

Title:

The presence of ascorbate induces expression of brain derived neurotrophic factor in SH-SY5Y neuroblastoma cells after peroxide insult, which is associated with increased survival.

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Short Title:

Induction of BDNF by ascorbate and peroxide in neuroblastoma cells.

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Abbreviations:

AD, Alzheimer's disease; AB, Amyloid beta; BDNF, Brain-derived neurotrophic factor; DNPH, 2,4-dinitrophenyl hydrazine; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid) 3-carboxy-4-nitrophenyl disulphide; IPG, immobilised pH gradient;

MTT, 3(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium; NADPH, β -nicotinamide adenine dinucleotide phosphate; SB3-10, N-decyl-N,N-dimethyl-3-ammonio-1-propanesulphonate; SSA, 5-sulphosalicylic acid; TBP, tributyl phosphine; TGS, Tris glycine SDS.

Keywords:

Ascorbate, SH-SY5Y, Hydrogen Peroxide, Neurotoxicity

Graphical Abstract:

Figure 4

Abstract:

Oxidative stress and free radical production have been implicated in Alzheimer's disease, where low levels of the antioxidant vitamin C (ascorbate) have been shown to be associated with the disease. In this study, neuroblastoma SH-SY5Y cells were treated with hydrogen peroxide in the presence of ascorbate in order to elucidate the mechanism(s) of protection against oxidative stress afforded by ascorbate. Protein oxidation, glutathione levels, cell viability and the effects on the proteome and its oxidized counterpart were monitored. SH-SY5Y cells treated with ascorbate prior to co-incubation with peroxide showed increased viability in comparison to cells treated with peroxide alone. This dual treatment also caused an increase in protein carbonyl content and a decrease in glutathione levels within the cells. Proteins, extracted from SH-SY5Y cells that were treated with either ascorbate or peroxide alone or with ascorbate prior to peroxide, were separated by 2DE and analysed for oxidation. Co-incubation for 24 hours decreased the number of oxidised proteins (e.g. Acyl CoA oxidase 3) and induced brain derived neurotrophic factor (BDNF) expression. Enhanced expression of BDNF may contribute towards the protective effects of ascorbate against oxidative stress in neuronal cells.

Introduction:

Oxidative damage to proteins, lipids and DNA has been observed in many chronic inflammatory and degenerative diseases [1,2]. These findings implicate oxidative stress in the disease processes, where oxidative stress arises from an imbalance between the rate of oxygen free radical generation, and their removal by low molecular weight scavengers and antioxidant enzymes [3]. The sources of oxygen radicals are diverse in pathophysiology, where they can arise from both endogenous and exogenous processes [4]. Three mechanisms have been proposed to account for elevated oxidative stress in neurodegenerative disease; the mitochondrial hypothesis where defects in the respiratory chain leads to electron loss and oxygen radical formation [5], the response to injury hypothesis where a brain inflammatory response may explain neuronal damage [6] and the generation of peroxides from beta amyloid fragments [7,8].

The brain is particularly vulnerable to oxygen radical damage, through the prevalence of oxidizable polyunsaturated fatty acids in membranes, the presence of redox active metal ions and the high metabolic requirement for oxygen [9]. Normally, neuronal cells accumulate high levels of the antioxidant ascorbic acid, and synthesize greater amounts of glutathione than other cells [10]. Indeed, glutathione and ascorbate are reciprocally linked, where glutathione is important in regenerating ascorbate from its oxidised form [4]. However, epidemiological evidence in elderly subjects has shown lower plasma levels of antioxidants such as ascorbate [11], and an inverse relationship exists between ascorbate levels and Alzheimer disease incidence [12, 13]. Furthermore, in a clinical trial, the antioxidant efficacy of vitamin E was augmented by co-supplementation with ascorbate [14].

