BIOCHEMICAL PHARMACOLOGY 72 (2006) 204–216

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Salicylate modulates oxidative stress in the rat colon: A proteomic approach

Janice E. Drew[a,*], *Sara Padidar*[a], *Graham Horgan*[c], *Garry G. Duthie*[a], *Wendy R. Russell*[a], *Martin Reid*[b], *Gary Duncan*[b], *Garry J. Rucklidge*[b]

[a] *Gut Health Division, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB Scotland, UK*
[b] *Proteomics Unit, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB Scotland, UK*
[c] *BIOSS, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB Scotland, UK*

ARTICLE INFO

Article history:
Received 13 March 2006
Accepted 6 April 2006

Keywords:
Mitochondria
Redox status
Phenolic compound
Partial least squares

Abbreviations:
CRC, colorectal cancer
cyGpx, cytosolic glutathione peroxidase
IBD, inflammatory bowel disease
NSAIDs non-steroidal anti-inflammatory drugs
PCA, principal component analysis
PDI, protein disulphide isomerase
PLS, partial least squares
ROS, reactive oxygen species
SDS, sodium dodecyl sulphate
TBARS, thibarbituric acid reactive substances

ABSTRACT

The dietary phenolic compound, salicylic acid, decreases oxidative stress and pro-inflammatory and potentially neo-plastic prostaglandins with a concomitant increase in glutathione peroxidase activity. Salicylic acid, a dietary plant-based phenolic compound and also the main metabolite of aspirin, may contribute to the colon protective effects of plant-based diets. Oxidative stress is a characteristic of pre-cancerous and cancerous colon and inflammatory bowel diseases (IBD) that increase colon cancer risk. The mechanism(s) whereby salicylic acid modulates potentially pro-cancerous activity associated with oxidative stress is further investigated here using a proteomic approach. A rat model of oxidative stress was supplemented with salicylic acid (1 mg/kg diet, mean plasma levels 310 ± 32 μmol/l). Soluble colon protein extracts were subjected to 2D PAGE. Salicylic acid modulated proteins, identified using MALDI-TOF and LC/MS/MS, are involved in protein folding, transport, redox, energy metabolism and cytoskeletal regulation. A partial least squares (PLS) analysis approach was used to assist biological interpretation of the altered protein profiles via their associations between previously published biochemical measurements of oxidative stress, prostaglandin levels and increased glutathione peroxidase activity. Early detection of altered homeostasis in colon may assist in identifying pre-pathological features preceding colon tumorigenesis and contribute to an understanding of epidemiological evidence supporting a protective effect of plant-based diets.

* *Corresponding author*. Tel.: +44 1224 712751; fax: +44 1224 716629.
E-mail address: jed@rri.sari.ac.uk (J.E. Drew).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.04.012

1. Introduction

Oxidative stress occurs as a consequence of excessive production and, or inadequate elimination of reactive oxygen species (ROS) in a biological system. The imbalance may be a consequence of reduced antioxidant capacity caused by disturbances in dietary intake, production and distribution of antioxidants, or an overabundance of ROS from an environmental or behavioural stressor, including tissue pathology. Improper regulation can lead to an excess of ROS and subsequent damage to cellular lipids, proteins or DNA and inhibition of normal cellular functions. Oxidative stress has thus been implicated in a growing list of human diseases, including colon pathologies, such as cancer, inflammatory bowel diseases (IBD) and diverticular disease [1–3]. Oxidative stress is also a characteristic of inflammation, with epidemiological studies suggesting a positive association of colorectal cancer (CRC) incidence in patients with IBD [4–7]. It is well established that tissues undergoing chronic inflammation are at an increased risk of undergoing malignant changes. Many cancers arise from sites of infection and chronic irritation as well as inflammation [8].

Pharmacological suppression of inflammation lessens the risk of CRC [9]. In subjects without IBD regular consumption of non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin, suldinac and celecoxib) reduces CRC risk [10–14]. Aspirin is metabolised to salicylic acid. Many plants of dietary significance contain salicylic acid, where it is employed as a phenol hormone mediator in the resistance of pathogenic attack and environmental stress [15]. Hence, salicylic acid is found in significant quantities in a plant-based diet. Since salicylic acid is common to both aspirin and fruits, vegetables and spices and both are known to protect against colon pathologies, including cancer, it is possible that this phenolic acid may be responsible for some of the beneficial effects of a plant-based diet on colon health [16–19].

Previous studies in our lab have shown that the dietary phenolic compound, salicylic acid, decreases oxidative stress and the synthesis of pro-inflammatory and potentially neo-plastic prostaglandins with a concomitant increase in cytosolic glutathione peroxidase (cyGpx) activity [20]. Altered cyGpx activity measured in this study was not associated with parallel changes in expression of the two cytosolic glutathione peroxidase gene transcripts, *Gpx*1 and *Gpx*2, in the colon [21]. Other studies in our lab using targeted gene arrays (Clontech Rat Stress gene array, 207 genes; http://www.ncbi.nlm.nih.gov/geo/, Accession no. GSE1063) also revealed a lack of significant gene changes in colon tissue in response to oxidative stress associated with elevations in indices of lipid peroxidation, a loss of membrane integrity and microanatomical colon tissue changes [22]. Since gene expression may not reflect alterations in protein profiles and activity, a proteomic approach was adopted in this study to elucidate salicylate modulation of oxidative stress in rat colon using protein extracts prepared from colon segments procured from the study reported previously [20].

2. Materials and methods

2.1. Animals and diets

The colons were procured from weanling male rats of the Rowett Hooded Lister strain offered a semi-synthetic diet as described in the previously reported study [20]. Briefly, the diets, offered 12 weeks ad libitum, were either sufficient in Vitamin E, 1000 mg Vitamin E/kg diet (as dlα-tocopherol acetate, Sigma) (+E diet), deficient in Vitamin E, less than 0.5 mg Vitamin E/kg (−E diet), or deficient in Vitamin E (less than 0.5 mg Vitamin E), but supplemented with 1000 mg salicylic acid/kg (−E + SA diet). After 12 weeks on the diet the rats were anaesthetised with isofluorane and plasma and colons were collected as described in the study reported previously [20].

2.2. Plasma salicylate measurement

Concentrations of salicylates in plasma ($n = 8$) were measured by HPLC with electrochemical detection using 4-methylsalicylic acid as an internal standard as previously described [23]. In brief, following adjustment of pH to 2.0 with hydrochloric acid (1 mol/l), the organic material was extracted twice with ethylacetate (2 ml), evaporated to dryness at 70 °C under nitrogen and reconstituted in 0.5 ml of the mobile phase containing 30 mmol/l citrate (pH 3.8) in 5% methanol containing 5.5 μl of EDTA (10mM). Reconstituted extracts (50 μl) were eluted with a ternary gradient programme to give maximum peak resolution substances being detected electrochemically at an oxidation potential of +1.1 V. The limits of detection for plasma salicylic acid was 5 nmol/l. The identities of the compounds being measured were confirmed by gas-chromatography mass spectrometry as described in Blacklock et al. [23].

2.3. Proteomic analysis

The distal colon was selected for proteomic analysis since this is the predominant location of pre-cancerous lesions and tumour formation in human sporadic colon cancers and in rats treated with colonotropic chemical carcinogens. Protein was extracted from 0.5 cm colon segments ($n = 6$ biological replicates per treatment group) by freeze milling frozen colon segments, followed by homogenisation with a hand held homogeniser in tris buffer (20 mM Tris–HCl, pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1 mM Na_3VO_4, leupeptin (4 μg/ml), toluene sulphonyl fluoride (60 μg/ml) and Triton X-100 (0.1%, v/v). The homogenate was centrifuged initially at 14,000 rpm and aliquots were extracted to assess prostaglandins in the study reported previously [20]. The homogenates were then subject to further purification by high-speed spin at 55,000 rpm. The resulting soluble proteins in the supernatant were decanted for estimation of protein concentration using a BioRad Bradford protein assay kit and γ-globulin as standard prior to 2D PAGE. Adjacent colon segments were used in the study reported previously to assess TBARS and cyGpx activity [20].

