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Dose-dependent modulation of the T cell proteome by ascorbic acid

Melissa M. Grant[1], Nalini Mistry[2], Joseph Lunec[2,3] and Helen R. Griffiths[1]*

[1]*School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK*
[2]*Genome Instability Group, Department of Cancer and Molecular Medicine, University of Leicester, Leicester LE2 7LX, UK*
[3]*King's College London, University of London, London SE1 1UL, UK*

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To investigate the hypothesis that the micronutrient ascorbic acid can modulate the functional genome, T cells (CCRF-HSB2) were treated with ascorbic acid (up to 150 μM) for up to 24 h. Protein expression changes were assessed by two-dimensional electrophoresis. Forty-one protein spots which showed greater than two-fold expression changes were subject to identification by matrix-assisted laser desorption ionisation time of flight MS. The confirmed protein identifications were clustered into five groups; proteins were associated with signalling, carbohydrate metabolism, apoptosis, transcription and immune function. The increased expression of phosphatidylinositol transfer protein (promotes intracellular signalling) within 5 min of ascorbic acid treatment was confirmed by Western blotting. Together, these observations suggest that ascorbic acid modulates the T cell proteome in a time- and dose-dependent manner and identify molecular targets for study following antioxidant supplementation *in vivo*.

Ascorbic acid: T cells: Proteomics: Phosphoinositol transfer protein

Many antioxidant micronutrients, such as ascorbic acid and α-tocopherol, are considered to exert their effects at least partially through changes in gene expression (Jackson *et al.* 2002) and these effects may be independent of antioxidant properties. For ascorbic acid, there is strong evidence supporting gene-regulatory effects which may relate to the capacity for ascorbic acid to act with either antioxidant or pro-oxidant effects depending on its environment (Podmore *et al.* 1998). Ascorbic acid has potent pro-oxidant activity in the presence of metal ions, where it acts as a reducing agent to provide catalysts for the Fenton reaction (Griffiths & Lunec, 2001). Thus, low concentrations of ascorbic acid enhance oxygen radical activity whilst high concentrations scavenge hydroxyl radicals, singlet oxygen and peroxy radicals.

In cardiomyocytes, ascorbic acid can maintain cell function and survival following hypoxia by inhibiting bax translocation to the nucleus, inhibiting the mitochondrial apoptotic pathway and stabilising Hif-1α (Vassilopoulos & Papazafiri, 2005). In addition, TNFα-induced caspase-9 activity is lowered by ascorbic acid. Similarly, stimulation of caspase-3 activity by high glucose in endothelial cells is suppressed by ascorbic acid (Ho *et al.* 2000). There is also *in vivo* evidence for a functional genomic effect of ascorbic acid; circulating apoptosis markers are decreased and endothelial function improves in congestive heart failure patients (Ellis *et al.* 2000; Rossig *et al.* 2001) following ascorbic acid supplementation. This suggests that ascorbic acid may have many other effects in addition to its accepted roles in reducing oxidative damage and promoting collagen synthesis.

We have previously demonstrated that proteomics is a valuable tool to dissect the sustained effects of ascorbic acid in a neuronal cell system (Grant *et al.* 2005); we showed induction of the pro-survival gene product, BDNF, in SH-SY5Y cells exposed to an oxidative stress in the presence of ascorbic acid. However, to date, there has not been a systematic examination of the early effects of ascorbic acid on the lymphocyte proteome. Thus we have undertaken a time- and dose-effect study of ascorbic acid on the transformed human T-cell lymphocyte cell line (CCRF-HSB2) using proteomics in an holistic attempt to analyse pathways affected by ascorbic acid.

Experimental methods

Cell culture

T cells (CCRF-HSB2) were maintained in RPMI 1640 with Glutamax1 (Gibco BRL, Grand Island, NY, USA) supplemented with fetal bovine serum (10 %, heat inactivated; Gibco). Cells were seeded at a density of 3×10^5 cells/ml in RPMI 1640 with Glutamax1 supplemented with fetal bovine serum (2 %, heat inactivated) and ascorbic acid (0, 10, 50 and 150 μM) was added. Cells were harvested after 5 min, 2, 8 and 24 h.

Flow cytometric DNA cell cycle analysis

Following treatment, PBS-washed cells were centrifuged at 100 ***g*** (Eppendorf centrifuge 5415D; Eppendorf, Hamburg, Germany) for 5 min, the supernatant fraction removed and the resulting cell pellet re-suspended in 1 ml hypotonic fluorochrome solution (propidium iodide (50 μg/ml) in 0·1 % sodium citrate and 0·1 % Triton X-100) to extract and stain nucleoids (Phillips & Griffiths, 2003). Samples were incubated

Abbreviations: 2DE, two-dimensional gel electrophoresis; PITP, phosphoinositol transfer protein; TTBS, Tween tri(hydroxymethyl)-aminomethane-buffered saline.

* **Corresponding author:** Professor Helen R. Griffiths, fax +44 121 359 5142, email h.r.griffiths@aston.ac.uk

in the dark at 4°C for 24 h before flow cytometric cell cycle analysis for propidium iodide fluorescence of individual nuclei. Measurements were made using an EPICS® XL-MCL flow cytometer (Beckman-Coulter, Inc., Miami, FL, USA) equipped with a 488 nm air-cooled Ar laser. The percentage of actual apoptosis was determined by quantifying the number of hypoploid (subdiploid) nuclei.

