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Oxidative Damage and Motor Neurone Disease Difficulties in the Measurement of Protein Carbonyls in Human Brain Tissue

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It has been suggested in the literature that elevated oxidative protein damage, measured as protein carbonyls, is present in the nervous system of patients with sporadic motor neurone disease (MND). However, the actual reported levels of brain protein carbonyls vary over a wide range. We show here that this is probably due to the use of different protocols for the carbonyl assay; results differ depending on when the dinitrophenylhydrazine reagent is added and at what stage in the procedure protein is assayed for the calculation of carbonyls on a unit protein basis. Using a range of different procedures, we were unable to confirm reports of elevated protein carbonyls in motor cortex from brains of patients with MND. We also measured thiobarbituric acid-reactive material in the brain samples using an HPLC-based TBA test in the presence of butylated hydroxytoluene. In general, there was no significant elevation of TBARS in MND motor cortex. However, four patients showed values higher than any of the control patients (both 'normal' control and 'disease control'). There was no correlation of TBARS with protein carbonyl values. We suggest that oxidative damage in motor cortex in sporadic MND, if it occurs, may be confined to a small group of patients and may affect different molecular targets in each patient.

Key words: motor neurone disease, superoxide dismutase, carbonyl assay, oxidative stress, dinitrophenylhydrazine, lipid peroxidation

INTRODUCTION

There is growing evidence that oxidative damage to lipids, proteins and DNA contributes to the pathology of neurodegenerative diseases, although its exact role in the disease process remains to be determined.[1–7] Considerable excitement about the possible role of oxidative stress in motor neurone disease (MND) was generated by reports of mutant superoxide dismutase enzymes in patients with the familial autosomal dominant form of this disorder.[8,9] Oxidative damage to proteins may be of particular importance in neurodegenerative disease,[1–4,7] and there have been two reports of elevated brain or spinal cord protein carbonyls in MND patients with normal

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SOD activity.[10,11] The carbonyl assay was developed as a 'general assay' of oxidative protein damage: it is based on the fact that several oxygen-derived species can attack amino acid residues (particularly histidine, arginine, lysine and proline) to produce carbonyl (>C=0) functions that can be measured after reaction with 2,4-dinitrophenylhydrazine.[7,12–15] The carbonyl assay has become widely used and many laboratories have developed individual protocols for it (e.g.[11,13–16]). Sometimes the exact assay procedures used in a particular laboratory are not specified in published papers (e.g.[10]) and even when they are, they often differ from those used originally by the group of Stadtman *et al.* (e.g.[11,16]). This variation in protocols may account for the fact that 'baseline' levels of protein carbonyls in brain tissue vary over a wide range (Table 1).

The object of the research in this paper was to investigate the reasons for the wide variation in reported results (Table 1) and to attempt confirmation of the reported elevated levels of protein carbonyls in MND patients.[10,11] As a further test of the hypothesis that oxidative stress is a common feature of MND, we examined the levels of lipid peroxidation end-products in motor cortex from patients and appropriate controls using an HPLC-based thiobarbituric acid test.[20]

MATERIALS AND METHODS

Reagents

Coomassie Blue protein reagent was from Pierce. All other reagents were of the highest quality available from Sigma Chemical Co., Poole, Dorset, UK.

Brain Tissue Sampling

At autopsy the brain was dissected as soon as possible to obtain tissues for rapid freezing. The brainstem was removed by a horizontal incision through the upper pons. The diencephalon was bisected and the cerebral hemispheres separated by incision in the sagittal plane. The left cerebral hemispheres were further subdisected by slicing 1 cm coronal sections, after removing the

TABLE 1 Selected Literature Values for Brain Protein Carbonyls

Samples studied	Carbonyl value (nmol/mg)	Comments	Reference
Human brain, frontal and occipital cortex	Alzheimer 4.51–7.14 Age-matched controls 4.00–6.43	Not clear whether DNPH reagent was added to protein in solution or after acid precipitation. Pierce BCA method used to measure protein, not A_{280}.	[7]
Human brain, frontal cortex	1.51 ± 0.80 controls 2.79 ± 1.13 MND	Method of Levine *et al* [13] used with unstated 'minor modifications'	[10]
Rat brain	1.6 ± 0.6 control 3.2 ± 0.8 Mg deficient	Method of Oliver *et al* [17] used. DNPH reagent added to precipitated protein. Pierce BCA assay used to measure protein instead of A_{280}.	[18]
Gerbil brain	~6.8 (control) 8.8–11.8 after 10 min ischaemia/reperfusion	Method of Oliver *et al* [17] used.	[19]
Human lumbar spinal cord	0.73 ± 0.63 controls 1.60 ± 1.23 MND 0.85 ± 0.62 other neurological disease controls	Modified version of assay used in which protein measured by Lowry method in initial supernatant	[11]

