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Depletion of glutathione does not affect electron transport chain complex activities in brain mitochondria: implications for Parkinson's disease and postmortem studies.

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Running title: Postmortem changes in glutathione levels

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Abstract

Glutathione is an important antioxidant in the brain that appears to be decreased, in conjunction with mitochondrial complex I activity, in Parkinson's disease patients. In postmortem analysis, measurement of glutathione levels and complex I activity can be delayed up to 20 h. We investigated if depletion of glutathione in the pre-weanling rat induces a reduction in complex I activity in brain mitochondria and the effect that postmortem delay has on glutathione levels and electron transport chain activities. Following injection with the gamma-glutamyl cysteine ligase inhibitor, buthionine sulfoximine (L-BSO), glutathione levels were decreased by 53% compared to the control values in whole brain homogenates. During postmortem delay of 24 hours, in which animals were kept at 4°C, levels of glutathione decreased in the control group by 58% and in the L-BSO treated group by 79%. However, during this period, there were no changes in mitochondrial electron transport chain complex I, II-III and IV activities in either group. These results suggest that a pre-existing deficiency of glutathione or a loss of glutathione during postmortem delay does not influence mitochondrial respiratory chain activities in the brain.

Keywords: Glutathione, Parkinson's disease, mitochondria, electron transport chain, complex I, antioxidant, postmortem.

Introduction

Parkinson's disease is characterised by a selective decrease in dopamine in the striatum caused by a degeneration of dopaminergic neurones in the zona compacta of the substantia nigra [1,2]. The primary cause of Parkinson's disease remains unknown, but several factors including oxidative stress and deficiencies in mitochondrial function have been associated with this debilitating disease [3]. Evidence for oxidative stress occurring in Parkinson's disease has come from studies that show a decrease (up to 40%) in levels of the cellular antioxidant, glutathione (GSH) [4-7]. GSH protects cells from oxidant damage [8,9] and has been shown to reduce levels of reactive free radicals, including semiquinones and the hydroxyl radical. Furthermore, in view of the susceptibility of the mitochondrial electron transport chain (ETC) to oxidative stress, loss of GSH is associated, in some cell types, to impaired activity of one or more of the respiratory chain complexes [10,11].

With regards to Parkinson's disease, post mortem studies reveal a loss of mitochondrial function, in particular at the level of complex I [12,13] and this defect is associated with a loss of GSH. Since studies upon brain material from patients with incidental Lewy body disease (thought to be presymptomatic Parkinson's disease) reveal GSH deficiency in the absence of mitochondrial dysfunction [7] it has been proposed, in view of the susceptibility of the ETC to oxidative damage, loss of GSH may be an early and contributing event to the mitochondrial dysfunction noted in Parkinson's disease [14-17].

To date, metabolic studies upon Parkinson's disease material have largely been performed upon post mortem material which can have incurred delays of up to 20 hours. Thus, in view of the vulnerability of the ETC to oxidative stress and the GSH deficiency reported in Parkinson's disease, we considered the possibility that a pre-existing GSH deficiency could elicit an artifactual oxidative inactivation of the ETC during a post mortem-delay. Therefore, in this study, we have depleted glutathione in rats and following postmortem delay, have investigated the maintenance of glutathione levels and the relationship to mitochondrial electron transport chain complex activities.

Materials and Methods

Materials. Ubiquinone-1 was a kind gift from Eisai Chemical Company, Tokyo, Japan. All other chemicals were supplied by either BDH, Dagenham, Essex, U.K. or Sigma Chemical Company, Poole, England. The animals used were adult (250 g) male Wistar rats, supplied by B and K Universal, Aldbrough, Hull, U.K.

Glutathione depletion. Animals were weighed every day and the following dosing protocol was used: 12-day-old pre-weanling rats were used. All animal experiments were carried out under a U.K. Home Office license according to Home Office guidelines. Pups were assigned to either a control or test group and received a single daily subcutaneous dose of L-buthionine sulfoximine (L-BSO) (3.8 mmol/kg) or iso-osmotic saline (controls) at 11.00 am. On day 11 of the injection regime the pups were killed at 9.30 am. After sacrifice (cervical dislocation), the carcasses were left at room temperature for 2 h and then transferred to a 4°C fridge. This procedure is similar to that which occurs during postmortem analysis of brains from Parkinson's disease patients [12,18,19]. Brains were removed at various intervals and used to produce homogenates following division of the brain along the mid-sagittal plane. The left hemisphere was homogenised (25% w/v in 15 mM o- phosphoric acid and following centrifugation (15,000 x g for 10 min) the supernatant was used for GSH 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. Control and test samples were prepared together at 4°C, to remove any effects of time on GSH status. All samples were stored at - 70°C until assayed.