2.4. 2D PAGE and analysis

Protocols for 2D PAGE of rat colon protein extracts have been optimised in previous studies [24]. The extracted protein

fractions (300 μg) were concentrated to a volume of 35 μl (concentration 8.57 μg/μl) using Vivaspin 0.5 ml concentrator devices (Vivascience, Germany) centrifuged at 12,000 × *g* at 4 °C. Each sample was then loaded onto a BioRad IPG strip (17 cm, pH 3–10) in 340 μl of 7M urea, 2M thiourea, 4% Chaps and 2% biolyte (BioRad) 3% DTT buffer to separate the proteins in the first dimension. A second dimension SDS-PAGE step was run on an 18 cm × 18 cm linear SDS polyacrylamide gradient as described previously [24]. The gels were then stained with colloidal Coomassie Blue staining as described by Anderson [25]. Gels ($n = 6$ representing six biological replicates of each protein extract per treatment group) were then rinsed in deionised water and brushed to remove particulate Coomassie Blue and imaged on a BioRad GS710 flat bed imager followed by image analysis using BioRad PD Quest Version 7.1. The gel with highest spot number and quality was selected as the match set standard. The PDQuest statistical analysis (*t*-test) were performed on a log transformed matched set data by pair wise comparisons of the Vitamin E deficient group with either the Vitamin E sufficient or Vitamin E deficient supplemented with salicylic acid groups at 95% confidence interval. SPSS version 13 was also used to assess differential signal intensity by one-way ANOVA.

2.5. MALDI-TOF mass spectrometric analysis

An Applied Biosystems Voyager-DE PRO mass spectrometer in reflectron mode was used to perform MALDI-TOF mass spectrometry of trypsin-digested proteins purified from 2D gels. Spectra were obtained using 500 shots of the appropriate laser power, and where appropriate spectra were accumulated and filed. Baseline corrections and de-isotoping of peptide mass peaks were made using a macro. The resulting peptide mass list was automatically generated from the most intense peaks. The peptide profiles were analysed using the Matrix Science (http://www.matrixscience.com/search_form_select.html) server-side tool. Mascot was used to match the mass spectra of peptide maps generated above against protein sequence databases (MSDB) setting the search criteria as follows: allowance of 0 or 1 missed cleavages; carbamidomethyl modification of cysteine; oxidation partial modification of methionine; a charged state of MH^+; and positive identities of at least 20% matched peptides covering at least 10% of the protein sequence. After visual examination of peptide matches with corresponding Mr and pI calculated from the 2D gels some gel purified proteins were selected for further confirmation of ID using LC/MS/MS if the Mr and pI differed markedly or if the significance scores assigned by MASCOT were low, but the other criteria were satisfactory. Mascot scores >55 were selected as significant identifications ($p < 0.05$).

2.6. Protein identification by nano LC/MS/MS

Samples from 2D PAGE gels were analysed using a nano LC system (LC Packings, Camberly, Surrey, UK) consisting of an 'Ultimate' nano LC system, pumping at 0.187 ml/min with a 625 splitter giving a column flow rate of 0.3 μl/min, a 'Famos' autosampler set to an injection volume of 5 μl and a 'Switchos' microcolumn switching device. The nanocolumn was a C18 PepMap 100, 15 cm × 75 μm i.d., 3 μm, 100 Å (LC Packings). HPLC grade solvents were used, 2% acetonitrile and 0.1% formic acid (A) and 80% acetonitrile and 0.08% formic acid (B). The gradient started at 5% B, going to 50% B over 30 min, then ramping to 80% B over a further 2 min, and holding for 10 min. The system was equilibrated at 95% A for 9 min prior to injection of subsequent samples. The solvent used by the 'Switchos' is 0.1% formic acid. The switching device was switched on after 3 min and off after 58 min. The flow rate of the Switchos was 0.03 ml/min. Mass spectrometry was then performed using a Q-Trap (Applied Biosystems/MDS Sciex, Warrington, UK) triple quadrupole fitted with a nanospray ion source using parameters described previously [24]. Individual ion scores >28 indicate identity or extensive homology ($p < 0.05$).

2.7. Partial least squares regression analysis

The burgeoning application and experience of the complexities of proteomic analysis has revealed issues with regard to verification and biological significance of the observed altered protein profiles. The use of Western blots to assess protein levels in an analogous fashion to the application of quantitative PCR to verify micoarrays is often not applicable [26]. Multiple protein expression forms resolved using isolectric focusing and gradient gels reveal altered protein profiles and spot patterns that cannot be replicated in a one-dimensional Western blot format. Western blot analysis also lacks the desired quantitative sensitivity, particularly if multiple bands are visible with the attendant possibility that these may be attributed to multiple protein expression forms or non-specific immunodetection. Proteomic analysis may be more readily applicable to analysis of altered protein profiles or patterns and less applicable to a purely or solely quantitative analysis. Thus alternative approaches are essential to assist interpretation and biological significance of altered protein profiles in proteomic experiments. One potential approach examined here is to determine overall significantly altered protein profiles (assessed here by a combination of ANOVA and *t*-test) and assess their relationship to biochemical measurements in the biological system being investigated as part of a systems approach. Thus biochemical data available from the prior study of the biochemical effects of salicylate on the oxidatively stressed rats used for proteomic analysis here was incorporated into a partial least squares (PLS) regression analysis. This approach permits reduction of the large number of variables involved and correlates significantly altered protein profiles and patterns with biochemical effects and associated dietary treatments. It is thus easier to interpret whether the observed cumulative altered protein profiles (albeit sometimes involving small quantitative shifts) make biological sense. Partial least squares analysis was applied using SIMCA-P + 10.5 Umetrics software to assess soluble colon protein profiles associated with indices of oxidative stress (plasma and colon TBARS), prostaglandin levels and cyGpx activity data and modulation by salicylic acid gathered from the previous study of the rats used for proteomics analysis [20]. PLS is a statistical technique, related to principal component analysis, which aims to find linear combinations of the variables within two sets of variables that best represent the associations between the two sets. In order to assess the significance of the correlation between the

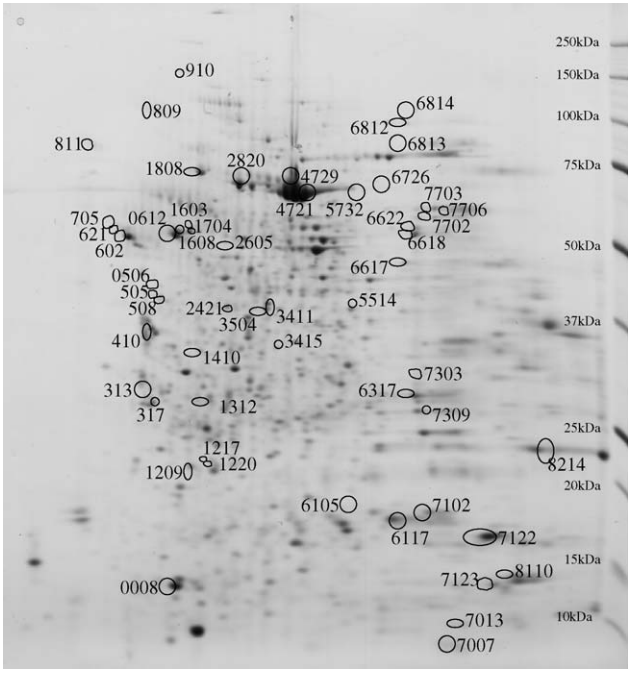

Fig. 1 – Schematic image of master 2D gel and location of 55 spots excised for MALDI-TOF and LC/MS/MS.

components obtained for the two sets of variables (proteomic and biochemical data) a randomisation test was carried out. For each of 1000 repetitions, the order of the biochemical data observations was randomised, the PLS analysis repeated and the correlation between the first components of each set of variables was recorded. This provided an empirical estimate of the distribution of the correlation under the null hypothesis of no association, against which the observed correlation could be compared. This was done for both the whole set of spot variables, and with only those selected for cutting (the significantly altered proteins identified by ANOVA and *t*-test). In this latter case, since the spot selection was based on the statistical significance of diet group differences, and the biochemical measurements were also significantly different between groups, this selection could lead to a significant correlation. The randomisation test was therefore repeated using the within-group residuals.