In vitro studies have confirmed the importance of ascorbate as a protective molecule against oxidative stress induced neurotoxicity. Studies by McGregor *et al.* [15] have demonstrated that kainiate induced excitotoxicity in the hippocampus can be ameliorated in the rat by dosing with vitamin C. A high concentration of ascorbate (1.5mM) prevented malondialdehyde formation in rat brain microsomes [16]. It has been shown that large amounts of ascorbate added to brain slices prevent subsequent oedema formation [17]. Li *et al.* [18] have also suggested that intracellular ascorbate is an effective antioxidant in neuronal cells (SH-SY5Y), and these findings support a role for ascorbate in protecting neurones from oxidant damage associated with various neurodegenerative diseases. Recent studies investigating the mechanisms of cytoprotection afforded by “antioxidants” such as ascorbate have suggested that their effects may not only lie in their capacity to reduce oxygen radicals, but also in their capacity to elicit expression of other cytoprotective molecules [19, 20]. Therefore, we have investigated the hypothesis that ascorbate protects against oxidative stress by preventing protein oxidation and inducing cellular protection mechanisms.

In this work, we have examined the protective effect of vitamin C against loss of glutathione and induction of protein carbonyls by hydrogen peroxide in neuroblastoma cells, and its capacity to elicit protection through the neuronal proteome using 2-dimensional gel electrophoresis.

Methods:

Cell culture:

Neuroblastoma cells (SH-SY5Y) were cultured using RPMI 1640 with Glutamax I (Gibco) supplemented with fetal bovine sera (15%, Gibco), non-essential amino acid solution (1%, Sigma) and penicillin (0.5U/ml, Gibco) and streptomycin (0.5mg/ml, Gibco). Cells were maintained in 75cm² flasks until confluent.

A 4-hour adaptive phase was allowed prior to start of treatments to allow for normalization of transient redox flux and loss of glutathione (GSH) that is associated with passage. SH-SY5Y cells were subject to 4 paradigms: 1, the true control, which had no oxidative stress or antioxidant added; 2, cells were treated with ascorbate (50-100µM) at 4h after seeding; 3, cells were treated with hydrogen peroxide (50-500µM) at 24h after seeding; and 4, cells were treated with ascorbate (100µM) 4h after seeding and then with hydrogen peroxide (100µM) at 24h after seeding. All four paradigms were incubated for 48h.

Cell viability:

Triplicate samples of SH-SY5Y cells (2×10^5 per well) were seeded in 96-well plates and subjected to the oxidative stress/ antioxidant conditions described. Cell viability was measured as described by Hansen *et al.* [21]. Briefly, two hours prior to the completion of the experiment 3(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium (MTT solution; 25µl, 5mg/ml) was added to the wells. The cells were then incubated at 37°C for 2h before addition of lysis buffer (100µl SDS (20%), dimethylformamide (50%), pH4.7 with acetic acid). Formazan release was read (570nm) after 16h at 37°C in a Dynex plate reader.

Protein assay:

Protein content was determined by the bichinchoninic acid assay [22].

Glutathione assay:

Triplicate samples of SH-SY5Y cells (2×10^5) were harvested after exposure to oxidative stress and antioxidants for the varying times and doses described. Glutathione levels were measured as described by Punchard *et al.* [23]. Briefly, cells were washed in PBS and pelleted prior to protein precipitation and extraction with sulfosalicylic acid (3.3 μ l, 100% w/v). After centrifugation, 125mM sodium phosphate with 6.3mM EDTA (96.6 μ l, pH 7.5) was added. Extracts (25 μ l) were aliquoted into 96-well plates. To each well NADPH (150 μ l, 0.3mg/ml, dissolved in 125mM sodium phosphate with 6.3mM EDTA pH7.5) and 5,5'-dithio-bis(2-nitrobenzoic acid) 3-carboxy-4-nitrophenyl disulphid (DTNB) (50 μ l, 6mM DTNB in 125mM sodium phosphate with 6.3mM EDTA pH7.5) solutions were added. Glutathione reductase (25 μ l, 20U/ml, Sigma) was added to each well and absorbance change (410nm) was recorded every 5min in a Dynex plate reader. Glutathione standards were prepared (0-2.5nmol/well) and treated in the same manner. Data are expressed as nmol glutathione per mg protein in order to correct for any variability in cell number dictated by cell treatment.

