DOI 10.1002/pmic.200300557 *Proteomics* 2004, *4,* 524–536

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Copper-associated liver disease: A proteomics study of copper challenge in a sheep model

Sheep display a variant phenotype with respect to their susceptibility to copper and derivative pathology. The North Ronaldsay sheep are acutely sensitive to environmental copper while the Cambridge breed is much more copper-tolerant. A study of protein expression in the liver of the two different breeds of sheep as a result of copper challenge would aid in the understanding of their differing pathophysiologies and contribute to knowledge of copper toxicosis in man. In this initial study, Cambridge breed sheep were challenged with oral copper and liver proteins were analyzed by two-dimensional (2-D) gel electrophoresis. Proteins whose expression pattern was modified by copper exposure were then identified by peptide mass fingerprinting using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. In conclusion, the pattern of changes in protein expression were consistent with an early adaptive response to oxidative challenge. This was followed by evidence of an impaired ability of the liver to compensate as copper loading increased, accompanied by oxidative stress-induced injury.

Keywords: Cambridge sheep / Copper toxicosis / Mass spectrometry / Two-dimensional gel electrophoresis / Wilson disease PRO 0557

1 Introduction

Wilson disease is a disorder of copper metabolism which occurs as an autosomal recessive disorder in adults of all ethnic groups [1]. Another copper-associated disease occurs in childhood and is variously named Indian childhood cirrhosis, Endemic Tyrolean idiopathic cirrhosis and sporadic idiopathic copper toxicosis. These diseases are pathologically and genetically distinct from Wilson disease and are all characterised by fibrosis leading to a life-threatening cirrhosis [2]. North Ronaldsay sheep, which have been identified as a possible model for this copper toxicosis of childhood, are particularly sensitive to environmental copper, which induces hepatocellular damage with exuberant fibrosis and incipient cirrhosis [3]. More copper-tolerant breeds of sheep such as the Cambridge and other domesticated breeds accumulate copper less easily and incur a more muted liver pathology lacking significant fibrogenesis [4]. These major differences in response to copper exhibited by different breeds of sheep may be due in part to differential uptake of the metal and/or excretion. More importantly, the divergent pathological response may derive from a differential expression of the constituent cellular components of the liver.

Genetic factors must ultimately determine these different types of response to copper in the two breeds, but at the cellular metabolic level it is the gene products that are responsible for the manifestation of the response. An exploration of copper challenge on protein expression in the liver of the two different breeds of sheep would be an important prelude to understanding their differing pathophysiologies. Further understanding of the molecular events in terms of protein expression would clarify the damaging effect of copper and contribute to the management of copper-associated liver disease in both man and animals. Proteomics aims to define changes in the levels of global protein expression in a physiological or pathophysiological system. In this paper, we report the first proteomics study of the response of hepatic soluble proteins in liver, specifically from copper-challenged Cambridge sheep.

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Abbreviations: GST, glutathione *S*-transferase; **ICP**, inductively coupled plasma; **IDH**, $NADP^+$-dependent isocitrate dehydrogenase

2 Materials and methods

2.1 Experimental animals

Nine Cambridge ewes were given either a copper-supplemented pelleted diet (Wynnstay Farmers, Llansantffraid, Powys, Clwyd, UK) containing 155 mg/kg copper (6 ani-

mals) or the same copper unsupplemented diet containing less than 50 mg/kg copper (3 animals). The concentrates were gradually introduced to the basal diet of hay and beet over a 3 month period to induce a non-fatal liver copper overload [5]. The trial was continued for a further month with all sheep receiving 500 g/day pelleted feed, whether copper-supplemented or not. Sheep were culled at 3, 3.5, and 4 months. The animals were euthanized with pentobarbitone and the livers immediately removed and perfused with heparinized saline. Samples were taken from both dorsal and ventral lobes since differential lobar accumulation of copper has been recorded in rats [6]. The samples were quenched in either liquid N_2 for subsequent 2-D gel analysis or over solid CO_2 for metal analysis. Additional liver samples were fixed in 2.5% v/v glutaraldehyde for electron microscopy and in 10% v/v formalin for routine histology [3]. Ethical statement: The animal experiments were carried out under licence granted by the UK Animal (Scientific Procedures Act) 1986.

2.2 Metal analysis

Liver samples (0.5 g) were weighed and dried for 72 h in an oven at 80°C. The dried tissues were reweighed and digested in 2 mL of SpA-grade concentrated nitric acid (Romil Cambridge, UK) in acid-washed pyrex glass boiling tubes on a hotplate. The digests were made up to 5 mL using Milli-Q water and then decanted into polypropylene screw-capped tubes. Elemental analysis was carried out using an Elan 6100 inductively coupled plasma mass spectrometer after appropriate dilution with Milli-Q water. Copper content is expressed as μg/g dry weight.