3. Results

3.1. *Plasma salicylates*

Rats fed the Vitamin E deficient diet supplemented with 1000 mg salicylic acid/kg had plasma salicylate levels of 310 ± 32 μmol/l (mean ± S.E.M.). Plasma salicylate levels were below detectable limits for rats not supplemented with salicylic acid.

3.2. *Proteomic analysis*

One thousand and thirty-nine spots were resolved from the soluble protein fractions extracted from the freeze milled distal colons (Fig. 1). Combinations of statistical approaches were then used to select resolved proteins showing altered expression patterns after normalisation of the spot densities. PDQuest proteomic analysis software was used to conduct pair-wise comparisons (*t*-test) of the Vitamin E sufficient and Vitamin E deficient supplemented with salicylic acid rats against those that were Vitamin E deficient. Three-way comparisons were then conducted using ANOVA. The combined statistical analysis yielded a total of 124 spots with significant altered expression (Fig. 2A). Spots jointly identified by *t*-test comparison and ANOVA as being significantly altered (Fig. 2A) were then subject to visual inspection to ensure the spot was present on replicate gels (at least three) and to eliminate those of insufficient quantity for downstream analysis (Fig. 2B). Additional spots significantly altered in either *t*-test comparisons or ANOVA were added after visual

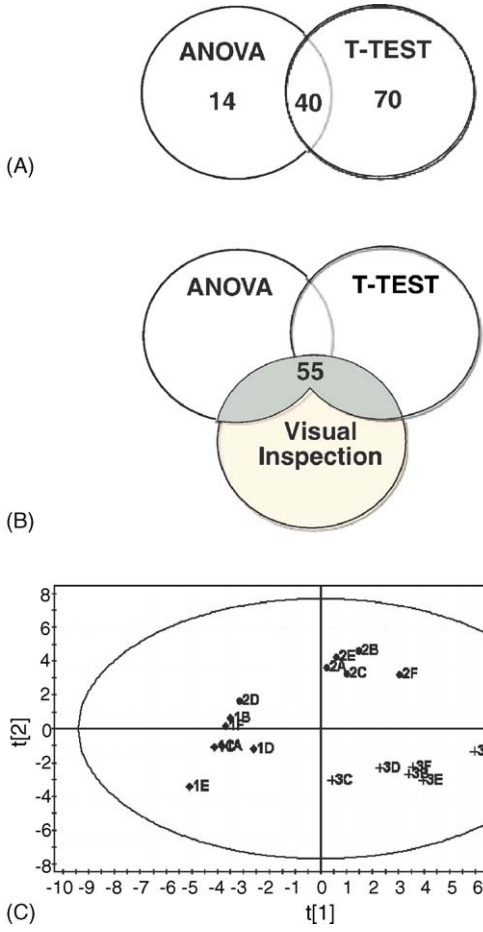

Fig. 2 – Statistical analysis and selection of significantly altered protein expression. Statistical analysis of 2D gels yielded 55 spots (A and B) that were significantly altered in response to the dietary treatments. Principal component analysis (C) confirms that the excised spots are significantly different in response to the different dietary treatments. Treatment 1 (◆) Vitamin E sufficient, treatment 2 (●) Vitamin E deficient, supplemented with salicylic acid and treatment 3 (+) Vitamin E depleted. Each label indicates the 2D gel source of the excised spots prepared from soluble colon extracts from individual biological replicates (*n* = 6, designated A–F).

inspection on the basis described (Fig. 2B). This resulted in the final selection of a subset of 55 spots ($p < 0.05$) ranging in pI from 4.3 to 9.3 with a size range from 8.8 to 106.9 kDa that were excised and subject to MALDI-TOF-MS and LC/MS/MS analysis (see below). Principal component analysis was applied to the selected sub-set to confirm that they clustered into distinct groups further supporting selection of groups that are statistically distinct (Fig. 2C). Rat ●2D (Fig. 2C) from the minus E plus salicylic acid treatment group was further assessed since the spot patterns on this gel appeared to be more consistent with those of the Vitamin E sufficient group (◆, treatment 1). However, analysis of the data revealed that the association was not significant. Our previous report identified an alleviation of oxidative stress on supplementation with salicylic acid. It is therefore not surprising that protein profiles from salicylic acid supplementation generate protein profiles that may be more closely aligned to Vitamin E sufficient as opposed to the Vitamin E deficient rats (+, treatment 3) (Fig. 2C).

Sixteen spots (Table 1) were positively identified solely from MALDI-TOF-MS spectra using the criteria specified above (2.4 MALDI-TOF-MS analysis). Further analysis and identification of a further 39 spots necessitated nanospray LC/MS/MS (Table 1). Together with LC/MS/MS a total of 55 of the selected proteins were identified, yielding 35 different proteins that were categorised into the following functional groups—redox signalling, cytoskeletal regulation, metabolism, protein folding and transport, cell adhesion and extracellular matrix and plasma proteins (Table 1). The fact that the 55 spots represent only 35 different proteins confirmed the presence of multiple protein expression forms. Altered profiles of the identified proteins are presented (Fig. 3). The identified proteins and functional significance in the rat colon model are discussed in further detail below.

3.3. *Partial least squares analysis of protein profiles and biochemical data*

Partial least squares analysis was performed to identify associations between the proteins identified as being statistically significantly altered in response to oxidative stress and salicylic acid supplementation and the biochemical data [20] that was measured in plasma and colon. This was viewed as an approach to address interpretation and biological significance of the altered protein profiles since Western blotting approaches are often not applicable to verify altered protein profiles and patterns obtained by 2D PAGE. PLS showed a positive relationship between the protein data from the spots selected as being significantly altered (see Fig. 1) in response to dietary treatment with the biochemical data. This relationship was explored further using coefficient plots of weights (w^*c plot) on the selected proteins and biochemical data (Fig. 4). Fig. 4 shows PLS weights of selected protein data (Y-axis) and biochemical data (X-axis), for the first two components.

The correlation between the first PLS components of the protein spot (whole set) and biochemical variables was 0.91. However, about 13% of the 1000 replicates of the randomisation test were as great or greater. When the protein spots were restricted to those cut for identification (see Fig. 1), the correlation increased to 0.93, and only five of the 1000 randomisation replicates exceeded this. This demonstrates statistical significance of the association when performing PLS on those protein spots identified as exhibiting significantly altered profiles, but not when non-significantly altered protein spots are included. When residuals from group means was used, the correlation was 0.94 and only two of 1000 randomisation replicates exceeded this. The biological significance of the relationships derived by PLS between the altered protein profiles and biochemical changes measured in response to salicylate modulation of oxidative stress are discussed below.

