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Depletion of Brain Glutathione is Accompanied by Impaired Mitochondrial Function and Decreased N-Acetyl Aspartate Concentration

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The effect of depletion of reduced glutathione (GSH) on brain mitochondrial function and N-acetyl aspartate concentration has been investigated. Using pre-weanling rats, GSH was depleted by L-buthionine sulfoximine administration for up to 10 days. In both whole brain homogenates and purified mitochondrial preparations complex IV (cytochrome c oxidase) activity was decreased, by up to 27%, as a result of this treatment. In addition, after 10 days of GSH depletion, citrate synthase activity was significantly reduced, by 18%, in the purified mitochondrial preparations, but not in whole brain homogenates, suggesting increased leakiness of the mitochondrial membrane. The whole brain N-acetyl aspartate concentration was also significantly depleted at this time point, by 11%. It is concluded that brain GSH is important for the maintenance of optimum mitochondrial function and that prolonged depletion leads also to loss of neuronal integrity. The relevance of these findings to Parkinson's disease and the inborn errors of glutathione metabolism are also discussed.

KEY WORDS: Glutathione; mitochondria; N-acetyl aspartate; oxidative stress.

INTRODUCTION

Reduced glutathione (GSH), in conjunction with glutathione peroxidase, is an important antioxidant which protects the cell against hydrogen peroxide accumulation and subsequent oxygen radical formation (1). In view of this antioxidant role, GSH deficiency states arising from inborn errors of GSH metabolism and possibly Parkinson's disease may lead to increased oxygen radical formation (2,3).

Within the cell, mitochondria are a potential source of hydrogen peroxide (4), but formation is kept to a minimum as a result of the high intra-mitochondrial GSH concentration (5). The concentration of mitochondrial GSH is maintained by a high affinity transport system that imports GSH into the mitochondria from the cytosol (6).

Depletion of mitochondrial GSH may result in impaired mitochondrial function, since all the complexes of the electron transport chain are reported to be susceptible to damage by oxygen free radicals, in vitro (7). Loss of brain GSH and impairment of mitochondrial function may be an important event in the pathogenesis of Parkinson's disease. In homogenates prepared from post mortem samples of substantia nigra from patients with Parkinson's disease, a decreased concentration of GSH and impaired mitochondrial complex I (NADH ubiquinone reductase) activity has been reported (3,8). However, in homogenates prepared from patients with incidental Lewy body disease (thought to be presymptomatic Parkinson's disease) there is GSH deficiency in the absence of complex I deficiency (3). These data suggest that GSH loss may occur early in the disease pro-

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cess. Whether this GSH depletion leads to the impaired mitochondrial function observed in Parkinson's disease is not known.

A number of compounds exist for the production of experimental models of GSH deficiency. Unfortunately some of these do not specifically affect GSH metabolism, e.g. diethyl maleate and 2-cyclohexene-1-one (9,10). In contrast, L-buthionine-S,R-sulfoximine (L-BSO) is a relatively specific inhibitor of GSH biosynthesis, and administration to experimental animals leads to GSH deficiency in the majority of tissues (9). A limitation of L-BSO is that it does not readily cross the blood brain barrier (11). However, preweanling rats lack a fully developed blood brain barrier and the peripheral administration of L-BSO to such animals leads to a marked depletion of brain GSH (11). Depletion of rat brain GSH, by L-BSO, is reported to lead to mitochondrial swelling and diminished citrate synthase activity (11). However, the effect of GSH depletion on the activity of the individual mitochondrial complexes was not investigated. Using 2-cyclohexene-1-one as a means of inducing oxidative stress in the rat brain, Benzi et al. (12) report sequential damage to the components of the electron transport chain with complex IV appearing to be the most susceptible.

Neuronal cells may be particularly susceptible to the effects of GSH depletion, since these cells, in contrast to glia, have relatively low concentrations of GSH (13,14). Such vulnerability may be reflected by the fact that depletion of GSH has been shown to induce apoptosis in cultured neurons (15).

In view of the above observations, we have used pre-weanling rats to investigate the effects of L-BSO induced GSH depletion on brain mitochondrial function (specific activities of the individual complexes and citrate synthase) and neuronal integrity, as reflected by the N-acetyl aspartate (NAA) concentration. NAA is believed to be located exclusively in neuronal cells (16-19), hence a reduction in brain concentration may reflect a loss of neuronal integrity.