Mitochondrial enzyme assays. All assays were performed at 30°C. Prior to analysis, samples were freeze-thawed and gently shaken 3 times to ensure mitochondrial lysis was complete. Complex I activity was determined using a modification of the method of Ragan et al. [20], and followed the oxidation of NADH at 340 nm using ubiquinone-1 as the electron acceptor. Complex II-III activity was determined as the antimycin A sensitive rate of cytochrome c (III) reduction at 550 nm, using the method of King [21]. Complex IV activity was determined by following the oxidation

of cytochrome c (III) at 550 nm [22] and was expressed as a first order decay rate constant. Citrate synthase activity was measured according to the method of [23].

Determination of glutathione

GSH was assayed by high performance liquid chromatography and electrochemical detection [6] and is expressed as nmol/g wet weight.

Results

Glutathione levels decrease during postmortem delay

Inhibition of gamma-glutamylsynthetase in pre-weanling rats over an 11-day period by L-BSO decreased levels of glutathione by 53% (Fig.1A). Following postmortem delay of 6, 12 and 24 h, glutathione levels decreased in the control rats by 27%, 37% and 58%, respectively. In the L-BSO treated rats, during the postmortem delay of 6, 12 and 24 h, glutathione levels decreased in the control rats by 71%, 72% and 79%, respectively. However, the ratio of glutathione in L-BSO treated rats to control values remained between 50 to 60% at each time-point during the total post-mortem delay period (Fig. 1B).

Glutathione depletion does not affect mitochondrial electron transport chain complex activities

Assay of mitochondrial respiratory chain complex I, II-III and IV activities revealed no changes in control or glutathione-depleted animals (Tables 1, 2 and 3). Similarly, the further reduction in glutathione during postmortem delay did not affect ETC activities. When these activities were expressed as ratios to citrate synthase activities no changes were observed indicating that when glutathione levels are decreased by up to 79% in the pre-weanling brain, there is no affect on ETC activities (Tables 1, 2 and 3).

Discussion

Administration of the gamma-glutamylcysteine synthetase inhibitor, L-BSO, over a period of 10 days decreased glutathione levels by 53% in brain homogenates of the pre-weanling rat (Fig.1). This reduction of glutathione is consistent with that found in

other studies using L-BSO [10,24] and is close to the reported 40% decrease in glutathione levels found in postmortem studies of Parkinson's disease patients [6]. Even though glutathione reduction is found only in the substantia nigra in Parkinson's disease, the general glutathione reduction found in whole brain homogenates from the L-BSO treated preweanling rat provides a useful model in studying the effects of postmortem delay of glutathione levels and mitochondrial respiratory chain control complex activities.

Following a postmortem delay of 24 hours, total glutathione levels in both the control and L-BSO treated rats were decreased by 58 and 79%, respectively (Fig. 1A), suggesting that glutathione undergoes degradation in the rodent brain when left at 4°C. It is not known if glutathione levels are higher in normal and Parkinson's disease patients before death and rapidly decrease during the postmortem period, however, the results with the rodent model of glutathione depletion suggest that such a possibility should be considered. The fact that the ratio of glutathione in L-BSO treated rats to glutathione in control rats does not change and remains between 50-60% during the delay period is important, as it demonstrates that the levels of glutathione depletion in the rodent brain decrease during the postmortem delay period at the same rate as in normal controls.

Postmortem delay-related changes in enzyme activity is well documented and may be an added factor when considering results of human tissue examination. For example, previous studies on postmortem delay showed that glutathione reductase and catalase were reduced by 40 % and 10 %, respectively, after a 24 hour delay while glutathione peroxidase activities remained normal [25]. Similarly, tyrosine hydroxylase, DOPA decarboxylase, monoamine oxidase and choline acetyl transferase activities were reduced by 10-45% after a 14 hour delay, while glutamic acid decarboxylase, Na-K-ATPase and lactate dehydrogenase remained stable [26]. In addition, postmortem delay may result in increased or decreased binding of neurotransmitters and ligands to receptors in the brain [27-30].