Sample preparation for two-dimensional gel electrophoresis

CCRF-HSB2 cells (5×10^6) were harvested, homogenised and incubated (at room temperature with rotation, 30 min) in tri(hydroxymethyl)-aminomethane HCl buffer (200 µl, pH 7·5, 40 mM) containing protease (1:100 Focus-Protease Arrest; Calbiochem, Nottingham, UK), phosphatase (imidazole (2 mM), sodium fluoride (1 mM) and sodium vanadate (1 mM); Sigma, Poole, Dorest, UK), and kinase (genistein (10 µM) and staurosporine (50 nM); Sigma) inhibitors. Nuclease was also incubated with cell lysates to remove nuclear DNA (1:100 Focus-Nuclease; Calbiochem). ASB 14 (1 %; Sigma) was included in the rehydration buffer (300 µl; urea (5 M), thiourea (2 M), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (2 %), caprylyl sulfobetaine (2 %), tri(hydroxymethyl)-aminomethane HCl (40 mM)) together with Destreak reducing agent (15 µl/ml; Amersham Biosciences, Little Chalfont, Bucks, UK) and incubated with lysates by rotation (room temperature; 30 min). Samples were centrifuged (13 000 rpm; 30 s) before analysis of protein content by using the RCDC protein assay (BioRad, Hercules, CA, USA). For two-dimensional gel electrophoresis (2DE), proteins were extracted from cells that had been treated in three separate experiments. Subsequently equal volumes of sample were pooled from each separate experiment and three replicates of the pooled samples were focused and subsequently electrophoresed under conditions previously described (Grant *et al.* 2005).

Focusing of samples

Extracted proteins (50 µg) were applied during rehydration to immobilised pH gradient strips (11 cm, pH range 4–7; BioRad) in rehydration buffer containing Biolytes 3/10 (0·5 %; BioRad) (180 µl, 16 h, 20°C). Proteins were focused on a Multiphor II with a DryStrip kit and power was supplied by an EPS 3500 XL power pack (Amersham Biosciences), cooled to 20°C (Grant cooling system). The running conditions were: a gradient of 0 V to 500 V over 500 Vh, 500 V to 3500 V for 3500 Vh and finally 3500 V for 90 kVh. After focusing the immobilised pH gradient strips were stored at −70°C until required.

Two-dimensional polyacrylamide gel electrophoresis

Immobilised pH gradient strips were equilibrated in equilibration buffer (Grant *et al.* 2005) before electrophoresis in the second dimension. They were then placed above a 10–20 % gradient gel (BioRad) and set in agarose (1 % in TGS buffer; BioRad). The proteins were electrophoresed in a BioRad Criterion kit for 70 min at 150 V, using TGS buffer. Gels were then stained with Ag (Yan *et al.* 2000) and images recorded using a GS 710 calibrated imaging densitometer. Subsequently, images were interrogated with PDQuest software (BioRad). Three replicate gels were analysed from the four time- and four dose-points (*n* 48) and these were used to create a master gel for all analyses. Changes in replicate spot intensity were subject to statistical analyses using paired comparison algorithms within PDQuest, allowing fold changes in expression to be identified. Protein spots which showed a fold change of at least 2, where the set included proteins spots whose quantity in treated cells was at least two times higher or lower than that of the corresponding spot in non-treated cells, were selected for identification. Those proteins which demonstrated time and dose responses within these subsets were selected for identification by MS.

Identification of proteins

Proteins were identified by mass fingerprinting. From Ag-stained gels, proteins of interest were excised and tryptically digested as described by Gharahdaghi *et al.* (1999). Peptides were extracted as described by Hellman *et al.* (1995) and desalted using C18 ZipTips (Millipore, Bedford, MA, USA). Mass fingerprints were generated on a Bruker Omni scan with N 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). Positive identification was based on standard MASCOT criteria for statistical analysis.

Western blotting

Protein samples (10 µg) were separated by one-dimensional SDS-PAGE (12·5 % gel) and electroblotted onto polyvinylidine fluoride membrane (Grant *et al.* 2005). After electroblotting, membranes were blocked in Tween 20 (1 %) tri(hydroxymethyl)-aminomethane-buffered saline (TTBS) containing 3 % bovine serum albumin for 2 h. Membranes were incubated with either a mouse monoclonal antibody against phosphoinositol transfer protein (PITP) (15:100 000, diluted in TTBS containing 0·3 % bovine serum albumin; Santa Cruz sc-13 569; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or mouse monoclonal antibody against actin (15:100 000, diluted in TTBS containing 0·3 % bovine serum albumin; Abcam Ltd, Cambridge, UK) overnight. The membranes were washed with TTBS (6 × 15 min) before incubation with a peroxidase conjugated secondary antibody (Sigma Aldrich) for 2 h (15:100 000, diluted in TTBS containing 0·3 % bovine serum albumin). Membranes were rinsed in tri(hydroxymethyl)-aminomethane-buffered saline (6 × 15 min) and then a chemiluminescent substrate (ECL Plus; Amersham Biosciences) was used to visualise detected proteins. The images were recorded using a GS 710 calibrated imaging densitometer. Bands were analysed and quantified using Scion software (National Institutes of Health, Bethesda, MD, USA).

Results and discussion

Ascorbic acid is an important intracellular antioxidant affecting redox status by maintaining thiol compounds, including glutathione, in their reduced state and in the regeneration of tocopherol from its oxidised form (Sies *et al.* 1992). Ascorbic acid has also been shown to enhance antioxidant defences of T

cells, and also increase T cell responsiveness to antigens, suggesting a role in regulating immune function (Wu *et al.* 2000). The importance of antioxidants in maintaining immune function is further supported by studies showing that oxidative stress may contribute to T cell hyporesponsiveness in a number of pathologies (Maurice *et al.* 1998). The potential for antioxidants such as ascorbic acid to modulate gene and protein expression and modulate cell function has been previously proposed to contribute to the range of biological processes affected by ascorbic acid, but there are few examples of a systematic approach to study the effects of micronutrients on the proteome (Choi *et al.* 2003; Lockwood, 2005).