DNPH: dinitrophenylhydrazine

midbrain. The motor cortex was identified in the appropriate coronal slices. The slices were sealed in polythene and snap frozen by immersion in melting Arcton surrounded by a liquid nitrogen bath. They were stored at – 80°C until required.

Patient Details

The patient groups comprised cases of motor neurone disease (MND) (n = 22), normal controls (n = 10) and disease controls (n = 11). The MND patients were diagnosed clinically by the standard diagnostic El Escorial criteria[21] and by detailed neuropathological examination. All the cases showed lower motor neurone loss in spinal cord and/or brainstem levels, together with characteristic ubiquitinated inclusion bodies.[22] The presence of upper motor neurone degeneration (loss of Betz cells or pyramidal tract degeneration) was present in 15 cases (amyotrophic lateral sclerosis ALS). In 7 cases upper motor neurone degeneration was not diagnosed either clinically or pathologically (progressive muscular atrophy PMA, El Escorial[21] 'suspected ALS'). Two cases with a family history of ALS were included in the series, one of whom had the GLU 100 GLY mutation in exon 4 of the gene encoding Cu/Zn superoxide dismutase. The MND group comprised 11 males and 11 females. The mean age was 61.5 ± 13.2 (mean ± SD) years (range 40–89 years). The mean delay from death to freezing of tissue was 17.5 ± 8.7 (mean ± SD) hours (range 9–48 hours).

The normal control group comprised 8 males and two females. The mean age was 69.1 ± 9.3 (mean ± SD) years (range 54–82 years). The mean delay from death to freezing of tissue was 18.4 ± 6.7 (mean ± SD) hours (range 10–31 hours). The causes of death in the normal control group were as follows: Ischaemic heart disease (5), respiratory failure (2) and carcinoma of the lung, rectum or uterus (3).

The disease control group comprised patients with neurodegenerative disorders who had a similar mode of death and agonal status compared to the MND group. This group included patients with the following diagnoses confirmed on neuropathological examination: Alzheimer's disease (4); Senile dementia of Lewy body type (3); Pick's disease (1); Olivopontocerebellar degeneration (1); Friedreich's ataxia (1) and Huntington's disease (1). This group comprised 5 males and 6 females, with a mean age of 65.6 ± 16.5 (mean ± SD) years (range 35–85 years). The mean delay from death to freezing of tissue was 17.7 ± 7.5 (mean ± SD) hours (range 9–34 hours).

Assay of Brain Protein Carbonyls

Two different methods were carried out on the brain protein samples. **Method A:** is based on the method described by Reznick and Packer.[16] **Method B:** is based on the method of Levine *et al.*[13] Full experimental protocols are given below, and the major differences between the two methods are written in italics in method B.

METHOD A

Brain tissue sample (200 mg) was homogenised in 2 ml of homogenising buffer (100 mM KH_2PO_4 – K_2HPO_4, pH 7.4 plus 0.1% digitonin) in a 2 ml glass homogeniser. The homogenised tissue was transferred to a plastic tube and a 10% (w/v) streptomycin sulphate solution added to give a final concentration of 1%. The solution was mixed and left to stand at room temperature for 10 minutes, followed by centrifugation for 10 min at 4000 r.p.m. The supernatant was removed and 0.8 ml divided equally into two 12 ml plastic centrifuge tubes. For each 0.1 ml of supernatant present, 0.4 ml of 10 mM dinitrophenylhydrazine (DNPH) in 2M HCl was added to one tube and 0.4 ml of 2M HCl to the other. The tubes were then incubated for 60 min on a rotator and the protein precipitated out by adding an equal volume of 20% (w/v) trichloroacetic acid. The protein precipitate was pelleted at 5000 r.p.m., the supernatant discarded and the pellet washed with 1.5 ml of a 1:1 ethyl acetate:ethanol mixture to

remove excess DNPH and soluble non-protein DNPH carbonyls. This was repeated three times. The final protein pellet was dissolved in 1.25 ml of 6M guanidine HCl and the absorbance of both (DNPH and HCl) solutions measured at 280 nm and 370 nm. Carbonyl values were calculated as in.[16]