4. Discussion

Biological interpretation of proteomic data is a challenging exercise considering the complexity of protein expression forms. Multiple products were detected for a single protein in this and other studies [24,26]. It would be expected also that the altered redox environment investigated by 2D PAGE in this study potentially represent a multitude of post-translational and altered protein forms either a consequence of functional responses or oxidative damage. This effectively excludes a one-dimensional approach to verify proteomic data. Thus alternative methods must be utilised to assist biological interpretation of proteomic data. Since biochemical data was available from the rats used here [20] for colon proteomic analysis, this study provided an opportunity for comparative examination of colon protein profiles with biochemical data using partial least squares analysis. Thus, changes in protein profiles in response to salicylic acid and oxidative stress were compared with respect to biochemical assays of the colon and plasma from the same rats. This approach permitted identification of potential mechanisms whereby dietary supplementation with salicylic acid modulates oxidative stress in the colon. The implications of the altered protein profiles observed in response to dietary treatments and their potential to influence colon pathology can be considered with regard to the altered biochemistry and the functional roles of the identified proteins. A moderate correlation between PG (prostaglandin levels), pTBARS (plasma TBARS) and TBARS (colon TBARS) is supported by the PLS analysis (Fig. 4). Likewise, there is also a moderate correlation between Gpx (cytosolic gluthathione peroxidase activity) and SA (plasma salicylates) (Fig. 4). This conclusion from interpretation of the PLS analysis is supported by our previous biochemical analysis of this group of rats in response to the dietary treatments [20]. The previous study indicated that oxidatively stressed rats (induced by Vitamin E deficiency) have elevated TBARS (a marker of oxidative stress) and prostaglandin levels in the colon. Conversely, salicylic acid supplementation of oxidatively stressed rats lead to a reduction in the markers of oxidative stress and prostaglandins and was associated with an elevation of colon cyGpx activity. Further interpretation of PLS analysis with regard to the selected proteins (see Fig. 4) can also be predicted. For example, PLS associates spots 317 (mitochondrial import stimulation factor S1), 1603 (lumican secretory interstitial proteoglycan precursor), 1609 (ATPase beta F1) and 7703 (M2 pyruvate kinase) with PG levels (see Fig. 4), indicating that an increase in the level of these proteins would be expected to be associated with an increase in PG

Table 1 – Proteins identified by MASCOT search of MALDI-TOF and LC/MS/MS spectra

Spot no.	Functional group	Mascot score*	ID	Matched (%)	Coverage (%)	Mass of ID (kDa)	Mass of spot (kDa)	pI of ID	pI of spot
0008*	Redox regulation	247	Thioredoxin (TRX-1)	N/A	N/A	11.88	12.56	4.9	5.1
0313*	Cytoskeletal regulation	474	Tropomyosin 5b	N/A	N/A	28.68	28.5	4.8	4.9
0317*	Protein folding and transport	279	Mitochondrial import stimulation factor S1 chain	N/A	N/A	**27.92**	**52.4**	4.7	5.3
0410	Cytoskeletal regulation	63	Tropomyosin	33	46	28.60	35.17	4.7	4.8
0505*	Metabolism	373	Ribonuclease inhibitor	N/A	N/A	**51.58**	**39.4**	4.6	4.9
0506*	Metabolism	470	Ribonuclease inhibitor	N/A	N/A	**51.58**	**41.11**	4.6	4.9
0508*	Metabolism	464	Ribonuclease inhibitor	N/A	N/A	**51.58**	**38.76**	4.6	5.0
0602*	Protein folding and transport	239	Calreticulin precursor	N/A	N/A	48.14	51.10	4.3	4.5
0612	Redox regulation Protein folding and transport	151	protein disulfide isomerase (PDI)	80	37	57.23	52.08	4.8	5.1
0621*	Protein folding and transport	120	Calreticulin precursor	N/A	N/A	4814	52.60	4.3	4.5
0705*	Protein folding and transport	90	Calreticulin precursor	N/A	N/A	48.14	54.62	4.3	4.4
0809*	Plasma proteins	88	Alpha-1 proteinase inhibitor III, variant 1 precursor	N/A	N/A	**166.59**	**105.2**	**5.68**	**4.8**
0811*	Cell adhesion and extracellular matrix	102	Cadherin 1	N/A	N/A	99.17	84.2	4.7	4.3
0910*	Plasma proteins	279	Alpha-1 proteinase inhibitor III precursor	N/A	N/A	165.04	145.7	5.7	5.1
1209*	Redox regulation	195	Thioredoxin peroxidase 1	N/A	N/A	21.94	20.58	5.4	5.3
1217*	Redox regulation	138	Thioredoxin peroxidase 1	N/A	N/A	21.94	21.6	5.3	5.5
1220*	Redox regulation	212	Thioredoxin peroxidase 1	N/A	N/A	21.94	21.23	5.4	5.5
1312*	Metabolism	352	Cathepsin b	N/A	N/A	28.36	26.9	5.1	5.5
1410*	Protein folding and transport	554	Annexin A6	N/A	N/A	**75.98**	**32.9**	5.4	5.4
1603*	Cell adhesion and extracellular matrix	139	Lumican secretory interstitial proteoglycan precursor	N/A	N/A	**38.65**	**52.36**	6.0	5.2
1609	Metabolism	57	ATPase Beta F1	38	32	50.74	45.46	5.0	5.3
1704*	Cytoskeletal regulation	70	Scinderin	N/A	N/A	**80.61**	**54.37**	5.6	5.3
1808	Protein folding and transport	166	Heat shock protein 70, 78 kDa glucose regulated	83	37	72.47	71.97	5.1	5.3
4721	Plasma proteins	100	Serum albumin precursor	51	41	70.67	65.72	6.1	6.5
4729	Plasma proteins	73	Serum albumin precursor	46	34	70.67	74.21	6.1	6.3
2421*	Cytoskeletal regulation	79	Scinderin			**80.61**	**37.27**	5.6	5.8
2605*	Redox regulation	125	Glutathione synthase (GSS)	N/A	N/A	51.03	49.12	5.9	5.7
2820*	Plasma proteins		Hemopexin precursor	N/A	N/A	**52.00**	**71.7**	**7.6**	**5.9**
3411*	Metabolism	560	NAD+ specific isocitrate-dehydrogenase a-subunit	N/A	N/A	40.04	37.73	6.5	6.2
3415*	Metabolism	93	Cytosolic malate dehydrogenase	N/A	N/A	36.63	37.73	5.9	6.2
3504*	Metabolism	604	Bisphosphate 3' nucleotidase 1	N/A	N/A	33.50	37.77	5.6	6.1
4729	Plasma proteins	73	Serum albumin precursor	46	34	70.67	74.21	6.1	6.3
5732	Plasma proteins	142	Serum albumin precursor	82	34	70.67	66.35	6.1	7.0

Table 1 (*Continued*)