Carbonyl ELISA:

Proteins (1 μ g per well) from treated and control SH-SY5Y cells were bound to a Nunc immunosorb 96 well plate and carbonyl groups were derivatized with reduced dinotrophenylhydrazine (DNPH) prior to identification by a mouse monoclonal anti-

DNP-antibody and secondary peroxidase coupled anti-mouse-antibody [24]. Data is expressed as nmol carbonyl per mg protein. This takes into account variability in cell number and protein loss through cell death.

Extraction of proteins:

SH-SY5Y cells (10×10^6) were harvested and then solubilized in rehydration buffer (urea (5M), thiourea (2M) CHAPS (2%), SB3-10 (2%), TBP (2mM), Tris HCl (40mM) and Biolytes 3/10 (0.5%)) by sonication (15min). The protein content of these preparations was determined by *RCDC* protein assay (BioRad).

Focussing of samples:

Immobilised pH gradient (IPG) strips (11cm, pH 3-10, BioRad) were used to separate proteins in the first dimension. Prepared proteins (50 μ g) were applied during rehydration to the IPG strips in rehydration buffer (180 μ l, 16h, 20°C). Proteins were focused on a Multiphor II with a DryStrip Kit and power was supplied by an EPS 3500XL power pack (Amersham Pharmacia Biotech), cooled to 20°C (Grant cooling system). The running conditions were: a gradient of 0V to 500V over 500Vh, 500V to 3500V for 3500Vh and finally 3500V for 90kVh. After focusing the IPG strips were stored at -70°C until required.

2DE:

Prior to the second dimension IPG strips were equilibrated in equilibration buffer (urea (6M), glycerol (20%), SDS (2%), tributyl phosphine (TBP;5mM), Tris (0.375M) pH 8.8) for 20min [25]. After equilibration IPG strips were placed above a 4-20% gradient gel (BioRad) and set in agarose (1%, in Tris/glycine/SDS (TGS)

buffer (BioRad)). The proteins were electrophoresed in BioRad Criterion kit for 70min at 150V, using TGS buffer (BioRad). Gels were then either stained with silver [26] or immunoblotted as described. Silver stained gels were analysed with PDQuest software (BioRad). Changes in protein expression were identified using Boolean interrogation. Protein spots were thus identified as (1) those which were present throughout control, ascorbate and/or peroxide treatment (2) those that showed altered expression, and (3) those which were only present upon treatment. Changes in expression were subject to statistical analyses within the software, allowing fold changes in expression to be identified. Protein spots that showed a fold change of 5, where the set included proteins spots whose quantity in treated cells was at least 5 times that of the corresponding spot in non-treated cells, were selected for further study.

Immunoblotting and detection of oxidized proteins:

Second dimension gels were transferred to a Hybond-P (PVDF) membrane (Amersham Pharmacia Biotech) using a BioRad Criterion Western Blotter kit for 2h at 170mA. After transfer the membrane was blocked in TBS-Tween-20 (0.5%) overnight. Membranes were then probed using an indirect method with for carbonyl groups with an anti-DNP antibody (1:2000, mouse monoclonal, Sigma) after sensitisation of carbonyl groups with 25mM DNPH. The primary antibody was detected with an anti-mouse peroxidase-conjugated antibody (1:4000, goat, Sigma). Oxidized proteins were visualized with *ECL plus* (Amersham Pharmacia Biotech). Films of each blot were analysed with PDQuest (BioRad) after scanning on a GS-710 Densitometer. After detection antibodies were removed with Restore buffer (Pierce)

and proteins on the blot were detected with colloidal gold stain (Aurodye Forte, Amersham Pharmacia Biotech).

Identification of proteins:

For all protein identification by mass fingerprinting, 6 gels were run and relevant spots from all gels were pooled to gain sufficient quantities. Second dimension gels were silver stained [26], proteins of interest were excised and tryptically digested as described by Gharahdagli *et al.* [27]. Peptides were extracted from the gel pieces as described by Hellman *et al.* [28]. Peptides were desalted with C18 ZipTips (Millipore) and mass fingerprints were produced at Alta Bioscience (Birmingham UK) by MALDI-TOF MS on a SIA (Manchester) LT3 LazerTOF with a nitrogen laser. The MS data were searched against a subset of human proteins in the MSDB database using the Mascot search program (Matrix Science, London, UK, www.matrixscience.com). Positive protein identification was based on standard Mascot criteria for statistical analysis.