The protein content of some of the final protein solutions was also measured by the Coomassie Plus Protein Assay reagent,[23] based on the absorbance shift from 465 to 595 nm that occurs when Coomassie Blue G-250 binds to proteins in an acidic solution. Calibration curves were obtained by diluting a stock solution of bovine serum albumin (BSA) into 6M guanidine hydrochloride.

METHOD B

The brain tissue sample (200 mg) was homogenised, treated with streptomycin sulphate and centrifuged at 4000 r.p.m. as described for method A. The supernatant was removed and 0.8 ml divided equally into two 1.5 ml Eppendorf tubes. *An equal volume of 20% (w/v) TCA was added so that the protein was precipitated. It was spun down at 4000 r.p.m., and the liquid remaining sucked off using a vacuum pump. 0.5 ml of 10 mM DNPH (in 2M HCl) was added to one tube and 0.5 ml of 2M HCl to the other. The tubes were then left standing for 1 h and then a further 0.5 ml of 20% (w/v) trichloroacetic acid was added to each tube.* The protein was spun down at 5000 r.p.m., the supernatant discarded and the pellet washed with 1.5 ml of a 1:1 ethyl acetate:ethanol mixture. This was repeated three times. The final protein pellet was dissolved in 1.25 ml of 6M guanidine HCl and the absorbance of both (DNPH and HCl) solutions measured at 280 nm and 370 nm.

Lowry Protein Assay

Brain homogenates were diluted in water to contain 25–500 μg/ml protein (usually a 1:50 dilution). Solution C (1 ml) was added to sample (0.2 ml), mixed and left to stand at room temperature for 10 min. Solution D (0.1 ml) was added and the solution mixed immediately. After 30 min, the absorbance was read at 750 nm. BSA was used to construct a calibration curve. Composition of solutions: C – Mix 50 ml of solution A with 1 ml of solution B (fresh) when required); A – 2% (w/v) Na_2CO_3 in 0.1M NaOH; B – 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) trisodium citrate; D – Sigma Folin and Ciocalteu's reagent diluted with water to make it 1M in acid (a ~2 fold dilution is normally required).

Measurement of TBA-reactive Material by HPLC

Brain tissue (100 mg) was added to 0.9 ml homogenisation buffer [KH_2PO_4-KOH buffer (pH 7.4) + 0.1% digitonin]. 100 μl of butylated hydroxytoluene (2g/litre in ethanol) was added and the tissue homogenised in a glass homogeniser. Two 0.25 ml samples were taken and 1.5 ml of 0.44 M H_3PO_4 was added to each. Samples were left standing for 10 min at room temperature and then 0.5 ml 1% (w/v) thiobarbituric acid (TBA) was added. Samples were heated at 90°C for 30 min, allowed to cool and centrifuged. The clear supernatant was taken and 20 μl injected onto a Spherisorb 5ODS2 (C_{18}) column with a guard column (Hiber C8). Elution was with 65% (v/v) 50 mM KH_2PO_4-KOH buffer pH 7.0 and 35% (v/v) methanol at a flow rate of 1 ml/min. Absorbance of the sharp peak at retention time 4.8 min was read at 532 nm.

RESULTS

Protein carbonyls were measured on brain cortex samples from 22 patients with motor neurone disease (MND) (15 with ALS, 7 with PMA), 10 normal controls (NC) and 11 disease controls (patients with other neurodegenerative disorders). One set of measurements was made by the method of Reznick *et al* [16], in which protein is reacted with

DNPH before precipitation and carbonyl determination (METHOD A). The samples were assayed in parallel as described by Levine *et al*,[13] in which protein is precipitated from the tissue homogenate and reacted with DNPH before being resuspended for carbonyl determination. We also measured the initial protein content of the homogenates using the Lowry assay.