EXPERIMENTAL PROCEDURE

Materials. Ubiquinone-1 was a kind gift of the Eisai Chemical Company (Tokyo, Japan). All other reagents were of analytical grade and were obtained from either BDH Ltd (Dagenham, England) or the Sigma Chemical Company (Poole, England). Sprague-Dawley rats were used throughout and were purchased from B and K Universal (Hull, England).

GSH Depletion. Animals were weighed every day and two dosing protocols followed:- (a). 14 day old pre-weanling rats were used. Pups were assigned to either a control or test group and were injected subcutaneously twice a day (9.00am and 5.00pm) with L-BSO (2.5 mmol/kg) or iso-osmotic saline (controls). Animals were killed either on day 4 or 5 of the injection regime at 2.00pm by cervical dislocation. (b) 12 day old pre-weanling rats received a single daily subcutaneous dose of L-BSO (3.8 mmol/kg) or saline at 11.00am. On day 11 of the injection regime the pups were killed at 9.30am. A single dose of L-BSO was used in this second protocol to minimise the risk of hind limb paralysis, weight loss and cataract formation associated with the prolonged use of high doses of L-BSO [20].

After sacrifice the brain was removed and used to produce homogenates or mitochondrial preparations. Homogenates were made following division of the brain along the mid sagittal plane. The left hemisphere was homogenized (25% w/v) in 15mM o-phosphoric acid and following centrifugation (15,000 *g* for 10 minutes) the supernatant was used for GSH and NAA analysis. The right hemisphere was homogenised (10% w/v) in isolation medium; 320 mM sucrose, 1 mM EDTA, 10 mM Tris (pH 7.4) and used for enzymatic analysis. Non-synaptic "free" mitochondria were isolated from pooled (2 or 3 animals) brains after 5 days of treatment and single whole brains after 10 days of treatment, as described by Lai and Clark (21) and reconstituted in isolation medium, for enzymatic analysis, or extracted with 15 mM o-phosphoric acid for GSH determination. Control and test samples were prepared together at 4°C, to remove any effects of time on GSH status. However, incubation of isolated brain mitochondria for 1 hour at 30°C does not result in loss of GSH (data not shown). All samples were stored at −70°C until assayed.

Enzymatic Analysis. All assays were performed at 30°C. Prior to analysis, samples were freeze-thawed and gently vortexed three times to ensure mitochondrial lysis was complete. Complex I (NADH-ubiquinone reductase. EC 1.6.99.3) and complex II/III (succinate cytochrome c reductase. EC 1.8.3.1) activities were determined as described by Schapira et al. (8). Complex IV (cytochrome c oxidase. EC 1.9.3.1) activity was assayed by the method of Wharton and Tzagoloff (22). Citrate synthase (EC 4.1.3.7) activity was measured according to the method of Shepherd and Garland (23). Monoamine oxidase (EC 1.4.3.4) activity was determined in purified mitochondrial preparations as described by Ragan et al. (24). The direct effect of GSH and L-BSO on complex I and complex IV activity was investigated by pre-incubation of purified mitochondria (protein content 1 mg) with 1 mM GSH or 1 mM L-BSO for 5 minutes at 30°C prior to enzymatic analysis. The protein content of brain homogenates and pooled mitochondria (5 day treatment) were determined by the method of Gornall et al. (25). In view of the lower yield of mitochondria isolated from single brains (10 day treated), the method of Lowry et al (26) was used for the measurement of protein in these preparations. Bovine serum albumin was used as the standard and comparable results were obtained with both methods.

Metabolite Analysis. The concentration of reduced glutathione was determined by HPLC and electrochemical detection (27). NAA was measured by HPLC and UV detection (16).

Statistical Analysis. All data is expressed as the mean ± SEM. Statistical significance for the comparison of two groups was assessed using Student's unpaired t-test. Multiple comparisons were made by one way analysis of variance followed by the least significant difference multiple range test.