To shed some light on the molecular effects of ascorbic acid on T cells, we have studied the proteome of CCRF-HSB2 T cells, derived from an acute human lymphoblastoid leukemia which has been extensively characterised (Adams *et al.* 1968), exposed to ascorbic acid for up to 24 h. Previous work has demonstrated that the major effects of ascorbic acid occur early with only 1 % of the effects on the proteome sustained beyond 18 h (Grant *et al.* 2005). To ensure that ascorbic acid was not having an adverse effect on T cell viability over 24 h, apoptotic nucleoids were determined by flow cytometry. Fig. 1 illustrates a typical experiment (*n* 3) showing that there was no significant increase in apoptotic nucleoids after 24 h treatment with up to 150 μM-ascorbic acid. The concentrations of ascorbic acid were selected to cover the range of plasma concentrations observed in 'normal' subjects; from 10 μM-ascorbic acid in nutritionally deficient subjects to 150 μM in those taking supplements (Carty *et al.* 2000). There are reports of ascorbic acid toxicity to cells in culture over physiological concentration ranges possibly due to mobilisation of intracellular redox-active Fe but other examples of cytoprotective effects of ascorbic acid against apoptosis-inducing agents have also been reported (Arteel *et al.* 1999; Ellis *et al.* 2000; Ho *et al.* 2000; Clement *et al.* 2001; Guidarelli *et al.* 2001; Rayment & Griffiths, 2002).

To investigate the hypothesis that ascorbic acid modulates the T cell proteome either through altering protein expression or modulating protein degradation and clearance, T cells were exposed to various doses of ascorbic acid from 10 to 150 μM over 24 h and the expressed proteins extracted for 2DE using immobilised pH gradient strips from isoelectric point 4–7. Initial analysis of two-dimensional gels by PD Quest consistently revealed over 300 spots per gel.

Further analysis centred on all proteins either raised or lowered at least two-fold between gels with an average of thirty-four spots showing a change in intensity (± 2·9 SEM; *n* 48 gels) following ascorbic acid treatment. These statistics

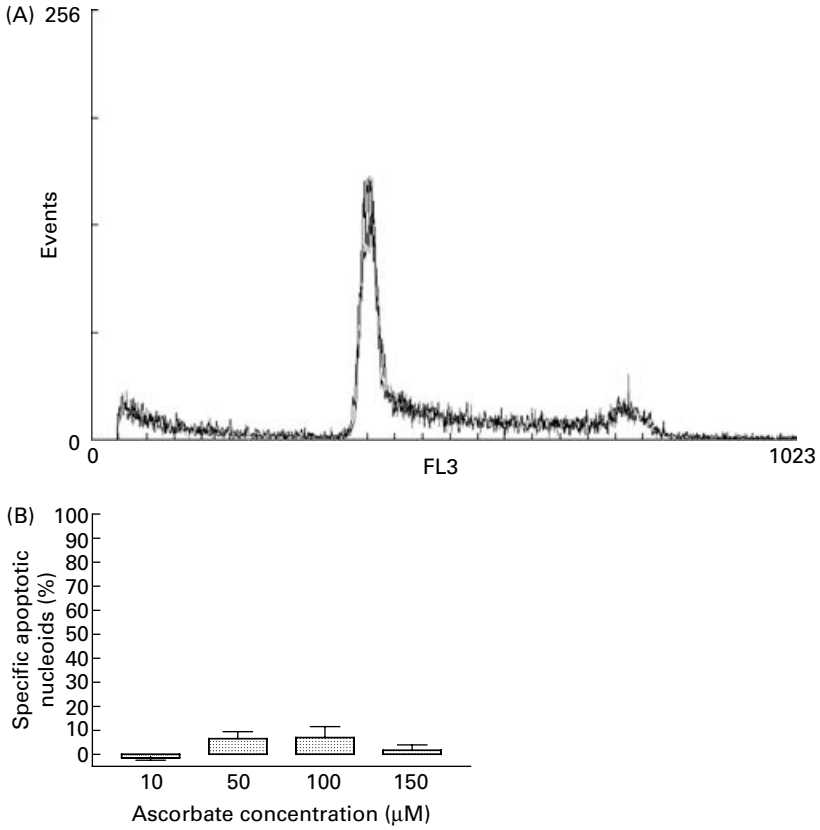

Fig. 1. Human T-cell lymphocyte CCRF-HSB2 T cells (3×10^5/ml) were incubated in a humidified 5 % CO_2–95 % air atmosphere at 37°C for 24 h with ascorbic acid (10–150 μM). Cell pellets were re-suspended in 1 ml hypotonic fluorochrome solution (p.19) before DNA cell cycle analysis by flow cytometry. For each sample, 20 000 nucleoids were counted. (A) Representative histograms from flow cytometric analysis of CCRF-HSB2 T cells treated with vehicle control (—) or 100 μM-ascorbic acid (▨) for 24 h. (B) Specific apoptotic nucleoid content of T cells was calculated for each experiment according to the formula: specific apoptosis = (T − C)/(100 − C) × 100, where T is the percentage of apoptotic events from treated cells, and C is the percentage of apoptotic events from control cells. The data are the arithmetic mean percentages of three individual experiments, with standard errors represented by vertical bars. None of the ascorbic acid effects were significant compared with vehicle treatment by one-way ANOVA followed by Dunnett's multiple comparison test.

confirm the high degree of reproducibility between gels. Fig. 2 shows the total T cell proteome master gel image of all samples locating the isoelectric point and molecular weight of spots that were significantly altered in intensity following ascorbic acid treatment. Secondary analysis for trends in time or dose response within this dataset was also undertaken, leading to identification of a total of forty-one proteins that were selected for mass spectral analysis. Table 1 describes the thirty-two proteins of these proteins showing altered expression over time and/or dose which were successfully identified by matrix-assisted laser desorption ionisation time of flight MS. The Table describes coverage as the percentage of the entire protein sequence that matched to tryptic peptide fragments identified by matrix-assisted laser desorption ionisation time of flight MS.