When Should Protein be Measured?

One approach is to use the A_{280} of the *final* protein solution in guanidine hydrochloride to calculate protein concentration, but sometimes protein carbonyl values have been based on the protein content of the *initial* brain homogenate, e.g. as determined by the Lowry method.[11] We found that it was also possible to determine protein content on the final suspension using the Coomassie Blue method. The results obtained correlated well with those obtained by A_{280} (Figure 1), which suggests that A_{280} measurement is a reliable way of determining final protein content. Interestingly, however, protein levels as determined by Coomassie blue (using a calibration curve of BSA in guanidine hydrochloride) were reproducibly less than those calculated from A_{280} values.

However, when the results of final A_{280} protein

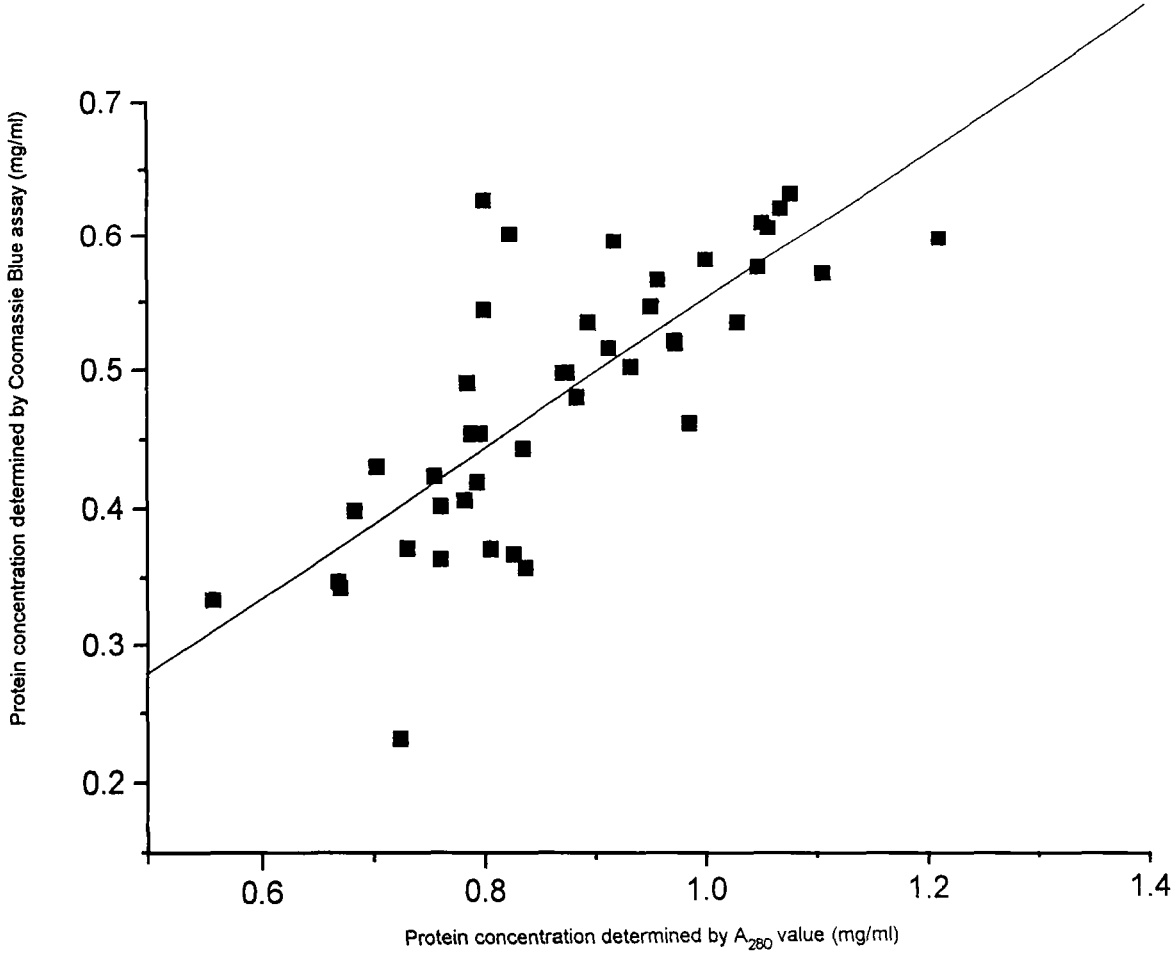

FIGURE 1 Correlation graph of MND brain cortex protein concentrations determined (on final solutions) by A_{280} and Coomassie blue assay on protein samples obtained from Method A. For simple linear regression ($y = A + B.x$), $r = 0.77$, $B = 0.54 \pm 0.07$ ($n = 43$).