Immunoblotting

Cell extract (20 μ g) was separated by SDS-PAGE on 12.5% polyacrylamide gels. After transfer to PVDF membrane BDNF protein was selectively detected using a mouse monoclonal antibody directed against human BDNF (Abcam). A monoclonal antibody to mouse IgG conjugated to peroxidase (Sigma) and the *ECL plus* system (APBiotech) was used to visualize BDNF signals. To demonstrate equal protein loading blots were analysed with an antibody against β -actin (Abcam).

Statistics

For biochemical assays, data are reported from three independent experiments. Data was evaluated statistically for multi-comparison using ANOVA with Dunnet's post test, where $p \leq 0.05$ was considered significant. For 2DE, cell extracts from six independent experiments were analysed.

Results:

Incubation of SH-SY5Y cells with hydrogen peroxide decreased cell viability in a dose dependent manner. No significant decrease in viability was seen following treatment with 100 μ M hydrogen peroxide (24h) but at 250 μ M a 69% reduction in viability that progressed to 92% after 500 μ M hydrogen peroxide treatment was observed (Figure 1). However, preincubation of SH-SY5Y cells with 100 μ M ascorbate for 24h prior to co-incubation with peroxide (100 or 500 μ M) protected cell viability over the following 24h period. Ascorbate alone (50 μ M and 100 μ M) did not have any significant effect on SH-SY5Y cell viability (Figure 1). None of the peroxide treatments described had any significant effect on the oxidation of proteins as seen by carbonyl ELISA, despite a trend to increasing carbonyl with increasing peroxide. Neither did ascorbate alone exert any significant change in the amount of oxidized proteins at 48h (Figure 2). Nevertheless, the presence of ascorbate (50 or 100 μ M) was seen to significantly inhibit carbonyl formation in peroxide (250 and 500 μ M) treated cells. Similarly, intracellular glutathione levels were not significantly altered at 48h by subjecting SH-SY5Y cells to the oxidative stress conditions described (Figure 3). However, co-incubation with ascorbate (100 μ M) and peroxide (500 μ M) did significantly reduce GSH content.

Separation of proteins by 2-dimensional polyacrylamide gel electrophoresis extracted from SH-SY5Y cells provided a more detailed analysis of the effects of ascorbate and peroxide (Figure 4). Proteins were immunoblotted after 2D-PAGE and probed for carbonyl groups, after derivitization with DNP, with antibody (anti-DNP), a marker of oxidative stress. In untreated cells, only 4 proteins were identified as oxidized under the conditions employed. The same proteins were oxidized in peroxide treated cells

and in cells pretreated with ascorbate. However, in extracts from cells treated with ascorbate followed by peroxide only one oxidized protein was detected (Table 1). Intensity changes in the four spots are reported in Table 2. The protein consistently oxidized (Figure 4) in all situations was identified by MALDI-TOF as collagen (sequence coverage 28%), from parallel silver stained gels. Only one of the other three proteins (δ) in the oxidized proteome was identified, from silver stained gels as acyl CoA oxidase 3 (sequence coverage 15%). There was insufficient protein per spot for mass fingerprinting of the other spots. After immuno-probing, blots were stripped of antibodies and proteins were stained with colloidal gold. Analysis of these expressed proteome blots showed that one protein in the SH-SY5Y cells treated with both ascorbate and hydrogen peroxide was induced by the treatment, in comparison to control cells and cells treated with only the stressor or the antioxidant (Table 1). Protein, extracted from parallel gels that were silver stained, allowed for identification of this induced protein by mass fingerprinting as brain derived neurotrophic factor (BDNF) (sequence coverage 29%). Further confirmation of this observation was achieved by immunoblotting (Figure 5), where little or no signal for BDNF was observed in control, ascorbate or peroxide treated cells despite equivalent protein loading (actin levels were equivalent through all treatment). In contrast, cells pretreated with ascorbate prior to exposure to hydrogen peroxide showed significant upregulation of BDNF expression.