In addition to the thirty-two identifiable spots, two further proteins were described as patented sequences, three were unknown and another three were hypothetical proteins. Of the thirty-two reported proteins, twenty-two protein spots changed in intensity with time and eight exhibited dose responses (Table 1). Further confirmatory evidence for the identity of these proteins was sought by comparing the predicted isoelectric point and molecular weight from database sequences and those obtained by 2DE.

Of the modulated proteins that were successfully identified, a large number were associated with signalling roles, while others had functions in immune function and sugar metabolism. The change in expression of these proteins by 'family' over time is illustrated in Table 2. Signalling protein responses appeared early, as either gain or loss of protein spots. It is unlikely that this reflects changes in protein expression but rather changes in protein partitioning in and out of the membrane or change in rate of protein turnover. Membrane proteins are poorly extracted for 2DE and increase in membrane association of signalling proteins may lead to an apparent loss at 5 min, whereas an increase in spot intensity may reflect an increase in mobilisation of the protein from the membrane compartment (Phillips *et al.* 2005). Only 1·3 % of the total proteome was modulated dose dependently within 5 min, with the remaining proteins showing no change after 5 min of ascorbic acid treatment, again demonstrating the reproducibility of the analysis technique and suggesting that such early changes in spot intensity are not experimental artifacts.

The movement from the insoluble membrane fraction to the cytoplasmic compartment may explain the change in the protein spots identified as 0503 cGMP phosophodiesterase β chain and 0112, PITP, which showed a dose response to ascorbic acid over the range of time points (Fig. 3). This was taken for further analysis by Western blotting, as an existing antibody was commercially available (Wang & Pan, 1991). At each time point, 10 and 50 μM-ascorbic acid increased levels of PITP protein above that seen when 0 or 150 μM-ascorbic acid was added to the T cells. Fig. 3 shows the intensities for spot 0112 over different times and doses of ascorbic acid treatment. Protein levels, detected by Western blotting for PITP (Fig. 4), mirror those seen by PDQuest analysis at 5 min whilst actin levels remained unchanged. Thus, Western blotting has authenticated proteomic analysis and has confirmed the hypothesis that ascorbic acid can modulate a signalling protein in the cytoplasmic T cell proteome, within 5 min.

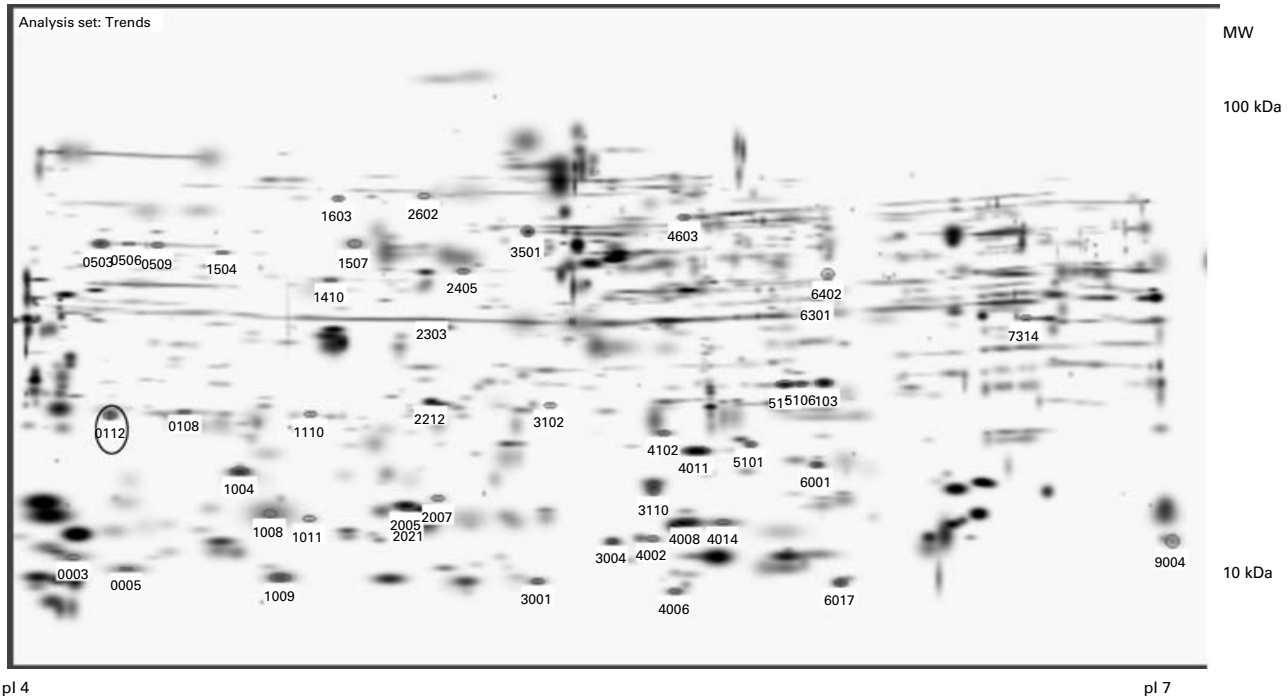

Fig. 2. Identification of a differentially expressed T cell proteome following ascorbic acid treatment. A master image of the two-dimensional PAGE (Ag stain) gel of the combined T cell proteome pre- and post-ascorbic acid treatments is shown. Proteins were separated in the first dimension across the isoelectric point (pI) range 4–7 before electrophoresis and separation in the second dimension according to size by SDS-PAGE. Ag-stained images were analysed and combined using PDQuest (BioRad, Hercules, CA, USA) to create a master gel illustrating all expressed proteins. Those protein spots seen to change in level by a factor of greater or equal to 2 upon treatment are identified by numbers. These forty-one spots were subject to matrix-assisted laser desorption ionisation time of flight MS analysis of protein identity. The spot (circled) was also analysed by Western blotting. MW, molecular weight.

