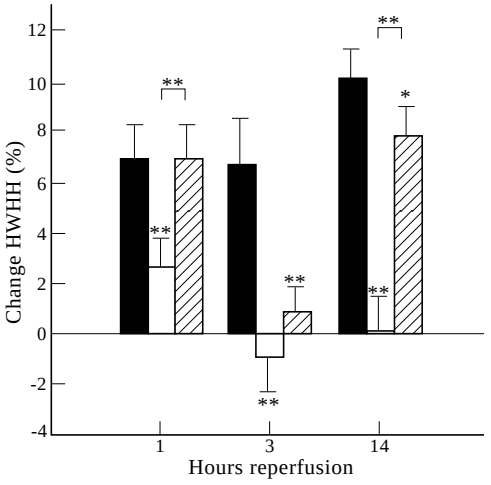

Fig. 1. Comparison of the effects of ischemia/reperfusion (solid bars), CHX administration (open bars) and CHX administration combined with ischemia/reperfusion (hatched bars) on the HWHH parameter of 5-NS-labeled lipid bilayer in cortical synaptosomal membranes from adult gerbils. Statistically different from ischemia/reperfusion alone: $*P<0.02$, $**P<0.001$; $n=4$–6 for each experiment. The connecting bars at 1 and 14 h show significant differences ($P<0.001$) between CHX administration and CHX administration combined with ischemia/reperfusion. All values of HWHH are statistically different ($P<0.02$) from the non-treated controls except those at 3 h (CHX and CHX plus ischemia/reperfusion) and 14 h (CHX administration).

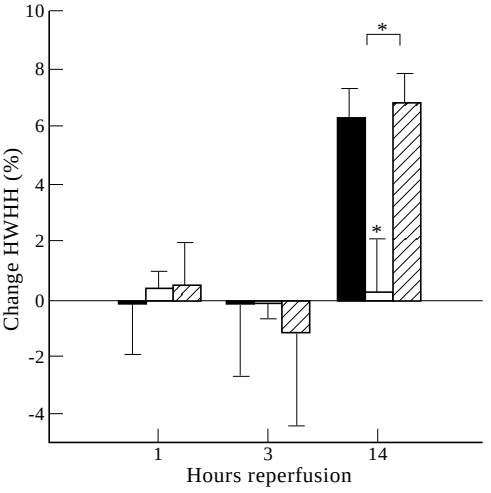

Fig. 2. Comparison of the effects of ischemia/reperfusion (solid bars), CHX administration (open bars) and CHX administration combined with ischemia/reperfusion (hatched bars) on the HWHH parameter of 5-NS-labeled cortical synaptosomal membranes from *aged* gerbils. Only the HWHH values at 14 h are statistically different from ischemia/reperfusion alone ($*P<0.005$); $n=5$ for each experiment. The connecting bar at 14 h represents a statistical difference between CHX treatment and ischemia/reperfusion combined with CHX treatment ($P<0.005$). The only significantly different HWHH value from non-treated controls occurred with the 14-h ischemia/reperfusion value.

motion and order of erythrocyte and synaptosomal membranes.[8–10,20,21]

Relative to untreated controls, CHX given alone to adult gerbils caused a significant initial increase in HWHH at 1 h reperfusion ($P<0.01$), which returned to control values by 3 h reperfusion and remained there throughout the 14-h reperfusion (Fig. 1). CHX treatment with ischemia/reperfusion showed no change when compared to ischemia/reperfusion alone at 1 or 14 h reperfusion. However, at 3 h reperfusion, the increase in HWHH with CHX administration followed by ischemia/reperfusion was completely nullified; at 14 h the HWHH increase was slightly, yet significantly, less than ischemia reperfusion alone ($P<0.02$).

Figure 2 shows the effects of CHX administration and CHX with ischemia/reperfusion on HWHH in aged gerbils. In contrast to adult gerbils, CHX caused no significant change in HWHH compared with non-treated control values at any time-point. Glutathione depletion combined with ischemia/reperfusion caused no significant change in HWHH when compared to ischemia/reperfusion alone.

DISCUSSION

Recent studies have indicated that damage to synaptosomal membrane proteins and lipids following ischemia/reperfusion is caused by free radical production.[20–22] Glutathione serves as a free radical scavenger in the antioxidant defense systems in most cells.[46] The mechanism of antioxidant protection during ischemia/reperfusion injury by glutathione is unclear.