Spot no.	Functional group	Mascot score*	ID	Matched (%)	Coverage (%)	Mass of ID (kDa)	Mass of spot (kDa)	pI of ID	pI of spot
5514*	Metabolism	697	Acyl-CoA dehydrogenase, short-chain specific, mitochondrial precursor (SCAD)	N/A	N/A	45.02	38.69	8.5	7.0
6105	Cytoskeletal regulation	68	Transgelin	43	52	22.51	17.89	8.9	7.2
6117	Cytoskeletal regulation	65	Transgelin	38	61	22.51	17.61	8.9	7.6
6317*	Metabolism	308	Carbonic anydrase II	N/A	N/A	29.14	27.54	6.9	7.6
6617*	Metabolism	234	Beta Enolase	N/A	N/A	47.14	45.34	7.7	7.5
6618*	Metabolism	245	Carbonic anhydrase II	N/A	N/A	**29.14**	**51.3**	6.9	7.5
6622*	Metabolism	232	Carbonic anhydrase II	N/A	N/A	**29.14**	**53.04**	6.9	7.5
6726*	Plasma proteins	1214	Serum albumin precursor	N/A	N/A	70.67	65.7	6.1	7.4
6812	Metabolism	62	Aconitate hydrate I	49	21	98.75	96.26	6.7	7.5
6813*	Metabolism	258	Mitochondrial aconitase precursor	N/A	N/A	86.16	84.57	7.9	7.5
6814*	Metabolism	295	Carbonic anhydrase II	N/A	N/A	**29.14**	**106.9**	6.9	7.5
7007*	Plasma proteins	137	Haemoglobin Beta 1	N/A	N/A	15.94	8.8	8.0	8.0
7013*	Plasma proteins	86	Hemoglobin alpha-1 and alpha-2 chains	N/A	N/A	**15.36**	**10.2**	7.9	8.1
7102*	Metabolism	377	Creatine kinase	N/A	N/A	**42.97**	**17.9**	**5.3**	**7.8**
7122*	Cytoskeletal regulation	412	Destrin	N/A	N/A	18.66	16.60	7.8	8.6
7123	Plasma proteins	106	Haemoglobin Beta 1	91	46	15.94	13.53	8.0	8.6
7303*	Metabolism	111	Alcohol dehydrogenase A chain	N/A	N/A	**40.40**	**30.46**	8.3	7.7
7309*	Redox regulation	669	Glutathione S-transferase yb-2 (GST)	N/A	N/A	25.73	26.02	7.3	7.9
7702	Metabolism	125	UDP dehydrogenase	79	40	55.54	55.89	7.5	7.8
7703	Metabolism	76	M2 Pyruvate kinase (PKM2)	59	29	58.31	58.49	7.2	7.8
7706	Metabolism	76	M2 Pyruvate kinase (PKM2)	59	29	58.31	58.30	7.2	8.1
8110	Cytoskeletal regulation	59	Profilin 1	48	55	15.15	14.52	8.5	8.9
8214	Cytoskeletal regulation	94	Transgelin	55	74	22.65	22.66	8.9	9.3

Mascot score, identification (ID) and percentage matched peptides of significantly altered spots are shown. A minimum of 20% matched peptides and 10% sequence coverage were the parameters used for protein identification. N/A = not applicable. Mass/pI of ID is the mass/pI of the identified protein specified in MSDB and Mass/pI of spot is that estimated from the 2D gel. Mascot scores >55 indicate

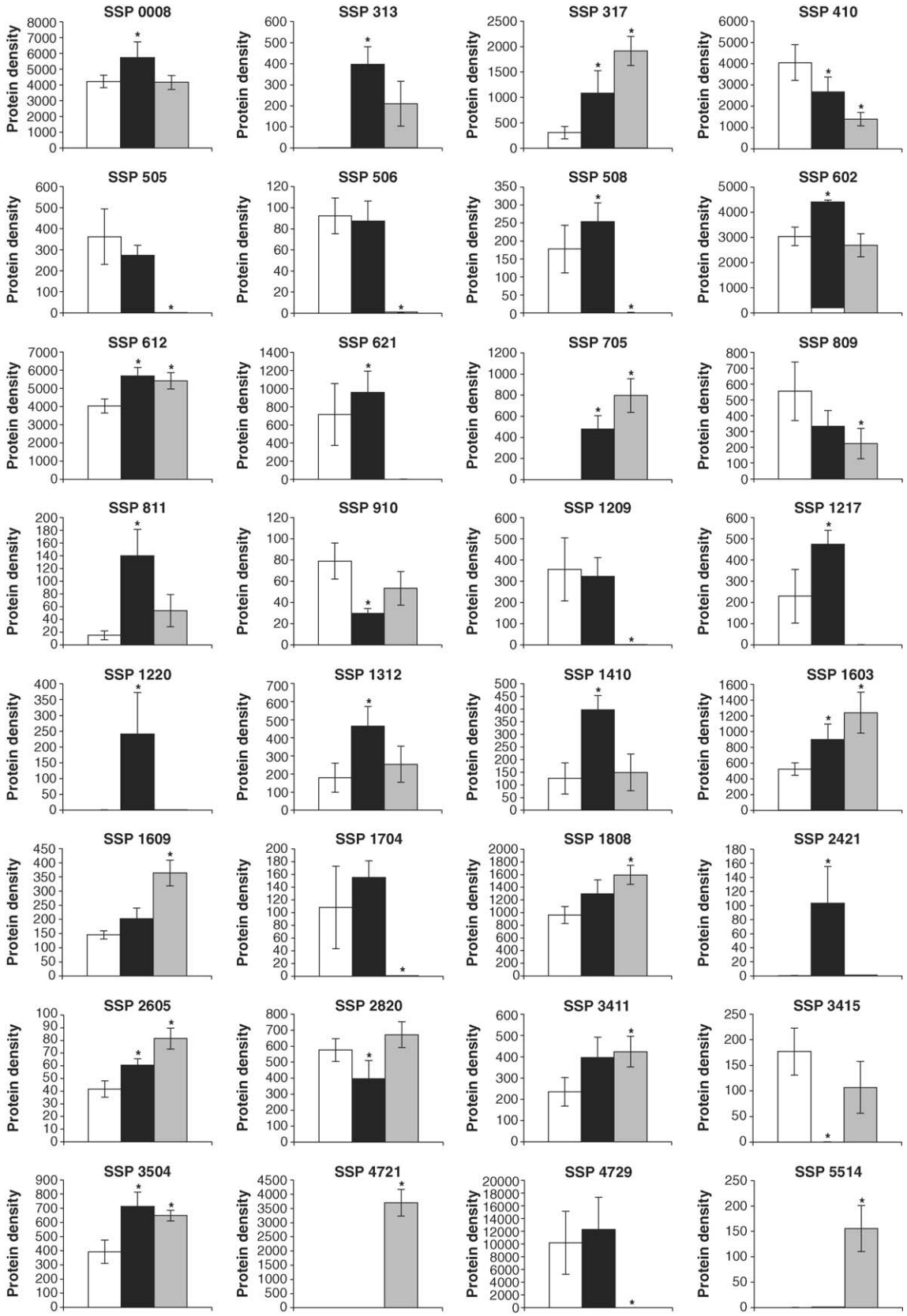

Fig. 3 – Histograms showing spot density measurements of the colon proteins significantly altered relative to the Vitamin E sufficient group. (□) Vitamin E sufficient; (■) Vitamin E deficient, supplemented with salicylic acid; (▩) Vitamin E deficient. Spot IDs and significant differences (*) are shown ($p < 0.05$).