Table 1. Identity of proteins showing two-fold change in spot intensity post-ascorbic acid treatment as determined by matrix-assisted laser desorption ionisation time of flight mass spectrometry*

SSP	Protein name	Coverage (%)	Change
0005	β-Globin fragment	75	Down over time
0108	Serine/threonine phosphatase 1γ	31	Lost at 8 and 24 h with 150 μM-ascorbic acid
0112	Phosphatidylinositol transfer protein†	34	Up at 5 min, dose-dependent 0–50 μM-ascorbic acid
0503	cGMP phosphodiesterase β chain	15	Dose-dependent loss at 5 min 0–50 μM-ascorbic acid
1004	Preproglucagon	42	Up regulated by 10 and 50 μM-ascorbic acid at all times
1008	T cell receptor α chain V region	66	Constitutive
1009	Ig heavy chain variable region	83	Lost with ascorbic acid and over time
1011	AIEBP	21	Lost over time
1110	HLA-B α chain	16	Increase with 10 and 50 μM-ascorbic acid
1410	Puromycin-sensitive aminopeptidase	21	Increase with 50 μM-ascorbic acid
1504	A-kinase anchor protein	15	Increase with time and dose of ascorbic acid; greatest effect at 24 h
2005	Fragment Zn finger protein	49	Loss with time and dose of ascorbic acid
2212	Fragment sugar transport protein	26	Increased at 8 h ascorbic acid treatment; dose response
2602	Ig heavy chain variable region	31	Loss with ascorbic acid; greatest at 2 and 8 h
3001	Kruppel-associated box protein	64	Dose–response change to ascorbic acid at all time points
3004	Fragment tyrosinase-related gene segment	29	Dose–response increase at 8 h ascorbic acid treatment
3102	Ig heavy chain variable region	35	Dose–response increase to ascorbic acid at 5 min, 2 and 8 h
3501	Protocadherin 9	19	Dose–response increase to ascorbic acid at 5 min. Decrease over time after 5 min
4002	E2 ubiquitin-conjugating enzyme	47	Increase after 10 and 50 μM-ascorbic acid; bell-shaped curve response to ascorbic acid
4008	K channel	71	Loss in expression with time
4011	ELE1	33	Increase at 2 and 8 h with 10 and 50 μM-ascorbic acid
4014	Fragment glycosyltransferase	28	Increase with 10 and 50 μM-ascorbic acid
4102	ASPSCR1	21	Increased by ascorbic acid at all times studied
4603	β Spectrin	18	Loss in expression with time
5105	Rho guanine nuclear exchange factor	14	Early loss with ascorbic acid in a dose-dependent manner; returned to baseline by 24 h
5107	Microphthalmia-associated transcription factor	33	Levels increase after 2 and 8 h with 10 and 50 μM-ascorbic acid
6001	Oestrogen receptor-associated protein	19	Increased at 2 and 8 h particularly after 10 and 50 μM-ascorbic acid
6017	AAC32739	100	No trend
6103	Fragment Ca sensor protein	29	Increased after 2 h
6301	Aconitate hydratase	16	Increased after 10 and 50 μM-ascorbic acid at 2 and 8 h
6402	Caspase recruitment domain protein	18	Lost at 2 and 8 h with ascorbic acid. Increased at 24 h
7314	Putative Zn finger protein	15	Loss with ascorbic acid from 5 min to 8 h

SSP, standard spot numbers (the number used to identify spots on the gel images; see Fig. 2); HLA, human leucocyte antigen.

* Identities are of protein spots picked from gels which showed intensities altered more than two-fold compared with control levels. Results are shown as SSP; identities were derived from mass fingerprinting using mascot searches and percentage coverage.

† Protein selected for further analysis.

PITP are ubiquitous proteins that transport lipids, such as phosphatidylinositol and phosphatidylcholine, between membranes and thus have important roles in signalling (Allen-Baume *et al.*, 2002). In this way, ascorbic acid may promote intracellular signalling cascades that are dependent on phospholipids. Other proteins involved in lipid transport include the Sec14 family, which include the α-tocopherol transport proteins (TAP and ATTP) and phospholipid transfer proteins that play an essential role in HDL metabolism (Azzi *et al.* 2002; Tan *et al.* 2005). We have also recently reported that α-tocopherol modulates expression of the major HDL protein, ApoA1 (Aldred *et al.* 2006), possibly through modulation of intracellular signalling.

Several other protein spots corresponding to proteins associated with signalling were also modulated by ascorbic acid; the loss of serine/threonine phosphatase 1γ and the increase of A-kinase anchor protein spots suggests that ascorbic acid treatment of T cells may favour phosphorylation. A-kinase anchor proteins play a role in controlling the activation of T cells and associate with cyclic nucleotide phosphodiesterases (Asirvatham *et al.* 2004). Indeed, cGMP phosphodiesterase β chain was decreased by ascorbic acid at 5 min and 2 h whereas the Ca sensor protein spot was increased at 2 h ascorbic acid treatment. The loss of the K^+ channel protein spot was observed at 2 h; this may represent an adaptive response to the increase in intracellular Na^+ driven by co-transport during ascorbic acid uptake by the anionic transporter (Park & Levine, 2000).

Several spots were identified as proteins involved in immune function; the spot corresponding to the antigen-processing enzyme, puromycin sensitive aminopeptidase (Levy *et al.* 2002), was increased by the presence of 50 μM-ascorbic acid at 2 h. This enzyme is responsible for trimming the final NH_2-terminal amino acids required for antigenic epitope processing (Stoltze *et al.* 2000). Similarly, human leucocyte antigen-B antigen and β spectrin (which controls CD45 surface display and IL-2 production; Pradhan & Morrow, 2002) were increased in spot intensity by ascorbic acid treatment from 2 h to 24 h.