The results of the present study demonstrate a significant decrease in the W/S ratio of MAL-6-labeled synaptosomal membrane proteins by glutathione depletion alone. This decrease, reflecting decreased segmental motion of membrane proteins, is consistent with the hypothesis that loss of glutathione diminishes the reduction of disulfide bonds that form within proteins during oxidative stress, thereby changing the conformation of membrane proteins to make them more rigid. Benzi *et al.*[5] have shown that there is an age-related depletion of the glutathione concentration in rat brain, with old animals having lower baseline glutathione concentrations than young animals. The same study showed that depletion of glutathione with CHX was also age-dependent over time, with no recovery of glutathione levels in old rats until 9 h after administration of the glutathione depletor. Our current results support the hypothesis that adult gerbils are able to recover from glutathione depletion, whereas aged animals cannot recover from glutathione depletion (Table 1, Table 2), suggesting that perhaps there is an overall decreased maintenance of antioxidant capacity in aged animals.

The results from glutathione depletion coupled with ischemia/reperfusion caused equal W/S ratio decreases at 1, 3 and 14 h reperfusion, with no recovery in W/S ratio over time in both adult and

aged gerbils. In contrast, adult animals subjected to loss of glutathione only were able to recover to a normal physical state of membrane proteins, while similarly treated aged animals were unable to do so. Taken together, these results suggest that glutathione depletion coupled with ischemia/reperfusion causes changes in membrane proteins which cannot be corrected in a glutathione-deficient environment. The protein-specific spin label used in these studies is known to bind to cytoskeletal and transmembrane proteins.[45] Both cytoskeletal and regulatory proteins are degraded during global ischemia,[29] and it is thought that degradation of cytoskeletal proteins may play an important role in the mechanism of delayed neuronal death after cerebral ischemia.[28] Such alterations may, in part, be related to changes in the physical state of synaptosomal membrane proteins observed in the current study.

Our laboratory has shown in human, rat and gerbil synaptosomal membranes that the baseline W/S values for MAL-6 are lower in old versus young tissue. This finding, combined with the demonstration that increased oxidative damage leads to a decrease in W/S ratio,[3,20,21,23,24] supports the hypothesis that there is an increase in baseline oxidatively damaged protein in old tissue versus young tissue. This result is consistent with previous spin labeling studies from our laboratory in which marked differences in aged versus young animals were observed basally and in response to hyperoxia.[24,25] This decrease in the W/S ratio of MAL-6 bound to synaptosomal membrane proteins could be a result of increased oxidative stress resulting from decreased antioxidant protection in old animals. As neurons age, their mitochondria become swollen and leak electrons, which are transferred to oxygen. In addition, mitochondria produce H_2O_2, which is harmful to tissue when reacted with transition metals to produce the highly reactive hydroxyl radical. Glutathione is known to protect the cell from oxygen radicals,[34] as well as reduce hydrogen peroxide.[26,27] As animals age, the number of oxidative events per unit time increases and the glutathione level decreases.[6] The combination of these two processes could lead to accelerated damage and reduction in glutathione levels which, in turn, would lead to further mitochondrial damage and ultimately an exponential increase in free radical production and tissue damage.

In contrast to the significant effects on the physical state of membrane proteins, administration of CHX alone or followed by ischemia/reperfusion has little additional effect, when compared to ischemia/reperfusion alone, on adult or aged synaptosomal membrane lipids. This result is consistent with the notion that glutathione provides considerably more protective effects on membrane proteins than lipids. With respect to ischemia reperfusion injury, IRI, one possible explanation for this observation is that the maximum change in HWHH is already seen with 10-min ischemia followed by subsequent reperfusion. An explanation for the apparent increased susceptibility of proteins over lipids to damage as a result of glutathione depletion could be based on the close proximity of cytoplasmic glutathione to membrane cytoskeletal proteins, i.e. this compound is located in the cytoplasm. If glutathione, a known free radical scavenger,[40] is removed, reactive oxygen species produced naturally in cells would be free to attack the first available cytoplasmic target, soluble and structural proteins, and, to a lesser extent, membrane lipids.

CONCLUSION

The results of these present studies are consistent with the concept that ischemia/reperfusion injury is associated with free radical damage, and that diminution of one key antioxidant defense system, glutathione, can have a profound influence on the subsequent membrane damage caused by ischemia/reperfusion. These results have significant implications with respect to potential therapeutic intervention in stroke, studies currently underway in our laboratory.

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