RESULTS

Administration of L-BSO had no appreciable effect on pup body weight gain for the duration of all the ex-

Table I. The Effect of L-BSO Administration on GSH Concentration

	Control	L-BSO	GSH depletion
Brain Homogenate (4 days administration)	1.24 ± 0.03 (7)	0.48 ± 0.01 (7)	61
Purified Mitochondria (5 days administration)	1.09 ± 0.08 (6)	0.36 ± 0.03 (4)	67
Brain Homogenate (10 days administration)	1.62 ± 0.03 (8)	1.03 ± 0.03 (7)	36
Purified Mitochondria (10 days administration)	0.80 ± 0.08 (6)	0.52 ± 0.02 (6)	35

Values expressed as μmol/g wet weight for brain homogenate and nmol/mg protein for mitochondria. GSH depletion is expressed as percentage decrease when compared to appropriate control. In all cases L-BSO administration resulted in a highly significant ($p<0.01$) loss of GSH. Number in parenthesis is sample size (n).

periments (control weight after 10 day dosing regime, 37.9 ± 1.28g; L-BSO animals, 35.2 ± 1.28g).

The whole brain and mitochondrial GSH concentrations reported here (Table I) for control animals is in close agreement with published observations (1,28). Mitochondrial complex activities were determined in whole brain homogenates and purified mitochondrial preparations and are shown in Figs. 1, 2, and 3.

L-BSO administration for 4 days caused a marked depletion of whole brain GSH as measured in the whole brain homogenates (Table I). This was accompanied by significant decreases in the activities of mitochondrial complexes I and IV, a 21% and 13% reduction respectively (Figs 1a and 3a). After 10 days of L-BSO administration, the whole brain GSH was only depleted by 36% (Table I). This may be due to maturation of the blood brain barrier in these animals or the fact that only a single daily dose of L-BSO was used. In these homogenate preparations, only the activity of complex IV was reduced, by 8%, in the pups receiving L-BSO (Fig. 3c). However, this loss was not statistically significant.

In order to pursue these observations further, "free" mitochondria were purified from animals receiving L-BSO for 5 or 10 days. Following this treatment, the intra-mitochondrial GSH concentration was depleted to an almost identical extent as the whole brain GSH in pups receiving comparable treatment regimes (Table I). In these mitochondrial preparations only a significant decrease in complex IV activity was observed (27% and 21% reduction after 5 and 10 days of L-BSO administration respectively) (Figs. 3b and 3d). Complex I and II/III activity were not significantly affected (Figs. 1b, 1d, 2b and 2d).

The addition of either 1 mM L-BSO or 1 mM GSH had no direct effect on complex I and complex IV activity, in vitro (Complex I activity in the presence of L-BSO was 91 ± 6 and for GSH, 90 ± 15; Complex IV activity in the presence of L-BSO was 107 ± 7 and for GSH, 105 ± 6. Data expressed as % of control activity, n=3).

In whole brain homogenates, L-BSO administration for 4 or 10 days had no effect on citrate synthase activity (Table II). In contrast, significant loss of citrate synthase activity was apparent in the purified mitochondrial preparations obtained from animals receiving L-BSO for 10 days (Table II), suggesting a loss of mitochondrial but not total cell citrate synthase activity. Alternatively, this apparent loss of activity may arise as a result of contamination by non-mitochondrial protein. In order to address this point the activity of monoamine oxidase, an outer mitochondrial membrane marker enzyme (24), was determined in the purified mitochondrial preparations. It is clear from Table III that the mitochondrial enrichment was virtually identical in the preparations obtained from control and L-BSO treated animals.

Both L-BSO treated and control animals exhibited a decrease in brain homogenate citrate synthase activity when the 10 day and 5 day treated animals were compared (Table II). This probably reflects the increase in total brain protein content over this developmental period (18 day old pups (4 day treated) 145 ± 5 mg/g wet weight; 23 day old pups (10 day treated) 194 ± 7 mg/g wet weight). However, if citrate synthase activities are measured in purified mitochondrial preparations and expressed per mg of mitochondrial protein a clear increase in enzyme expression is observed, consistent with previously published data (29).

Administration of L-BSO for 4 days had no effect on the brain NAA concentration (Table IV). However, after 10 days of treatment the concentration of NAA was significantly reduced by 11% (Table IV).

DISCUSSION

L-BSO is a well established agent for causing depletion of GSH in experimental animals (9). Consequently, administration of high doses for prolonged period of time leads to weight loss, hind limb paralysis and cataract formation due to severe GSH depletion (20). In order to minimise the risk of these signs becoming apparent, but to study the effects of GSH depletion on brain mitochondrial function beyond 5 days a single daily dose of L-BSO was used. Using this dosing regime marked depletion of brain GSH, though not as great as that observed for the 4/5 day dosing protocol, was possible for 10 days in the absence of the above effects.