Four proteins involved in carbohydrate metabolism were up regulated between 2 and 8 h of treatment with ascorbic acid, namely preproglucagon, sugar transport protein, ASPCR1, glycosyl transferase and aconitate hydrolase. The dose-dependent increases in spot intensity between 0 and 50 μM-ascorbic

Table 2. Trend analysis of ascorbic acid effect on protein spot intensity according to protein function 'family'*

Protein 'family'	Change in spot intensity	Protein identities of spots which change in intensity with time and concentration of ascorbic acid treatment			
		5 min	2 h	8 h	24 h
Apoptosis and cell cycle	Decrease	–	Caspase 3 recruitment domain (10–150 μM)		–
	Decrease	–	–	AIEBP (10–150 μM)	
	Increase		Preproglucagon (10–50 μM)		–
	Increase		Fragment glycosyl transferase (10–50 μM)		–
	Increase		ASPRC1 (10–150 μM)		
	Increase	–	Aconitate hydratase (10–50 μM)		–
Carbohydrate	Increase	–	–	Fragment sugar transport (10–150 μM)	–
	Constitutive		T cell α chain V region		
	Increase	–	HLA-B chain (10 and 50 μM)		
	Increase	–	Aminopeptidase (50 μM)		
Immune	Decrease	–	–		Spectrin
Signalling	Decrease	cGMP phosophodiesterase (10–150 μM)		–	–
	Decrease	–	K^+ channel (10–150 μM)	–	–
	Decrease	–	Serine threonine phosphatase γ (10–150 μM)		
	Increase	PITP (10–150 μM)	–	–	–
	Increase		A-kinase anchor protein (10–150 μM)		
	Increase	–	Oestrogen receptor-associated protein (10–150 μM)		–
	Increase	–	Fragment Ca^{2+} sensor protein (10–150 μM)		
Transcription	Decrease		Zn finger proteins (10–150 μM)		–
	Decrease	–	ELE (10–50 μM)		–

HLA, human leucocyte antigen.
*The identity of spots identified by matrix-assisted laser desorption ionisation mass spectrometry which show variation at each time and dose of ascorbic acid treatment are grouped according to function. The concentrations which elicited changes are shown.

acid treatment may be attributed to the similarities in structure between ascorbic acid and glucose. As glucagon secretion is not traditionally ascribed to cells other than islet cells and some cells of the nervous system, the identification of high levels of preproglucagon in these T cells together with further modulation of these levels by ascorbate may be a phenomenon of the cell line used and may have little physiological relevance *in vivo*. Moreover, the precursor hormone only exerts biological activity after processing and secretion.

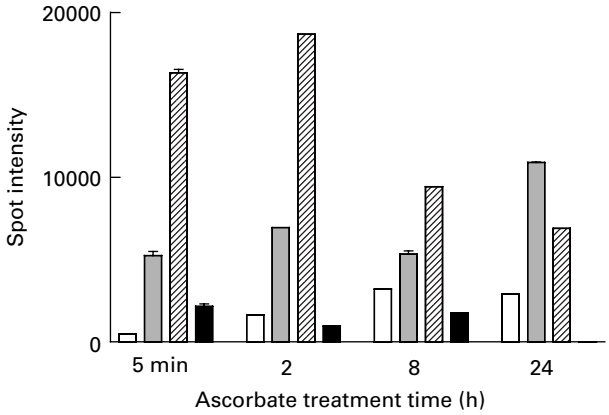

Fig. 3. Protein spot 0112 (see Fig. 2) shows time and dose responses to ascorbic acid which are evident within 5 min of treatment. CCRF-HSB2 T cells (3×10^5/ml) were incubated in a humidified 5 % CO_2–95 % air atmosphere at 37°C for 24 h with ascorbic acid (at concentrations of 0 μM (□), 10 μM (▩), 50 μM (▨) and 150 μM (■) over 5 min to 24 h. Each experiment was performed in triplicate and the samples pooled. Proteomes from pooled T cell lysates corresponding to each treatment were separated on three independent gels. Following Ag staining, gels were scanned using PDQuest (BioRad, Hercules, CA, USA) and the average spot intensity (and 95 % CI) over the three replicate gels was calculated and is illustrated in histogram form.

A further two proteins were modulated by ascorbic acid which are classified as apoptosis- or cell cycle-related genes: the caspase recruitment domain protein showed a loss in spot intensity from 2 to 8 h, but an increase at 24 h; AIEBP, which influences the cell cycle, was switched off from 8 h. These data suggest that whilst short-term exposure to ascorbic acid may be protective, prolonged exposure to high doses of ascorbic acid may prime the cell for apoptosis, possibly due to the induction of DNA damage as previously shown by Anderson *et al.* (1997). However, we saw no induction of apoptosis measured as subdiploid nuclei or caspase 3 activity under these conditions.

Several proteins related to transcriptional activation were also modulated by ascorbic acid; spots corresponding to Zn finger proteins showed a loss in intensity, whereas the spots corresponding to nuclear hormone receptors and transcription factors were increased. In contrast, the spot identified as ρ guanine nuclear exchange factor expression was decreased from 2 to 24 h in a dose-dependent manner.

We and others have previously shown that ascorbic acid uptake is significant within minutes (Bowers-Komro & McCormick, 1991) and is maximal into leucocytes at 15 min. This supports the possibility that ascorbic acid may induce early signalling effects directly. In addition, the potential for ascorbic acid to modulate the redox state of extracellular receptor domains may also contribute to rapid changes in protein compartmentalisation and therefore direct or indirect signalling within the cytoplasm (Griffiths & Lunec, 2001). We cannot preclude the possibility that the changes in expression may be attributable to modulation of labile Fe either intra- or extracellularly or that an ascorbic acid metabolite, such as the oxidation product dehydroascorbate, may be exerting a role in changing the protein expression profile,