Discussion:

The present study shows that peroxide induced cell death in a dose dependent manner in SH-SY5Y neuroblastoma cells. Ascorbate counteracted peroxide toxicity with the most significant protection afforded by 100 μ M ascorbate. At this concentration of ascorbate, cellular respiration was apparently enhanced over control indicative of growth promotion. Whilst little protective effect of ascorbate was seen against 250 μ M peroxide (probably due to greater variability in cell death according to confluence of cells prior to treatment) a significant protective effect was seen against protein carbonyl formation under extreme conditions, where greater than 50% of the cells were dead. When the cells were treated with 100 μ M peroxide or less, no change in carbonyl was seen at 24h, possibly reflecting early removal of oxidized proteins by the 20S proteasome, which predominantly removes and degrades oxidized proteins. Grune *et al.* [29] showed that hydrogen peroxide induced protein degradation over control in C9 liver cells, which was inhibited by lactacystin, indicating a pathway through via the 20S proteasome. Davies [30] also demonstrated that hydrogen peroxide increased protein degradation in C9 cells, which could be prevented by immunoprecipitation of the 20S proteasome. Furthermore, Ernst *et al* [31] demonstrated that an increase in carbonyl content in OLN 93 cells is associated with an increase in proteolytic activity, when treated with hydrogen peroxide (100 μ M).

The fate of ascorbate and glutathione are linked within the cell. Recycling of dehydroascorbate by the loss of glutathione produces ascorbate once more that can 'mop-up' reactive oxygen species within a cell. Uptake of ascorbate into the cell is via the GLUT receptor after the extracellular conversion of ascorbate to dehydroascorbate or by direct up-take of ascorbate via sodium dependent ascorbate transfer or glutamate–ascorbate heteroexchange [32-34]. However, the former is much more

efficient. Indeed, Long and Halliwell [35] showed that the addition of ascorbate to cell culture medium could produce hydrogen peroxide. Herein, we detected a drop in glutathione levels after addition of peroxide to cells and at the same time an increase of protein. These changes may stimulate the cell into a mildly stressed state that may elicit protection against oxidative stress, ultimately resulting in increased cell viability.

The analysis of protein oxidation and expression, as seen by 2D-PAGE, showed a reduction in the number of oxidized proteins (four proteins to one) and an induction of one protein when cells were pretreated with ascorbate followed by peroxide. Two of the oxidized proteins were identified: collagen was constitutively oxidized; whilst the oxidation of acyl CoA oxidase 3 was reduced by ascorbate. The abundance of collagen as a mechanical cellular component with a low turnover may explain the consistent oxidation of this protein, or this may be an illustration of the oxidation of new collagen being formed within the cells as is the norm with collagen synthesis. Acyl CoA oxidase 3 is involved in β oxidation, the metabolism of fatty acids. One of the products of the enzyme reaction is hydrogen peroxide. This may be the cause of the oxidative damage even in untreated cells. In addition this enzyme has an important role in the detoxification of lipid peroxides [36]. The inclusion of ascorbate and hydrogen peroxide may induce repair mechanisms that allow for a higher turnover of protein and hence the reduction of oxidation. Pratt *et al.* [37] recently illustrated that protein turnover can occur at an average rate of 2.2%/h, with highest degradation rates of 10%/h. After 24h, based on these figures, a turnover of 10% would leave only 8% of the original population of a particular protein. If the turnover was increased by the conditions described here any initially damaged proteins would be cleared rapidly.

Indeed, Ullrich and Grune [38] have demonstrated that 70% of oxidized nuclear proteins are removed after 24h, in comparison to 20% in control cells.