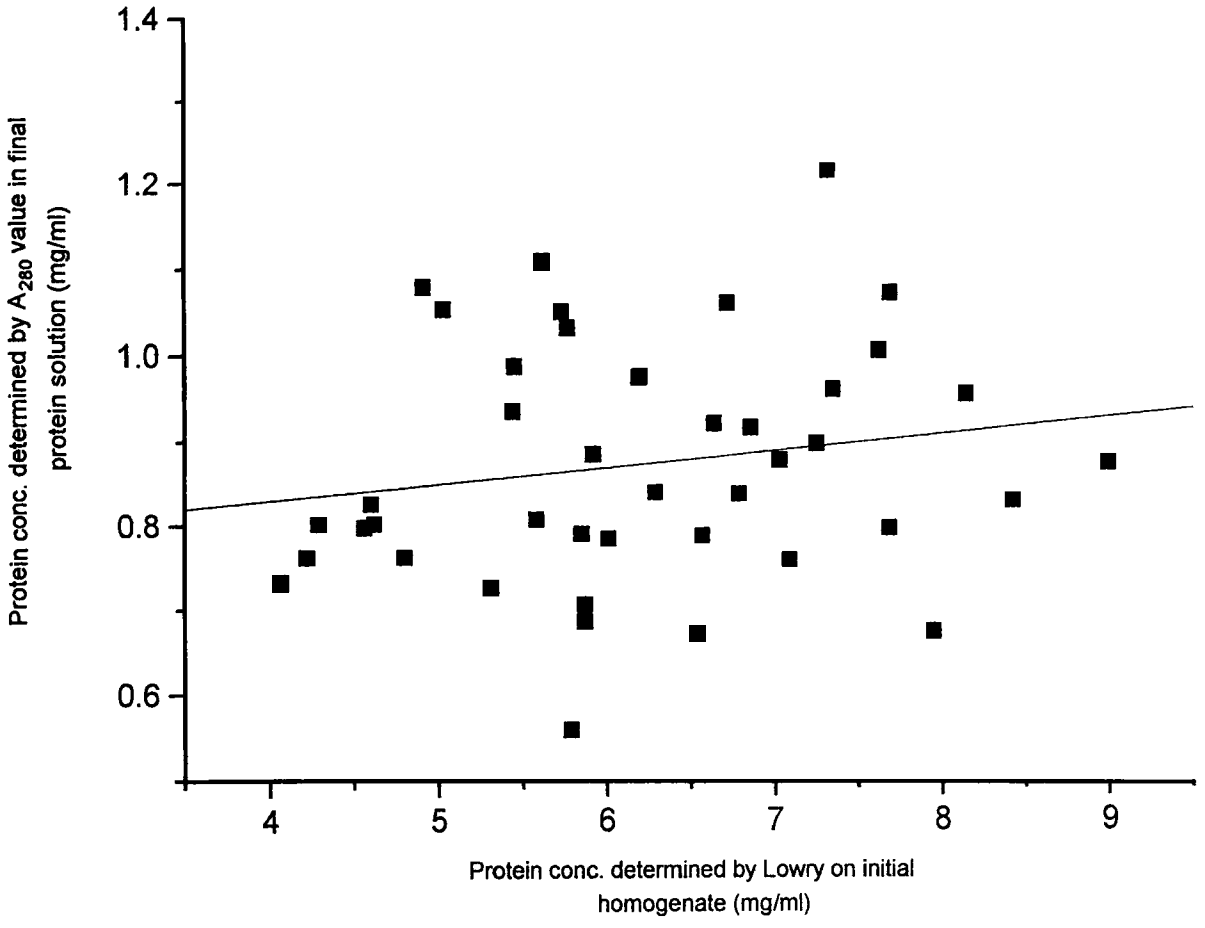

FIGURE 2 Correlation graph between MND brain cortex protein concentrations determined by Lowry assay on initial tissue homogenate and A_{280} values of the final solution. For simple linear regression r = 0.165 (n = 43).

or Coomassie blue determinations were compared with those of Lowry measurements on the initial homogenate, the correlation was poor (Figure 2). We conclude, in agreement with Reznick and Packer,[16] that losses of protein are variable during the extraction and washing procedures, i.e. it is questionable to calculate protein carbonyl values on the basis of protein determinations in the initial tissue homogenate.