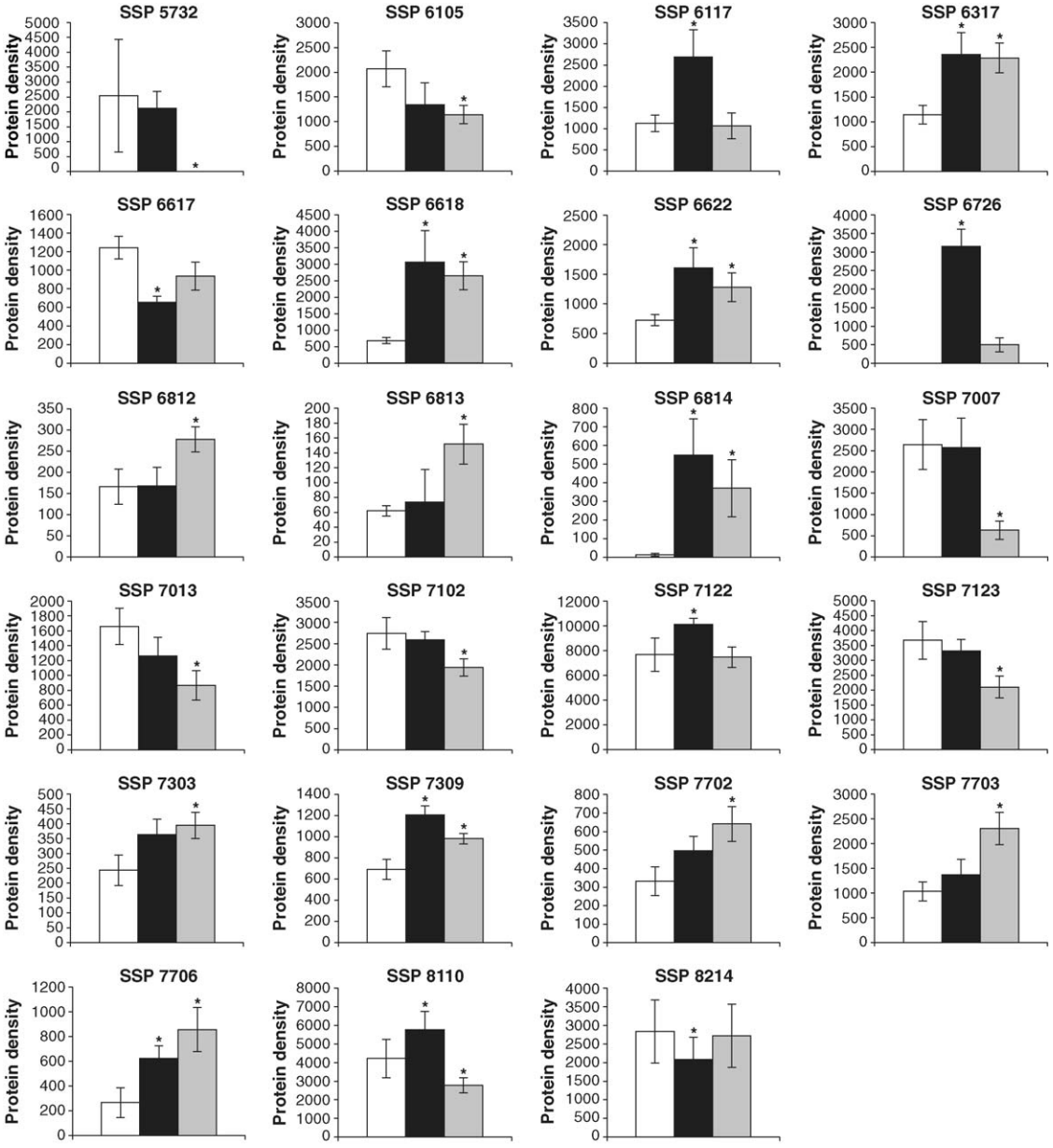

Fig. 3. (Continued).

levels. Interestingly, plasma pyruvate kinase activity was previously observed to be elevated in oxidatively stressed rats (Vitamin E deficient group) when colon prostaglandin levels were increased. Conversely, salicylic acid supplementation reduced plasma pyruvate activity with a corresponding decrease in colon prostaglandins [20] as also supported by the biochemical variable SA that is negatively associated with spot 7703 (Fig. 4). Prostaglandins and mitochondria are both sensitive to oxidative stress. Notably three of the four proteins are associated with mitochondria (spots 317, 1609 and 7703).

The PLS w*c plot can thus be used to predict relationships between *x* and *y* variables (Fig. 4). An arrow directed through the origin towards the biochemical variable (*x* variable) of interest, e.g. PG, permits identification of the *y* variables that are related to the biochemical variable of interest. Hence, for example, spot 410 (tropomysosin), is negatively correlated with prostaglandin levels. A previous study in pre-cancerous rat colon tissue indicated altered patterns and profiles of tropomyosin isomers associated with elevated prostaglandin levels [24]. Conversely, proteins 2820 (hemopexin precursor) and 4721 (serum albumin precursor) would not be predicted to influence prostaglandin levels because they meet the arrow close to the origin. Distance from the origin reflects a corresponding increase in the quantitative relationship between *x* and *y* variables. It is not surprising to learn that these are both plasma proteins that are potentially influenced by salicylic acid supplementation (see further discussion on these proteins below). This would also be predicted from the w*c plot by aligning an arrow through the origin towards SA.

4.1. *Salicylic acid modulation of redox signalling*

Comparison of Vitamin E sufficient rats with Vitamin E deficient or Vitamin E deficient supplemented with salicylic

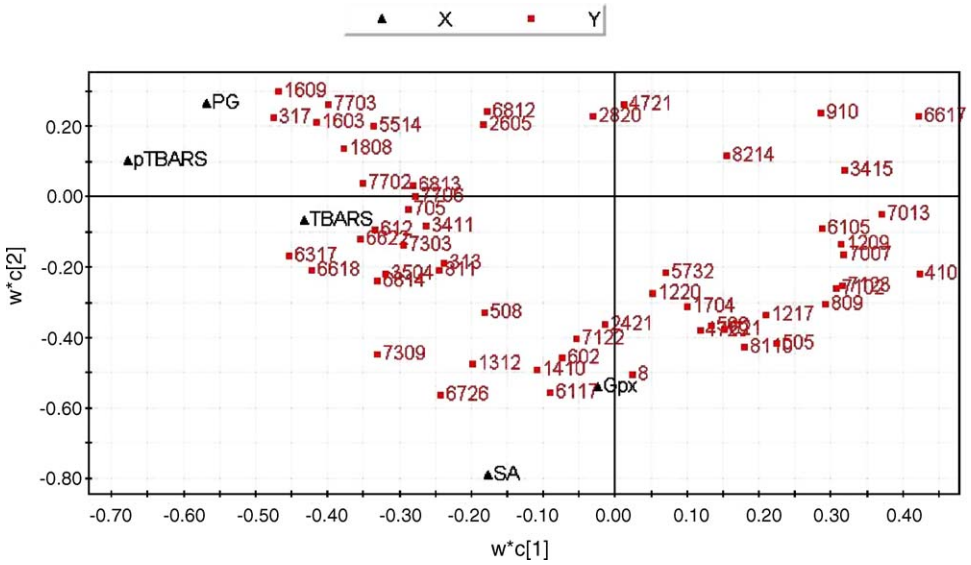

Fig. 4 – Partial least squares (PLS) analysis coefficients of weights (w*c plot) of selected protein data (Y) (the 55 spots selected as being significantly altered in response to the dietary treatments) and biochemical data (X) for the first two components. The w*c plot indicates a moderate correlation between prostaglandins (PG) and TBARS (plasma TBARS = pTBARS and colon tissue TBARS = TBARS); a moderate correlation between salicylic acid (SA) and Gpx activity. Adjacent spots indicate those proteins most strongly correlated with the biochemical parameter. Furthermore, the w*c plot suggests that protein(s) diagonally opposite the biochemical marker via a line directed through and at a distance from the origin is negatively correlated with that biochemical marker.

acid shows parallel and opposing changes in regulation of redox signalling proteins in the colon (Fig. 3). This implies that salicylic acid can regulate redox signalling via similar pathways to Vitamin E, but may do this indirectly through modulation of interactive components of the redox signalling system. The changes observed provide evidence that salicylic acid may alter regulation of the redox environment to alleviate the oxidative imbalance caused by Vitamin E depletion. Seven of the identified proteins are involved in the two major redox pathways of thioredoxin and glutathione (Table 1). These proteins are involved in numerous functions required for maintenance of the redox environment.

Thioredoxins are low molecular weight (10–12 kDa) proteins with oxidoreductase activity. Their numerous functions include activation of redox sensitive transcription factors such as NF(B, p53 and AP-1 [27–29], peroxiredoxin electron donor [30], reduction of protein disulphide targets [31] (see also Section 4.3 below) and regulation of apoptosis. Reduced thioredoxin can inhibit redox sensitive apoptosis by complexing apoptosis signal regulating kinase-1 (ASK-1). Thioredoxin oxidised by ROS dissociates from ASK-1 and activates apoptotic signal transduction [32].

Thioredoxin peroxidase 1 (peroxiredoxin 2) reduces peroxides and is reduced back to its active form via the thioredoxin–thioredoxin reductase system. It is thought to participate in the signalling cascades of growth factors and TNF-α by regulating the intracellular concentrations of hydrogen peroxide. The spot density of this protein was reduced relative to Vitamin E sufficient rats. Oxidative stress inactivates this enzyme by over oxidising its cysteine groups [33]. Thioredoxin peroxidase 1 is thus thought to be a highly sensitive indicator of oxidative stress [34] and its recovery from oxidative stress has been found to be the fastest of all the peroxiredoxins [35]. Its elevation relative to Vitamin E deficient rats supports salicylic acid modulation of oxidative stress [25].