One protein was induced by in cells incubated with ascorbate followed with hydrogen peroxide. This was identified as BDNF by MALDI-TOF MS and immunoblotting. The immunoblot indicates that there is minimal/no expression of BDNF in any cells except the cells receiving combined ascorbate and hydrogen peroxide within the detection limits of the assays utilised in this study. BDNF supports cell survival via a variety of pathways downstream of Trk B interaction, including upregulation of superoxide dismutase, glutathione reductase, glutathione peroxidase [39], and activation of the Ras-MAPkinase survival pathway [40,41]. Furthermore, BDNF has been previously shown to activate glutathione reductase in SH-SY5Y cells ensuring protection against ROS and the resultant oxidative stress [42]. We investigated the levels of BDNF receptor in neuroblastoma cells by ELISA and found low levels of Trk B (BDNF receptor) were expressed in untreated SH-SY5Y cells. Furthermore, addition of BDNF (10ng/ml) for 24h reduced apoptosis (assessed by flow cytometry) induced by 100 μ M peroxide from 35% to 25% compared to cells treated with peroxide alone (data not shown).

These findings suggest that the protective effect of ascorbate *in vitro* may partly be due to the increased expression of BDNF, which may in turn activate a cascade of antioxidant defence mechanisms. The importance of ascorbate in eliciting this protective pathway *in vitro* remains to be determined.

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Legends

Table 1. Effect of peroxide and ascorbate on the number of proteins showing oxidation and increased expression.

Table 2. Relative intensities of the four proteins affected by oxidation determined using PD-Quest. Data shown are from one representative carbonyl blot.

Figure 1. Effect of peroxide and ascorbate on cell viability measured by MTT assay SH-SY5Y cells (2000 per well) were seeded in 96 cell plates. Cells were treated with hydrogen peroxide (0-500 μ M, 24h), in the presence or absence of ascorbate (50-100 μ M, 48h). Cell viability was measured as described by Hansen *et al.* (1989) [16]. Statistical significance test by ANOVA with Dunnet's post test (* $p < 0.05$, ** $p < 0.01$).

Figure 2. Effect of peroxide and ascorbate on carbonyl content measured by carbonyl ELISA. Protein (25 μ g) was extracted from SH-SY5Y cells (2×10^5) that had been treated with hydrogen peroxide (0-500 μ M, 24h), in the presence or absence of ascorbate (50-100 μ M, 48h) were utilized in a carbonyl ELISA as described by Carty *et al.* (2000) [19]. Statistical significance test by ANOVA with Dunnet's post test (* $p < 0.05$, ** $p < 0.01$).

Figure 3. Effect of peroxide and ascorbate on intracellular GSH levels measured by GSH assay. (** $p < 0.01$). Glutathione levels were determined in SH-SY5Y cells (2×10^5) that had been treated with hydrogen peroxide (0-500 μ M, 24h), in the presence or absence of ascorbate (50-100 μ M, 48h) as described by Punched *et al.*

(1994) [18]. Statistical significance test by ANOVA with Dunnet's post test (* $p < 0.05$, ** $p < 0.01$).

Figure 4.(a) 2D-PAGE of ascorbate (100 μ M, 48h) and peroxide (100 μ M, 24h) treated SH-SY5Y neuroblastoma cells. (b) magnified areas of 2D-PAGE shown in (a) showing oxidized proteins. (c) magnified areas of 2D-PAGE shown in (a) showing gold stained proteins highlighting an induced protein. (d) Mass spectrum for oxidized protein α (shown in b) identified as collagen. (e) Mass spectrum for oxidized protein δ (shown in b) identified as acyl CoA oxidase3. (f) Mass spectrum for induced protein (shown in c) identified as BDNF.

Figure 5. Immunoblots of cell extracts following incubation with ascorbate (100 μ M, 48h) and /or peroxide (100 μ M, 24h). Protein was separated on 12.2% SDS-PAGE gels and transferred to PVDF membrane. Membranes were probed with anti-human BDNF, where human BDNF was run as a positive control. Blots were probed for β -actin to demonstrate equal protein loading.

Sample	Number of oxidized proteins	Number of proteins with increased expression of proteins
Control	4	-
100μM peroxide	4	-
100μM Ascorbate + 100μM peroxide	1	1
100μM Ascorbate	4	-

	α	β	γ	δ
Control	260	25	92	90
100 μ M peroxide	91	21	67	73
100 μ M Ascorbate + 100 μ M peroxide	671	-	-	-
100 μ M Ascorbate	314	11	16	58