Protein Carbonyls in Human Brain Cortex

Figure 3 summarizes the results obtained. It may be seen that the levels of protein carbonyls in cortex vary over a wide range depending on which assay procedure is used. Table 2 summarizes the mean and SD values. No significant difference was found between the normal controls, the MND cases and the 'other disease' controls, whichever assay procedure was used. There was also no significant difference between the ALS and PMA sub-groups of the MND patients.

Measurement of Lipid Peroxidation End-products

Figure 4 shows the results of HPLC-based TBARS determination upon brain cortex samples. There

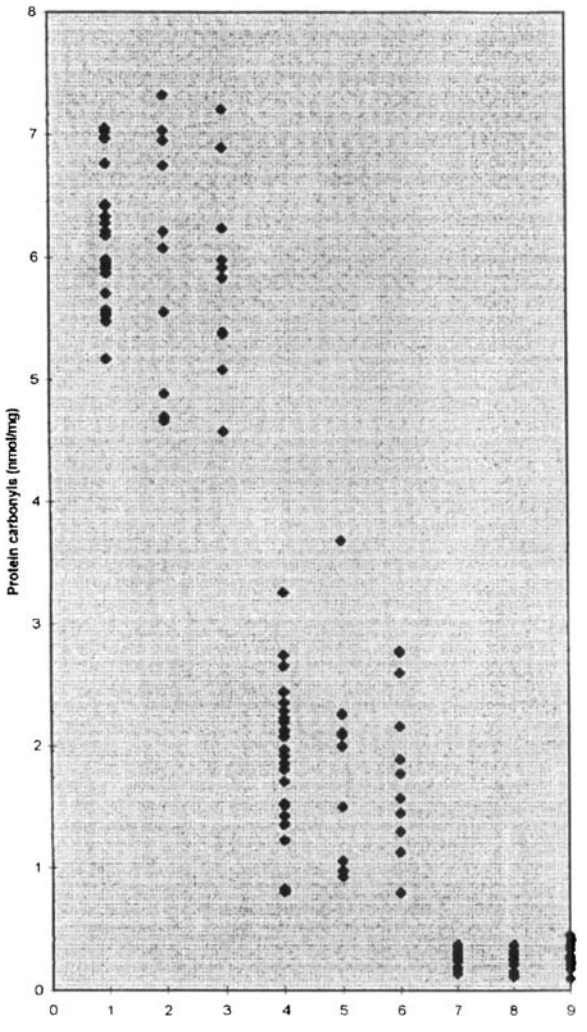

FIGURE 3 Protein carbonyl values obtained by using Method A and Method B on motor cortex samples from patients with motor neurone disease (MND), normal controls (NC) and patients with other neurodegenerative diseases (DC). key: *method A* 1 MND, 2 NC, 3 DC; *method B* 4 MND, 5 NC, 6 DC; *method B but carbonyl calculation based upon protein concentration of initial brain homogenate as determined by the Lowry method* 7 MND, 8 NC, 9 DC.