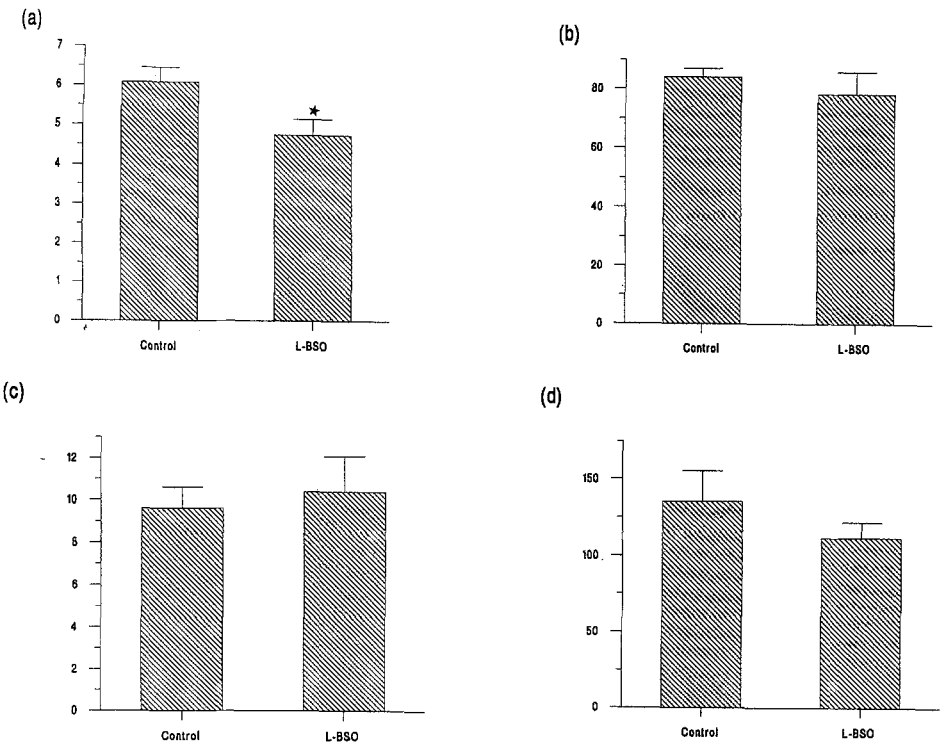

Fig. 1. The effect of L-BSO administration on complex I activity in; (a) Brain homogenates after 4 days of treatment. (b) Purified brain mitochondria after 5 days of treatment. (c) Brain homogenates after 10 days of treatment. (d) Purified brain mitochondria after 10 days of treatment. * Denotes statistically different ($p<0.05$) from appropriate control group. Activity expressed as nmol/min/mg protein. n is as in Table I.

Whilst L-BSO administration is considered to be a relatively specific agent for effecting GSH depletion (9,10), exposure of rats to L-BSO via their drinking water has been reported to lead to induction of the hepatic drug metabolising enzymes, UDP-glucuronosyltransferase and glutathione S-transferase (30). However, the effect of L-BSO on these enzymes when administered by routes other than the drinking water is considerably reduced (30).

The sub-cutaneous administration of L-BSO to preweanling rats was an effective means of depleting whole brain of GSH. Furthermore, this was accompanied by an almost identical degree of depletion of GSH compartmentalised within the mitochondria. Using this model, the present study demonstrates that the activity of complex IV was reduced by L-BSO administration and that the magnitude of this damage was more pronounced in purified "free" non-synaptic mitochondrial preparations than whole brain homogenates prepared from pups receiving comparable treatments. This difference may arise from the fact that, in contrast to the mitochondrial preparations utilised which reflect the non-synaptic component, homogenates represent the whole brain, where biochemical compartmentalisation has been lost, and contain mitochondria that are of synaptic and non-synaptic origin.

In contrast to complex IV, the activity of complex I was only significantly reduced in brain homogenates from animals receiving L-BSO for 4 days. Since this complex was not affected in the homogenates obtained from pups after administration of L-BSO for 10 days and no significant loss of activity could be demonstrated in the purified mitochondrial preparations, it is possible that marked GSH depletion, of the order of 60%, is required before significant complex I damage occurs and that this damage is more closely associated with the synaptic mitochondria present in whole brain homogenates.