Glutathione is an important antioxidant present in the majority of living cells and is the most abundant intracellular thiol [36]. Glutathione protects against oxidative damage, facilitates formation of DNA, reacts with toxic compounds and participates as a co-enzyme to glyoxylase, glutathione peroxidase, glutathione S-transferase yb-2 and glutathione-dependent formaldehyde dehydrogenase [37]. Oxidative stress causes a decrease in glutathione levels as a consequence of participation in reduction of peroxides with glutathione peroxidase [38]. Glutathione synthase catalyses the production of more glutathione via a negative feedback mechanism [39] and was elevated in the Vitamin E deficient rat colon. Glutatione S-transferase Yb-2 may be a protective mechanism to alleviate oxidative stress and is correspondingly elevated in the Vitamin E deficient and Vitamin E deficient supplemented with salicylic acid rat colon (Fig. 3).

4.2. *Salicylic acid modulation of cytoskeletal proteins*

A number of actin binding proteins involved in regulation of the cytoskeleton showed altered regulation (Table 1). The actin cytoskeleton is an important target of oxidative stress that leads to accumulation of oxidised actin and inappropriate disulphide bond formation. The actin skeleton is central to a number of cell functions, such as cell growth and differentiation, cell signalling and gene expression and is thus intimately linked to human disease.

Vitamin E deficiency appears to alter tropomysosin isomers. Altered tropomyosin isomer expression was observed in a previous study in colon from rats treated with the alkylating agent, dimethylhydrazine [24] and altered tropomysosin profiles are associated with colon pathology [40,41]. Salicylic acid supplementation of Vitamin E deficient rats appears to regulate some of the identified actin-binding proteins in colon (schinderin, profilin-1) in a similar way to rats sufficient in Vitamin E, with opposing effects on expression levels in Vitamin E deficient rats. This would appear to indicate that oxidative stress has an adverse effect on proteins involved in regulating the actin skeleton directly and that salicylic acid modulation of oxidative stress helps to maintain regulation of the actin skeleton. A number of the actin binding proteins were characterised by altered profiles perhaps indicating changes in modification of these proteins. A number of these proteins are prone to gluthionylation [42], which occurs after oxidative stress and in addition to altered isomer expression or truncated proteins may contribute to the multiple IDs (generated by varying Mr and, or pI) resolved on the 2D gels. Salicylic acid potentially opposes modifications generated by oxidative stress, supported by the opposing effects on the density of the multiple spots observed for these proteins.

4.3. *Salicylic acid modulation of protein folding*

It is well recognised that oxidative stress invokes heat shock proteins and chaperones to deal with aberrant folding and damaged proteins generated in the cytosol. Oxidative stress appears to result in elevation of HSP70 (1808) (Fig. 3) and one of the three detected calreticulin spots (0705) (Fig. 3) in Vitamin E deficient rats. Salicylic acid appears to reduce elevation of these particular spot densities (0705 and 1808) in Vitamin E deficient rats. This may be an indication of a reduced requirement of these proteins to deal with aberrant folding or damaged proteins generated in the cytosol of Vitamin E deficient rats supplemented with salicylic acid. Alternative calreticulin spots with altered spot densities in response to salicylic acid and multiple locations (0602 and 0621), may be related to activation of calreticulin by post-translational modifications.

The redox environment must be maintained to ensure optimal formation of disulphide bond formation and responses to protein misfolding. A large number of disulphide bonded proteins are involved in molecular chaperoning, glycolysis, cell growth, cytoskeletal structure and signal transduction [43]. Disulphide regulation is a major player in protein folding and protein disulphide isomerase (PDI) and thioredoxin are key players in this process [31,43]. Vitamin E depletion (with/without salicylic acid supplementation) appears to result in elevation of PDI (a redox enzyme). However, the concomitant up-regulation of TRX with salicylic acid supplementation may provide opportunities for further interaction of these proteins in regulating redox signalling and protein folding processes.

4.4. *Salicylic acid modulation of energy metabolism*

A number of enzymes involved in regulating energy metabolism showed altered protein profiles in response to Vitamin E depletion and salicylic acid modulation (Table 1; Fig. 3). Many are involved in mitochondrial metabolism indicating the importance of regulation of redox balance to ensure optimal mitochondrial function. It was noted that mitochondrial transport may also be influenced, as a mitochondrial import stimulation factor was elevated in Vitamin E depleted colon. As observed for altered levels of proteins involved in redox signalling, comparison of Vitamin E sufficient rats and Vitamin E deficient supplemented with salicylic acid show both parallel and opposing changes in regulation of energy metabolising proteins in the colon (Table 1). A number of these enzymes are regulated by thiol reduction and require an optimal redox environment to ensure activity. ATPases are damaged by oxidative stress as are mitochondria and are notably depressed in carcinomas [44,45]. It is therefore not surprising to find that Vitamin E deficient rats have elevated levels of ATPase FI sub-unit that is transported to mitochondria where it functions in ATP synthesis coupled proton transport [46]. Similarly, increased levels of NAD + specific isocitrate dehydrogenase α-subunit in Vitamin E deficient rats may be linked to replenishment of thioredoxin in mitochondria [47]. Pyruvate kinase, a marker of oxidative stress is higher in Vitamin E deficient rats compared to those supplemented with salicylic acid. Salicylic acid appears to reduce elevation of a number of these proteins involved in energy metabolism relative to Vitamin E depleted rats, alternatively salicylic acid produces the opposite effect perhaps indicating effects involved in alleviating the oxidative stress caused by Vitamin E depletion.

4.5. *Salicylic acid modulation of plasma proteins*

The presence of blood plasma proteins may simply represent contamination of the colon extracts with traces of blood or may indicate altered profiles in plasma in response to salicylic acid. There was evidence of altered profiles of serum proteins with several proteins in close vicinity being identified as serum albumin precursor (Fig. 1; Table 1). The altered profile of the resolved proteins in response to dietary treatment may indicate underlying changes in the systemic circulation in response to salicylic acid absorption from the gastrointestinal tract.

5. Concluding remarks

Salicylic acid modulates oxidative stress and associated potentially pro-cancerous activity in the colon. This may be via mechanisms linked to redox signalling. The changes observed may assist in identification of pre-pathological features preceding colon tumorigenesis and contribute to an understanding of epidemiological evidence supporting a protective effect of plant-based diets.

Acknowledgements

The authors would like to thank Gwen Baxter (Dumfries Royal Infirmary, Scotland) for technical assistance on measurement of salicylic acid. This work was supported by the Scottish

Executive Environment and Rural Affairs Department (SEERAD), Scotland.