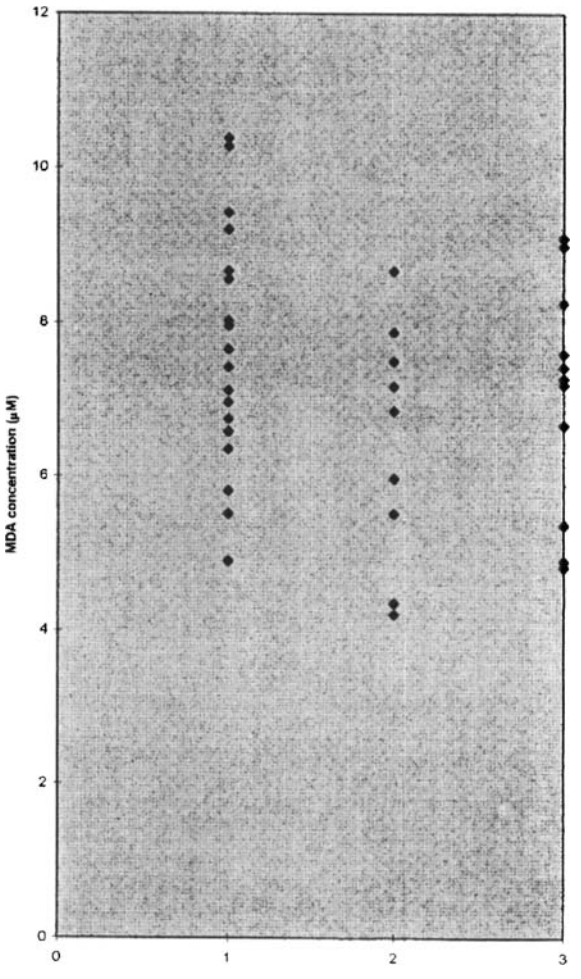

FIGURE 4 Lipid peroxidation in brain cortex from motor neurone disease (MND) patients, normal controls (NC) and disease controls (DC). Results are expressed as μM malondialdehyde equivalents. Values were (mean ± SD) MND 7.70 ± 1.54; NC 6.60 ± 1.53; DC 7.05 ± 1.50. key 1 = MND, 2 = NC, 3 = DC.

was a suggestion of elevation in the MND subjects, in that 4 of these subjects showed values higher than in either of the control groups. However, there was no statistically-significant difference between the groups when all samples were considered ($p>0.05$ in all cases). We attempted to correlate protein carbonyl levels with TBARS, but no significant correlation was observed in any group of subjects (e.g. Figure 5). In particular, the 4 patients with especially high brain TBARS did not show high protein carbonyl values.

DISCUSSION

The carbonyl assay is a widely used assay to measure oxidative protein damage, but some authors have criticized it (e.g.[24]). However, the data in Table 1 suggest that more comparison and

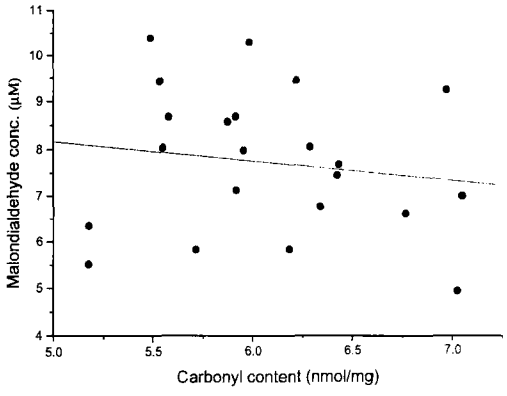

FIGURE 5 Lack of relationship of protein carbonyl content to lipid peroxidation (as assessed by TBARS) in brain cortex from motor neurone disease patients. For simple linear regression, r = 0.16.

standardization of the different methods used by various laboratories are required.

Protein Determination in the Carbonyl Assay

Carbonyl content values in proteins are expressed as nmol/mg of protein. Therefore it is necessary to determine the protein concentration of the samples. This can either be done on the initial supernatant by using a protein assay (e.g. Pierce [Table 1] or Lowry) or on the final guanidine hydrochloride solution by measuring A_{280} or by the Coomassie blue method used here. A_{280} measurements would be expected to be prone to interference by the many biologically-important substances other than proteins that absorb at this wavelength. However, we would expect such substances to be removed by the multiple washing procedures. The Coomassie blue method applied to the final protein suspensions gave results that correlated well with A_{280}, although were about 50% lower. We do not know the reason.

Whereas Levine *et al*[13] state that protein recovery is generally excellent throughout the carbonyl assay procedure, our comparison of protein determinations in the initial supernatant with A_{280} values suggests that considerable and variable protein loss occurs; the several washing stages with ethanol:ethyl acetate may be particularly prone to variable losses of protein. Reznick and Packer[16] state that about 10–15% of protein is lost in the various washing steps and they recommend that protein levels are determined in the actual final suspensions (by the A_{280} value), after all washings are finished. Our data support this view and suggest that use of the A_{280} value to determine protein concentration of the final protein suspension is preferable to calculating carbonyl values based upon measuring protein concentration in the initial supernatant.