Whilst, the exact mechanism for the observed loss mitochondrial function reported in this study is not known, a relationship between oxidative stress and mitochondrial damage has been reported in a variety of model systems; copper overloading in rats results in loss of hepatic GSH and diminished complex IV activity (31). Iron loading of PC12 cells leads to loss of cellular GSH, complex I and complex IV activity (32). Vitamin E deficiency, in rats, leads to increased oxidative stress and a characteristic myopathy accompanied by decreased muscle complex I and IV activity (33). In ad-

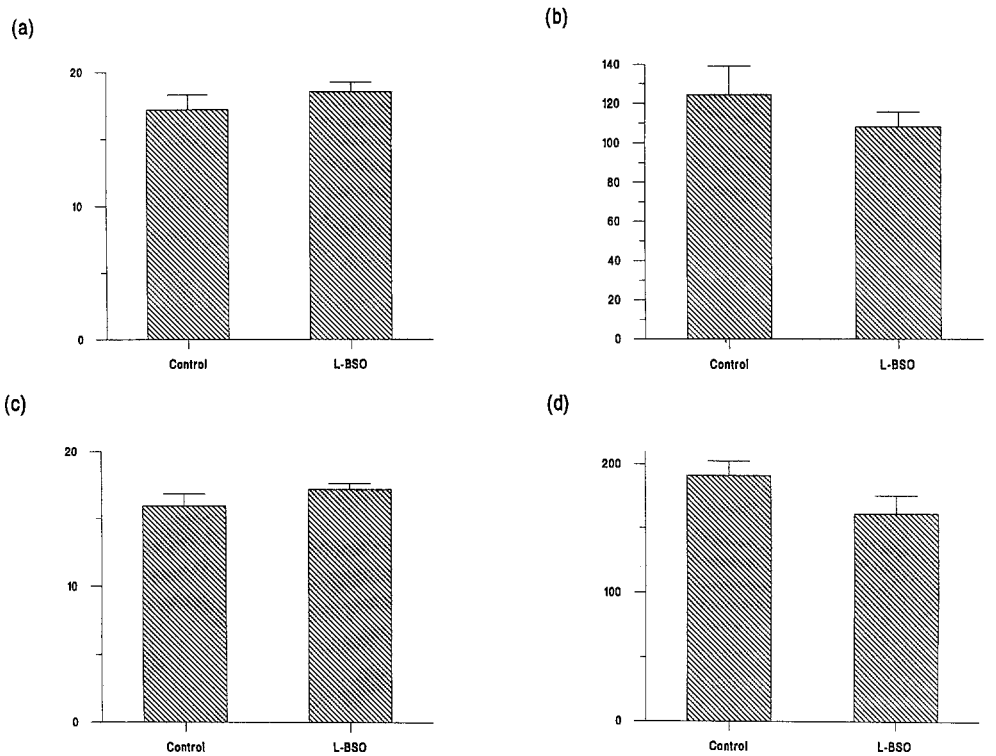

Fig. 2. The effect of L-BSO administration on complex II/III activity. (a)-(d) as for Figure 1. Activity expressed as nmol/min/mg protein. n is as in Table I.

dition, all four mitochondrial complexes are inactivated by the exposure of heart sub-mitochondrial particles to oxygen free radicals, in vitro, with complex IV being the least susceptible (7). In contrast to the findings of Zhang et al. (7), our study suggests that mitochondrial complex IV is the most susceptible complex to damage following depletion of brain GSH, in vivo. Furthermore, this finding is in agreement with the observations of Benzi et al. (12), who used 2-cylohexene-1-one to induce oxidative stress in the rat brain.

The susceptibility of complex IV to damage as a result of GSH depletion, in vivo, may arise in part from its dependence on cardiolipin for maximal activity (34). Cardiolipin is a polyunsaturated phospholipid located in the inner mitochondrial membrane and under conditions of high oxidative stress, due to the unusually high content of unsaturated bonds, it is prone to lipid peroxidation (34).