REFERENCES

[1] Borek C. Dietary antioxidants and human cancer. Integr Cancer Ther 2004;3:333–41.
[2] Seril DN, Liao J, Yang GY, Yang CS. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. Carcinogenesis 2003;24: 353–62.
[3] Rhodes JM, Campbell BJ. Inflammation and colorectal Cancer: IBD-associated and sporadic cancer compared. Trends Mol Med 2002;8:10–6.
[4] Ekbom A, Helmick C, Zack M, Adami HO. Increased risk of large-bowel cancer in Crohn's disease with colonic involvement. Lancet 1990;336:357–9.
[5] Langholz E, Munkholm P, Davidsen M, Binder V. Colorectal cancer risk and mortality in patients with ulcerative colitis. Gastroenterology 1992;103:1444–51.
[6] Bargen JA. Chronic ulcerative colitis associated with malignant disease. Dis Colon Rectum 1994;37:727–30.
[7] Choi PM, Zelig MP. Similarity of colorectal cancer in Crohn's disease and ulcerative colitis: implications for carcinogenesis and prevention. Gut 1994;35:950–4.
[8] Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860–7.
[9] Eaden J, Abrams K, Ekbom A, Jackson E, Mayberry J. Colorectal cancer prevention in ulcerative colitis: a case–control study. Aliment Pharmacol Ther 2000;14:145–53.
[10] Thun MJ, Namboodiri MM, Heath Jr CW. Aspirin use and reduced risk of fatal colon cancer. New Engl J Med 1991;325:1593–6.
[11] Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresaier R, et al. A randomised trial of aspirin to prevent colorectal adenomas. New Engl J Med 2003;348:891–9.
[12] Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, et al. Treatment of colonic and rectal adenomas with suldinac in familial adenomatous polyposis. New Engl J Med 1993;328:1313–6.
[13] Ladenheim J, Garcia G, Titzer D, Herzenberg H, Lavori P, Edson R, et al. Effect of suldinac on sporadic colonic polyps. Gastroenterology 1995;108:1083–7.
[14] Steinbach G, Lynch PM, Phillips RKS, Wallace MH, Hawk E, Gordon GB, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. New Engl J Med 2000;342:1946–52.
[15] Pierpoint WS. Can aspirin help identify leaf proteins active in defence responses? Ann Appl Biol 2002;140:233–9.
[16] Donaldson MS. Nutrition and cancer: a review of the evidence for an anti-cancer diet. Nutr J 2004;3:19.
[17] Steinmetz KA, Potter JD. Vegetables, fruit and cancer. I. Epidemiology. Cancer Causes Control 1991;2:325–57.
[18] Paterson JR, Lawrence JR. Salicylic acid: a link between aspirin, diet and the prevention of colorectal cancer. Quart J Med 2001;94:445–8.
[19] Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, et al. A randomised trial of aspirin to prevent colorectal adenomas in patient with previous colorectal cancer. New Engl J Med 2003;348:883–90.
[20] Drew JE, Arthur JR, Farquharson AJ, Russell WR, Morrice PC, Duthie GG. Salicylic acid modulates oxidative stress and glutathione peroxidase activity in the rat colon. Biochem Pharmacol 2005;70:888–93.
[21] Drew JE, Arthur JR, Farquharson AJ, Russell WR, Morrice PC, et al. Novel sites of cytosolic glutathione peroxidase expression in colon. FEBS Letters 2005;579:6135–9.
[22] Drew JE, Mercer DK, Mayer C, Farquharson AJ, Morrice PC, Arthur JR, et al. Oxidative stress in colon tissue induced by Vitamin E depletion. Biochem Soc Transact 2004;32:979–81.
[23] Blacklock CJ, Lawrence JR, Wiles D, Malcolm EA, Gibson IH, Kelly CJ, et al. Salicylic acid in the serum of subjects not taking aspirin. Comparison of salicylic acid concentrations in the serum of vegetarians, non-vegetarians, and patients taking low dose aspirin. J Clin Pathol 2001;54:553–5.
[24] Drew JE, Rucklidge GJ, Duncan G, Lufty A, Farquharson AJ, Reid MD, et al. A proteomics approach to identify changes in protein profiles in pre-cancerous colon. Biochem Biophys Res Commun 2005;330:81–7.
[25] Anderson L. Two dimensional electrophoresis–operation of the ISO-DALT system Rockville MD, USA: Large Scale Biology Press; 1991.
[26] Yang JW, Juranville JF, Hoger H, Fountoulakis M, Lubec G. Molecular diversity of rat brain proteins as revealed by proteomic analysis. Mol Divers 2005;9:385–96.
[27] Abate C, Patel L, Rauscher FJ, Curran T. Redox Regulation of fos and jun DNA-binding activity in vitro. Science 1990;249:1157–61.
[28] Qin J, Clore GM, Kennedy WP, Hutch R, Gronenborn AM. Solution structure of human thioredoxin in a mixed disulfide intermediate complex with its target peptide from the transcription factor NF-kappa B. Structure 1994;3:289–97.
[29] Ueno M, Masutani H, Arai RJ, Yamauchi A, Hirota K, Sakai T, et al. Thioredoxin-dependent redox regulation of p53-mediated p21 activation. J Biol Chem 1999;274:35809–15.
[30] Nordberg J, Arner ESJ. Reactive oxygen species, antioxidants, and the mammlian thioredoxin system. Free Rad Biol Med 2001;31:1287–312.
[31] Lundstrom J, Holmgren A. Protein disulfide-isomerase is a substrate for thioredoxin redctase and has thioredoxin-like activity. J Biol Chem 1990;256:9114–20.
[32] Hodges NJ, Smart D, Lee AJ, Lewis NA, Chipman JK. Activation of c-jun-N-terminal kinase in A549 lung carcinoma cells by sodium dichromate: role of dissociation of apoptosis signal regulating kinase-1 from its physiological inhibitor thioredoxin. Toxicology 2004;197:101–12.
[33] Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K, et al. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. J Biol Chem 2002;277:38029–36.
[34] Cesaratto L, Vascotto C, D'Ambrosio C, Scaloni A, Baccarani U, Paron I, et al. Overoxidation of peroxiredoxins as an immediate and sensitive marker of oxidative stress in HepG2 cell and its application to the redox effects induced by ischemia/reperfusion in human liver. Free Rad Res 2005;39:255–68.
[35] Chevallet M, Wagner E, Luche S, van Dorsselaer A, Lieze-Wagner E, Rabilloud T. Regeneration of peroxiredoxins during recovery after oxidative stress. J Biol Chem 2003;278:37146–53.
[36] Filomeni G, Rotilio G, Ciriolo MR. Cell signalling and the glutathione redox system. Biochem Pharmacol 2002;64:1057–64.
[37] Anderson ME. Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 1998;111:1–14.
[38] Mullineaux P, Creissen GP. Glutathione-reductase: regulation and role in oxidative stress. In: Oxidative stress and the molecular biology of antioxidant defenses. USA: Cold Spring Harbor Laboratory Press; 1997.
[39] Meister A, Anderson ME. Glutathione. Ann Rev Biochem 1983;52:711–60.
[40] Geng X, Biancone L, Dai HH, Lin JJ, Yoshizaki N, Dasgupta A, et al. Tropomyosin isoforms in intestinal mucosa:

production of autoantibodies to tropomyosin isoforms in ulcerative colitis. Gastroenterology 1998;114:912–22.
[41] Lin JL, Geng X, Bhattacharya D, Yu JR, Reiter RS, Sastri B, et al. Gastroenterology 2002;123:152–62.
[42] Fratelli M, Demol H, Puype M, Casagrande S, Eberini I, Salmona M, et al. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. Proc Natl Acad Sci USA 2002;99:3505–10.
[43] Ellgaard L, Ruddock LW. The human protein disulphide isomerase family: substrate interactions and functional properties. EMBO Rep 2005;6:28–32.
[44] Cuezva JM, Krajewsja M, Lopez de Heredia M, Lrajewsja S, Santamaria G, Kim H, et al. The bioenergetic signature of cancer: a marker of tumour progression. Cancer Res 2002;62:6674–81.
[45] Isidoro A, Martinez M, Fernandez PL, Ortega AD, Santamaria G, Chamorro M, et al. Alteration of the bioevergetic phenotype of mitochondria s a hallmark of breast, gastric, lung and oesophageal cancer. Biochem J 2004;378:17–20.
[46] Fillingame RH. Coupling H + transport and ATP synthesis in F1F0-ATP synthases: glimpses of interactingparts in a dynamic molecular machine. J Exp Biol 1997;200:217–24.
[47] Jo SH, Son MK, Koh HJ, Lee SM, Song IH, Kim YO, et al. J Biol Chem 2001;276:16168–76.