In the case of Parkinson's disease, the possibility that decreased concentrations of glutathione in the brain may have secondary implications for complex I activity has

been proposed [15,17,16,31,14,32] however measurement of the respiratory chain complex I, II-III and IV activities indicated that depletion of glutathione in the rat brain did not effect these activities (Tables 1, 2 and 3). This finding is in agreement with similar studies involving stereotaxic administration of L-BSO into adult rat brain [33]. While mitochondrial swelling, reductions in citrate synthase have been reported in non synaptic mitochondria prepared from rats that were pre-treated with L-BSO for 10 days [24,10], no effects on complex I activities were found in either homogenates or synaptic mitochondria.

In the case of complex IV, reduced activities (27%) were found in gradient-purified synaptic mitochondria but not in homogenates [10], indicating that enrichment of brain mitochondrial fractions from glutathione depleted rats reveals complex IV defects. However, the consequences of such a complex IV deficiency on oxidative phosphorylation in brain mitochondria may be limited as complex IV activities must be inhibited by more than 60% in synaptic and non synaptic mitochondria before rates of respiration and ATP synthesis are severely affected [34-36, 37].

During the postmortem delay period, glutathione levels were decreased, although this was found not to affect complex I, II-III or IV activities. These results do not support suggestions that the complex I defect observed in postmortem studies of the parkinsonian brain is associated with reduced levels of glutathione that may occur during the disease progression or the postmortem delay period. The results presented in this study differ from other studies that selectively depleted glutathione levels in cell culture models and observed decreases in complex I activities [36,15,16, 31,14,32]. In PC12 cells treated with L-BSO, glutathione levels were reduced by 90 % with a subsequent 50% decrease in complex I activity and an ablation of complex I energy thresholds [36,38]. Reduced complex I activities are mainly specific to studies with neuronal cultures in which glutathione concentrations are almost totally ablated and may reflect a reliance on glycolytic metabolism in neurons and a reduction in mitochondrial metabolism, compared to the *in vivo* situation where mitochondria supply the majority of ATP in the neuron. One recent report suggested that glutathione depletion in adult mice using L-BSO induces a 40 % decrease in brain complex I activity [39] however, other studies have shown that L-BSO does not

cross the blood brain barrier in adult mice or rats and has no effect on brain levels of glutathione [40]. In fact, only after acute and repeated intracerebroventricular infusion will L-BSO deplete glutathione levels [41] and even then, will not cause any nigrostriatal degeneration [42]. In a transgenic mouse model in which glutathione levels were reduced by 30-40%, complex I activity was found to be reduced by 26% in dopaminergic synaptosomes [31] however, the use of 2,6-dichlorophenolindophenol in the complex I assay may be problematic as 2,6-dichloroindophenol is capable of conjugating with mitochondrial thiol groups [43] and reduced glutathione [44].

Glutathione depletion in the pre-weanling rat is a limited model as it may not necessarily reflect the effects of glutathione depletion in the aged or parkinsonian brain. The relative variations in levels of GSH in different brain regions are cortex>cerebellum>hippocampus>striatum>SN [45-47], however, GSH profiles change through the lifespan, from high values during growth dropping to a maturation plateau and then decreasing 30% during aging [45]. Following preparation of synaptosomes from mouse brain, depletion of glutathione *in vitro* decreased ATP levels and viability of synaptosomes from aged mice but not from young mice [48]. Nevertheless, following *in vivo* depletion of glutathione and a 24 h postmortem delay in which *in* glutathione levels are decreased by 79 %, approximately 2-fold more than in Parkinson's disease, there was no effect on complex I activity or complex II-III and IV activities.

These results suggest that the cause of complex I deficiency in Parkinson's disease is still unknown but is unlikely to be a postmortem artifact arising from a pre-existing glutathione deficiency state. Reduced complex I activities in mitochondria from Parkin and PINK1 knockout animals [49,50] as well as in leukocytes from patients with parkin mutations have been identified [51], although interestingly, in parkin/DJ-1/PINK1 triple knockout mice no such deficiencies are present [52]. While such findings reveal possible causes of mitochondrial dysfunction in familial Parkinson's disease, the cause of complex I deficiency in the sporadic disease remains unknown.