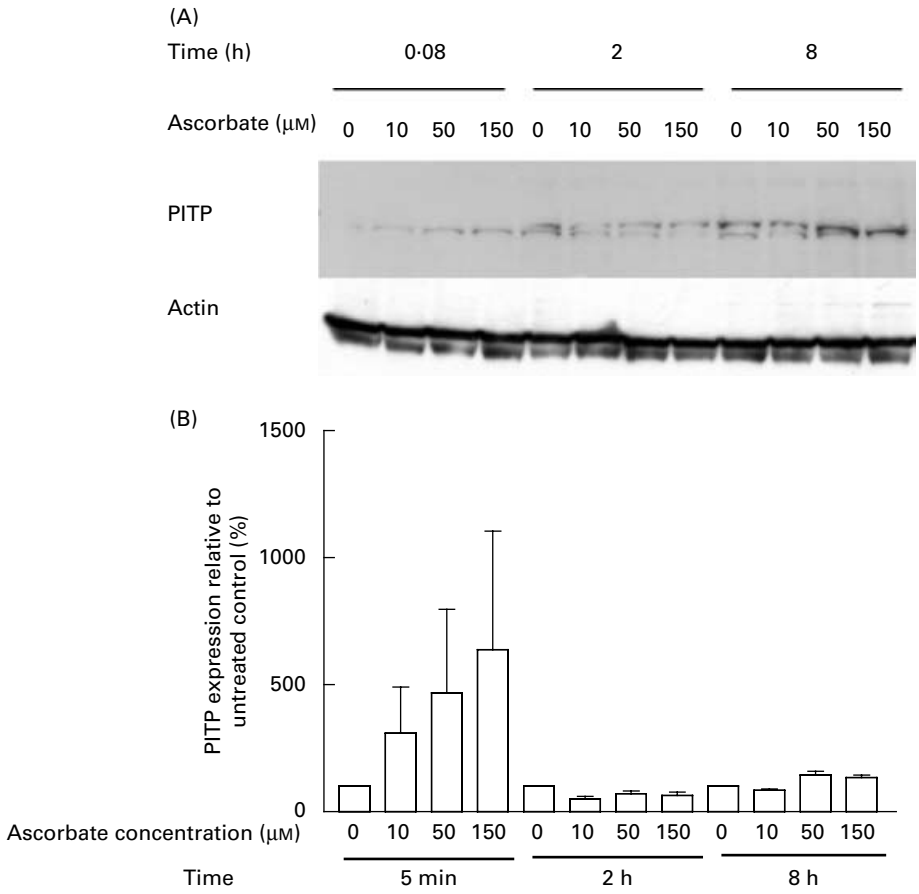

Fig. 4. Phosphoinositol transfer protein (PITP) protein levels in the extracted proteome are increased within 5 min by ascorbic acid. Changes in spot intensity for 0112 (see Fig. 2), which was identified as PITP by matrix-assisted laser desorption ionisation time of flight MS, were confirmed by Western blotting. (A) Western blot of PITP and actin as loading control. (B) Intensity of PITP in T cell lysates following ascorbic acid treatment for varying times; PITP in lysates was identified by Western blot, determined by scanning densitometry and shown as percentage PITP levels in ascorbic acid-treated cells compared with control cells with correction for actin expression (*n* 3).

where cells may only be exposed to the reduced form of ascorbate for a matter of minutes. Therefore, it can be reasonably expected that the physiological effects of an increase in dietary ascorbate may modulate the T cell proteome in a similar manner and this is currently under investigation in a human intervention study.

For the majority of modulated proteins, dose-dependent trends in spot intensity were observed between 10 and 50 μM-ascorbic acid. The lower quintile average of plasma ascorbic acid recorded in the EPIC study was 20 μM; in contrast, 50 μM-ascorbic acid lies between the average of the third and fourth quintiles for plasma vitamin C (Khaw *et al.* 2001). Therefore, these studies suggest that changes in ascorbic acid concentration over a physiological range may affect the T cell proteome and identify potential mechanisms of ascorbic acid effects on T cell function. These markers are currently under investigation in an *in vivo* ascorbic acid intervention study.

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References

Adams RA, Flowers A & Davis BJ (1968) Direct implantation and serial transplantation of human acute lymphoblastic leukemia in hamsters, SB-2. *Cancer Res* **28**, 1121–1125.

Aldred S, Sozzi T, Mudway I, Grant MM, Neubert H, Kelly FJ & Griffiths HR (2006) Vitamin E supplementation elevates pro-apolipoprotein A1 levels in the plasma of normal subjects. *Proteomics* **6**, 1695–1703.

Allen-Baume V, Segui B & Cockcroft S (2002) Current thoughts on the phosphatidylinositol transfer protein family. *FEBS Lett* **531**, 74–80.

Anderson D, Phillips BJ, Yu TW, Edwards AJ, Ayesh R & Butterworth KR (1997) The effects of vitamin C supplementation on biomarkers of oxygen radical generated damage in human volunteers with "low" or "high" cholesterol levels. *Environ Mol Mutagen* **30**, 161–174.

Arteel GE, Bribya K & Sies H (1999) Protection against peroxynitrite. *FEBS Lett* **445**, 226–230.

Asirvatham AL, Galligan SG, Schillace RV, Davey MP, Vasta V, Beavo JA & Carr DW (2004) A-kinase anchoring proteins interact with phosphodiesterases in T lymphocyte cell lines. *J Immunol* **173**, 4806–4814.

Azzi A, Ricciarelli R & Zingg JM (2002) Non-antioxidant molecular functions of alpha-tocopherol (vitamin E). *FEBS Lett* **519**, 8–10.

Bowers-Komro DM & McCormick DB (1991) Characterization of ascorbic acid uptake by isolated rat kidney cells. *J Nutr* **121**, 57–64.

Carty JL, Bevan R, Waller HL, Mistry N, Cooke M, Lunec J & Griffiths HR (2000) The effects of vitamin C supplementation on protein oxidation in healthy volunteers. *Biochem Biophys Res Comm* **273**, 729–735.