Previous studies using L-BSO have shown that depletion of brain GSH for 9 days causes mitochondrial enlargement and a reduction in citrate synthase activity in purified mitochondrial preparations (11). The observed mitochondrial swelling may be due to the inactivation of complex IV seen in the present study, since energy deficiency states are reported to cause mitochondrial swelling (35). In our study, no significant effect on citrate synthase activity was observed for either the whole brain homogenates or the purified mitochondrial preparations, after 4-5 days of L-BSO administration. However, after 10 days there was a significant decrease in citrate synthase activity in the purified mitochondria obtained from the animals receiving L-BSO but no corresponding change in brain homogenate activity. Since the mitochondrial enrichment, as reflected by monoamine oxidase activity, was virtually identical in the preparations obtained from control and L-BSO treated animals, it is possible that this loss of citrate synthase activity arises from increased mitochondrial leakiness. Such increased permeability would mean that the enzyme could be lost from the mitochondrial matrix in vivo, or during the purification process. Recently, oxidative damage has been reported to lead to the opening of a pore in the mitochondrial membrane resulting in increased permeability to solutes (36). Furthermore, in cardiac global ischaemia, the specific loss of citrate synthase activity, following mitochondrial purification, is suggested to arise as a result of increased mitochondrial leakiness (37). In this light, it is possible that such a

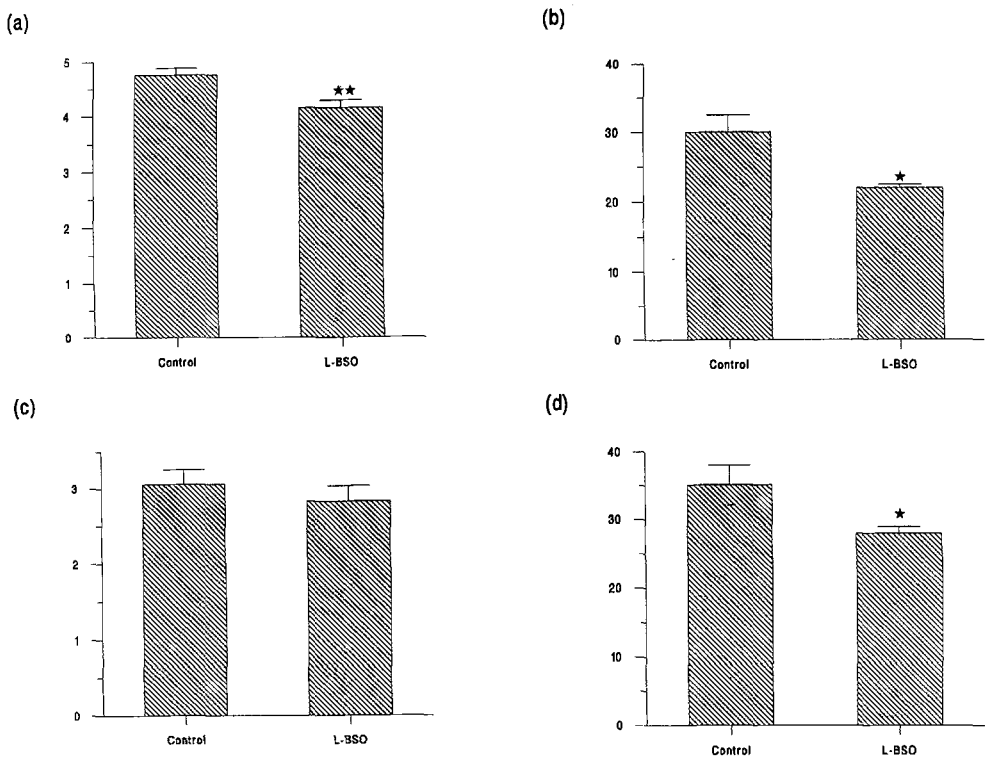

Fig. 3. The effect of L-BSO administration on complex IV activity. (a)-(d) as for Figure 1. * $p<0.05$ when compared to appropriate control group. ** $p<0.02$ when compared to appropriate control group. Activity expressed as first order rate constant/min/mg protein. n is as in Table I.