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Abbreviations: L-BSO = L-buthionine sulfoximine; Complex I = NADH:ubiquinone oxidoreductase; EC 1.6.5.3; Complex II-III = succinate: cytochrome c reductase; EC 1.3.5.1 + EC 1.10.2.2; Complex IV = cytochrome c oxidase; EC 1.9.3.1.

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Figure Legends

Fig. 1 Postmortem changes in whole brain levels of glutathione. (a) Preweanling rats were treated with or without L-BSO for 10 days. Following postmortem delay, where animals were left at 4°C, GSH levels were measured in homogenates of the right hemisphere. (b) GSH levels in L-BSO treated rats were expressed as ratios to control GSH levels. Data are expressed as the mean of activities obtained from 3 rats $\pm$ SEM. * $p < 0.01$

Table 1. Complex I activity is not affected by postmortem delay. Following post-mortem delay, the left hemisphere of animals treated $\pm$ L-BSO were homogenised and assayed for complex I activity and expressed as a ratio to citrate synthase activities as described in the Materials and Methods section. Data are expressed as the mean of activities obtained from 3 rats $\pm$ SEM.

Post Mortem Delay (h)	Complex I (nmol/min/mg)		Complex I /Citrate Synthase Ratio	
	Control	L-BSO	Control	L-BSO
0	32.47 $\pm$ 3.85	31.28 $\pm$ 5.44	0.088 $\pm$ 0.012	0.089 $\pm$ 0.016
6	28.13 $\pm$ 1.27	30.90 $\pm$ 1.48	0.078 $\pm$ 0.004	0.086 $\pm$ 0.006
12	31.81 $\pm$ 0.53	32.91 $\pm$ 2.45	0.087 $\pm$ 0.002	0.851 $\pm$ 0.007
24	34.48 $\pm$ 0.74	34.03 $\pm$ 0.85	0.095 $\pm$ 0.004	0.088 $\pm$ 0.001

Table 2 Complex II-III activity is not affected by postmortem delay. Following post-mortem delay, the left hemisphere of animals treated $\pm$ L-BSO were homogenised and assayed for complex II-III activity and expressed as a ratio to citrate synthase activities as described in the Materials and Methods section. Data are expressed as the mean of activities obtained from 3 rats $\pm$ SEM.

	Complex II-III (nmol/min/mg)		Complex II-III /Citrate Synthase Ratio	
	Control	L-BSO	Control	L-BSO
Post Mortem Delay (h)				
0	38.97 $\pm$ 1.92	36.23 $\pm$ 2.63	0.105 $\pm$ 0.008	0.103 $\pm$ 0.008
6	34.69 $\pm$ 0.15	37.34 $\pm$ 1.26	0.097 $\pm$ 0.002	0.104 $\pm$ 0.004
12	35.31 $\pm$ 0.97	38.93 $\pm$ 2.14	0.096 $\pm$ 0.016	0.101 $\pm$ 0.006
24	33.53 $\pm$ 0.89	35.30 $\pm$ 1.31	0.092 $\pm$ 0.004	0.091 $\pm$ 0.003

Table 3 Complex IV activity is not affected by postmortem delay. Following post-mortem delay, the left hemisphere of animals treated $\pm$ L-BSO were homogenised and assayed for complex IV activity and expressed as a ratio to citrate synthase activities as described in the Materials and Methods section. Data are expressed as the mean of activities obtained from 3 rats $\pm$ SEM.

	Complex IV (K(min ⁻¹ .mg ⁻¹))		Complex IV/ Citrate Synthase Ratio	
Post Mortem Delay (h)	Control	L-BSO	Control	L-BSO
0	5.362 $\pm$ 0.140	5.161 $\pm$ 0.237	0.015 $\pm$ 0.001	0.015 $\pm$ 0.001
6	4.457 $\pm$ 0.152	4.401 $\pm$ 0.081	0.012 $\pm$ 0.001	0.012 $\pm$ 0.000
12	4.446 $\pm$ 0.935	4.877 $\pm$ 0.221	0.012 $\pm$ 0.003	0.013 $\pm$ 0.001
24	5.123 $\pm$ 0.162	4.882 $\pm$ 0.164	0.014 $\pm$ 0.001	0.013 $\pm$ 0.000

Figure 1A

Figure 1B