Precipitation of Protein before Addition of DNPH Reagent

In the method described in,[13] protein is dried or precipitated from solution and DNPH reagent is added to a protein pellet. This is done when dealing with low amounts of protein in order to concentrate the proteins. By contrast, Reznick and Packer[16] use larger amounts of proteins and DNPH is reacted with soluble proteins. Our data show that precipitating the protein before addition of DNPH (Method B), alters the results significantly compared to omitting the precipitation step. However, we found that the protein concentrations estimated by A_{280} were very similar and

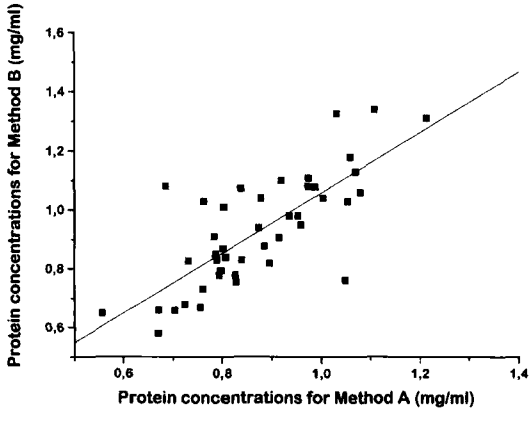

FIGURE 6 Protein concentrations as determined by A_{280} in the final guanidine hydrochloride suspension of brain samples from MND patients. A comparison of method A and method B. Simple linear regression ($y = A + B.x$) r = 0.76, B = 1.02 ± 0.14.

TABLE 2 Human Brain Protein Carbonyls in motor neurone (MND), normal (NC) and disease control (DC) Subjects Measured by Different Procedures

	Carbonyls (nmol/mg protein) ± mean DF		
Method used	MND	NC	DC
A	6.07 ± 0.56	6.02 ± 1.02	5.81 ± 0.77
B	1.93 ± 0.61	1.89 ± 0.83	1.84 ± 0.67
B*	0.28 ± 0.06	0.25 ± 0.09	0.28 ± 0.11

* (based on Lowry protein in initial brain homogenate)

highly correlated for both methods (Figure 6). This suggests that the difference in carbonyl values may relate to incomplete reaction with DNPH when the protein is in solid form. We recommend reaction of the protein with DNPH in solution whenever possible.

Is There Increased Oxidative Protein Damage in MND?

Our data show that the variations in baseline protein carbonyl levels in brain tissue reported in the literature (Table 1) are probably due to the use of different methodology and emphasize the need for a full description of experimental protocols in papers dealing with this topic. However, whatever assay method we used, we could not confirm the report of an elevation of brain protein carbonyls in cortex from MND patients.[10] Unfortunately, this report did not spell out the details of the methodology used.

We also examined another marker of oxidative stress, lipid peroxidation, in the MND samples. Peroxidation was assessed by the HPLC-based TBA test. Again, no statistically-significant differences were seen. However, 4 of the MND patients showed TBARS levels higher than any of the control subjects. To some extent our data resemble those in,[11] in which only a percentage of MND patients showed elevated protein carbonyls in the spinal cords. In our studies, however, the patients with high TBARS values did not show particularly high protein carbonyl values in cortex, i.e. these two markers of oxidative damage were poorly correlated. One possibility is that there are different sub-groups of MND patients showing elevations in different parameters of oxidative stress. It is particularly interesting that patients with SOD defects (familial MND) did not show elevated protein carbonyls in either of the previous literature reports.[10,11]

In conclusion, our data emphasize the difficulty of measuring parameters of oxidative protein damage in brain tissue. Much more attention to methodology is required before we can be certain that oxidative damage is really elevated in MND. In general, severe oxidative stress produces demonstrable rises in damage to all major classes of biomolecules (e.g. as is seen in patients with active rheumatoid arthritis[25]). It seems that oxidative stress in the motor cortex, if it exists in MND, is not, in general, of that degree of severity.

Acknowledgements

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