Table II. Citrate Synthase Activity in Whole Brain Homogenates and Purified Mitochondria and the Effects of L-BSO Administration

	Brain Homogenates		Purified Mitochondria	
	Control	L-BSO	Control	L-BSO
4/5 days administration	242.3 ± 6.1	244.4 ± 6.6	992.2 ± 20.3	932.1 ± 50.7
10 days administration	215.8 ± 10.0	215.5 ± 6.9	1531.5 ± 99.2	1254.0 ± 86.4*

Activity is expressed as nmol/min/mg protein. * Significantly different ($p<0.05$) when compared to the appropriate control group. L-BSO was administered for 4 days to pups from whom homogenates were made and for 5 days to animals from whom mitochondria were purified. Age at sacrifice was 18 and 19 days respectively. Animals receiving L-BSO or vehicle for 10 days were 23 days old at sacrifice. For both preparations there were also significant ($p<0.05$) developmental changes in activity. n is as in Table I.

mechanism contributes to the 80% loss of citrate synthase activity reported for mice muscle mitochondria purified after 9 days of L-BSO treatment and the 75% loss from mitochondria purified from rat lens epithelia after 4 days of L-BSO administration (38,39). Such an increase in the permeability of the mitochondrial membranes may also theoretically lead to dissipation of the proton gradient. Recently, using isolated liver mitochondria, fatty acid hydroperoxides, the intermediate products of lipid peroxidation, have been shown to cause permeability changes in the mitochondrial membrane the extent of which is dependent on the mitochondrial GSH concentration (40). The result of this increased permeability is an uncoupling of oxidative phosphorylation (40).

In addition to the effect of L-BSO on citrate synthase, marked developmental changes in activity of this enzyme were observed. For whole brain homogenates, the recorded decrease in activity is likely to be attributed to an increase in total brain protein over the time period

Table III. The Effect of L-BSO Administration on Monoamine Oxidase Activity in Purified Brain Mitochondria

	Control	L-BSO
5 Days administration	2.04 ± 0.23	1.99 ± 0.28
10 Days administration	2.37 ± 0.26	2.31 ± 0.14

Values expressed as nmol/min/mg protein. n is as in Table I.

Table IV. The Effect of L-BSO Administration on Whole Brain NAA

	Control	L-BSO
4 Days administration	4.45 ± 0.17	4.43 ± 0.05
10 Days administration	4.44 ± 0.18	3.94 ± 0.09*

Values expressed as μmol/g wet weight. * Significantly different ($p<0.05$) when compared to the appropriate control group. n is as in Table I.

of the study. This notion is supported by the fact that if the data were to be expressed on a wet weight basis there is an apparent increase in enzyme expression with age. The data from the purified mitochondrial preparations confirm that there is a developmental increase in citrate synthase activity relative to mitochondrial protein, in agreement with the findings of Land et al. (29).

The damage to complex IV, seen in this study, could in theory further increase the degree of cellular oxidative stress, since it is the terminal enzyme complex of the mitochondrial electron transport chain and is involved in the transfer of electrons from cytochrome c to oxygen resulting in the formation of water. Consequently, it is possible that a reduced capacity of this complex will, in addition to impaired ATP production, lead to an incomplete reduction of oxygen, increased free radical formation and further mitochondrial damage. A consequence of this impaired mitochondrial function may be cell death due to energy deficiency.

Neuronal cells have a relative paucity of GSH and hence may be particularly vulnerable to the effects of GSH depletion. Our observation that the putative marker of neuronal integrity, NAA, is reduced in concentration in the brain of animals receiving L-BSO for 10 days may suggest that neuronal damage is occurring as a result of prolonged GSH depletion. NAA has been shown to be synthesised in the mitochondria in an energy dependent manner (41). Therefore decreased NAA concentrations may arise as a consequence of neuronal energy deficiency. Whether such ATP depletion leads to neuronal cell death is not known, but MPP^+, a potent inhibitor of the electron transport chain, and GSH depletion can both induce apoptosis in cultured neurons (42,15). Alternatively, as immature animals have been used in this study and NAA is known to exhibit a developmental profile (41,43,44,45) reduced NAA concentrations may reflect delayed neuronal development.

In conclusion, our results suggest that GSH is important for maintaining brain mitochondrial function and depletion of GSH leads to loss of complex IV activity, increased permeability of the mitochondrial membrane to citrate synthase and loss of neuronal integrity. In addition, these findings may have important implications for our understanding of the pathogenesis of Parkinson's disease and the neurological impairment associated with the inborn errors of GSH metabolism. Extrapolation to the situation in Parkinson's disease is difficult, since immature animals have been used in this study and the data regarding brain mitochondrial function in Parkinson's disease has been derived solely from tissue homogenates, i.e. the activity of the mitochondrial complexes has yet to be determined in purified mitochondrial preparations prepared from the substantia nigra of such patients. Furthermore the effects of GSH depletion in the developing brain may be different to that in the aging brain.

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