Choi J, Conrad CC, Dai R, Malakowsky CA, Talent JM, Carroll CA, Weintraub ST & Gracy RW (2003) Vitamin E prevents oxidation of antiapoptotic proteins in neuronal cells. *Proteomics* **3**, 73–77.

Clement MV, Ramalingam J, Long LH & Halliwell B (2001) The *in vitro* cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxid Redox Signal* **3**, 157–163.

Ellis GR, Anderson RA, Lang D, Blackman DJ, Morris RH, Morris-Thurgood J, McDowell IF, Jackson SK, Lewis MJ & Frenneaux MPJ (2000) Neutrophil superoxide anion-generating capacity, endothelial function and oxidative stress in chronic heart failure: effects of short- and long-term vitamin C therapy. *J Am Coll Cardiol* **36**, 1474–1482.

Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS & Mische SM (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**, 601–605.

Grant MM, Barber VS & Griffiths HR (2005) 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. *Proteomics* **5**, 534–540.

Griffiths HR & Lunec J (2001) Ascorbic acid in the 21st century – more than a simple antioxidant. *Env Tox Pharm* **10**, 173–182.

Guidarelli A, De Sanctis R, Cellini B, Fiorani M, Dacha M & Cantoni O (2001) Intracellular ascorbic acid enhances the DNA single-strand breakage and toxicity induced by peroxynitrite in U937 cells. *Biochem J* **356**, 509–513.

Hellman U, Wernstedt C, Gonez J & Heldin CH (1995) Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal Biochem* **224**, 451–455.

Ho FM, Liu SH, Liau CS, Huang PJ & Lin-Shiau SY (2000) High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3. *Circulation* **101**, 2618–2624.

Jackson MJ, Papa S, Bolanos J, *et al.* (2002) Antioxidants, reactive oxygen and nitrogen species, gene induction and mitochondrial function. *Mol Aspects Med* **23**, 209–285.

Khaw K, Bingham S, Welch A, Luben R, Wareham N, Oakes S & Day N (2001) Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective population study. European Prospective Investigation into Cancer and Nutrition. *Lancet* **357**, 657–663.

Levy F, Burri L, Morel S, Peitrequin SL, Levy N, Bachi A, Hellman U, Van den Eynde BJ & Servis C (2002) The final N-terminal trimming of a subaminoterminal proline-containing HLA class I restricted antigenic peptide in the cytosol is mediated by two peptidases. *J Immunol* **169**, 4161–4171.

Lockwood TD (2005) The transfer of reductive energy and pace of proteome turnover: a theory of integrated catabolic control. *Antioxid Redox Signal* **7**, 982–998.

Maurice MM, van der Voort EA, Leow A, Levarht N, Breedveld FC & Verweij CL (1998) CD28 co-stimulation is intact and and contributes to prolonged ex vivo survival of hyporesponsive synovial fluid T cells in rheumatoid arthritis. *Eur J Immunol* **28**, 1554–1562.

Park JB & Levine M (2000) Intracellular accumulation of ascorbic acid is inhibited by flavonoids via blocking of dehydroascorbic acid and ascorbic acid uptakes in HL-60, U937 and Jurkat cells. *J Nutr* **130**, 1297–1302.

Phillips DC & Griffiths HR (2003) Ceramide induces a loss in cytosolic peroxide levels in mononuclear cells. *Biochem J* **375**, 567–579.

Phillips GR, Florens L, Tanaka H, Khaing ZZ, Fidler L, Yates JR III & Coleman DR (2005) Proteomic comparison of two fractions derived from the transsynaptic scaffold. *J Neurosci Res* **81**, 762–775.

Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P & Lunec J (1998) Vitamin C exhibits pro-oxidant properties. *Nature* **392**, 559.

Pradhan D & Morrow J (2002) The spectrin-ankyrin skeleton controls CD45 display and interleukin 2 production. *Immunity* **17**, 303–315.

Rayment SJ & Griffiths HR (2002) Effects of antioxidant vitamins C and E on gene expression in the vasculature. In *Oxidative Stress and Aging: Diagnostics, Intervention and Longevity*, pp. 659–672 [H Rodriguez and R Cutler, editors]. Boca Raton, FL: CRC Press.

Rossig L, Hoffmann J, Hugel B, Mallat Z, Haase A, Freyssinet JM, Tedgui A, Aicher AM, Zeiher S & Dimmeler S (2001) Vitamin C inhibits endothelial cell apoptosis in congestive heart failure. *Circulation* **104**, 2182–2187.

Sies H, Stahl W & Sundquist AR (1992) Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. *Ann N Y Acad Sci* **30**, 7–20.

Stoltze L, Schirle M, Schwarz G, Schroter C, Thompson MW, Hersh LB, Kalbacher H, Stevanovic S, Rammensee HG & Schild H (2000) Two new proteases in the MHC class I processing pathway. *Nat Immunol* **1**, 413–418.

Tan KC, Shiu SW, Wong Y & Tam S (2005) Plasma phospholipid transfer protein activity and subclinical inflammation in type 2 diabetes mellitus. *Atherosclerosis* **178**, 365–370.

Vassilopoulos A & Papazafiri P (2005) Attenuation of oxidative stress in HL-1 cardiomyocytes improves mitochondrial function and stabilizes Hif-1alpha. *Free Radic Res* **39**, 1273–1284.

Wang F & Pan YC (1991) Structural analyses of proteins electroblotted from native polyacrylamide gels onto polyvinyldiene difluoride membranes. A method for determining the stoichiometry of protein-protein interaction in solution. *Anal Biochem* **198**, 285–291.

Wu CC, Doriarajan T & Lin TL (2000) Effect of ascorbic acid supplementation on the immune response of chickens vaccinated and challenged with infectious bursal disease virus. *Vet Immunol Immunopathol* **74**, 145–152.

Yan YX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH & Dunn MJ (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* **21**, 3666–3672.