2.3 2-DE of soluble liver proteins

Perfused liver (300–500 mg wet weight) was thawed and sectioned with a scalpel blade to facilitate homogenization. To each 300 mg tissue was added 1 mL of 40 mM Tris, 2 mM $MgCl_2$, pH 8.0, containing Complete™ EDTA-free protease inhibitor cocktail (Roche), and the tissue was homogenized for 1 min using a Ystral homogeniser (SIC, Eastleigh, Hampshire, UK). Deoxyribonuclease 1 (DNase 1 from bovine pancreas, 2000 Kunitz units per mg of protein; Sigma, Poole, UK) and ribonuclease A (RNase A, 80 units/mg protein; Sigma) were added to a concentration 100 units and 0.8 units per mL of homogenate, respectively, and the homogenates were incubated at room temperature for 30 min. The homogenates were centrifuged at 29 000 rpm ($\sim 85\,000 \times g$) for 1 h at 20°C using a T865 rotor in a Sorvall Combi ultracentrifuge. The supernatant fractions were decanted and either applied directly to IPG strips or stored at −20°C for future analysis. The protein content of the supernatant fraction was measured using a Coomassie dye binding assay (Pierce, Cheshire, UK). Liver supernatants (typically 10–20 μL) containing 200 μg of protein were solubilized in 7 M urea, 2 M thiourea, 4% w/v CHAPS, 20 mM DTT, 0.2% v/v carrier ampholytes (pH 5–8), with a trace of bromophenol blue dye. Samples were incubated at room temperature for 1 h before centrifugation at 8000 rpm for 5 min. Samples (185 μL) were applied to 11 cm, pH 5–8 IPG strips (Bio-Rad, Hemel Hempsted, UK). Active rehydration of the strips was at 50 V for 500 Vh, and 1000 V for 2000 Vh, followed by a voltage ramping step of 1000 V–8000 V for 24 000 Vh and a final focussing step of 8000 V for 25 000 Vh. All focussing steps were at 20°C in a Protean IEF cell (Bio-Rad). Proteins resolved in the first dimension strips were reduced and alkylated prior to second-dimensional electrophoresis. Strips were incubated for 15 min in a solution containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 2% w/v DTT, followed by 15 min in the same solvent containing 2.5% w/v iodoacetamide in place of the DTT. The second-dimensional electrophoresis (SDS-PAGE) was performed on a Hoefer SE 600 system (Amersham Biosciences). After laying the strip on top of a 12.5% polyacrylamide gel and sealing it with agarose, electrophoresis was carried out for 30 min at 15 mA per gel, at which time a constant current of 30 mA per gel was applied until the dye front reached the lower end of the gel. Proteins were stained overnight with 0.08% w/v Coomassie Brilliant Blue G-250 in 40% v/v methanol, 10% v/v acetic acid and were visualized by background destaining in a solution of 10% v/v methanol, 10% v/v acetic acid.

2.4 Image analysis of 2-DE gels

Gels were scanned using an Epson 1680 pro flat bed scanner (266 dpi, 24-bit colour) and images were saved in an uncompressed TIFF file format. The gels were compared using the PDQuest 7.0 gel analysis software (Bio-Rad). Protein spots whose levels had changed by 2-fold or more were placed into analysis sets and selected for excision by the ProteomeWorks spot cutter (Bio-Rad). Excised gel spots were robotically destained, digested with trypsin and spotted onto a MALDI target plate by a MassPREP Workstation (Micromass, Manchester, UK).

2.5 Characterization of protein spots by MALDI-MS

Peptide mass fingerprinting of protein digestion products was performed on a M@LDI R mass spectrometer (Micromass, Manchester, UK). Protein databases were searched using the Mascot search engine [7]. The monoisotopic masses of the tryptic peptides were compared to

the SWISS-PROT and NCBI mammalian databases [8, 9] using a mass tolerance of 150–200 ppm, allowing for up to one missed tryptic cleavage, and with carbamidomethylation of cysteine residues as a fixed modification and oxidation of methionine as a variable modification.

3 Results and discussion

3.1 Copper content of liver

Liver copper accumulation was recorded from 3 months in both dorsal and ventral lobar compartments. The copper content of the two compartments were indistinguishable from one another at any one time point. A moderate liver copper overload had occurred at 3 months in the two trial sheep of 474 and 445 μg/g compared with the control sheep of 260 μg/g (copper content averaged across dorsal and ventral lobes). By 3.5 months this value had risen to 976 and 1142 μg/g (control sheep 279 μg/g), and was sustained at a similar value of 1017 and 1095 μg/g at 4 months (Fig. 1).

3.2 Changes in the liver proteome

Proteins were resolved in the first dimension by isoelectric focusing across the pH range 5–8, which gives excellent resolution of large numbers of liver proteins. In the second dimension, proteins were electrophoresed in linear 12.5% w/v polyacrylamide gels which resolved proteins over a mass range of 6.5–200 kDa. The location of known proteins in the gels correspond to their locations on a recently published 2-DE reference map of bovine proteins [10]. For each 2-DE experiment (performed in triplicate), a control sample prepared from an unsupplemented sheep liver was analyzed at the same time as liver samples prepared from two individual copper-challenged sheep. Quantitative information on differentially expressed proteins was obtained by image analysis using PDQuest software. Representative 2-D gels of soluble liver proteins isolated from a control and one each of the copper-challenged sheep at moderate and high copper-loading, respectively, indicate the reproducibility of the separation and the complexity of the protein pattern over this p*I* range (Fig. 2). Circled on the gels are proteins that were differentially expressed (more than a 2-fold difference) as a result of copper challenge. These spots were excised from the gel and identified where possible through peptide mass fingerprinting using MALDI-MS.

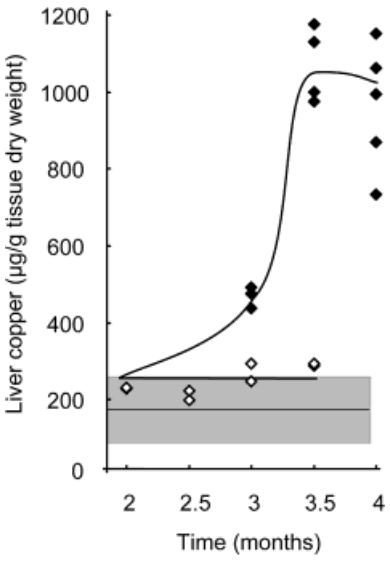


Figure 1. Copper content in liver of normal and challenged sheep**.** Samples from the perfused dorsal and ventral lobes of control (open symbols) or copper challenged (closed symbols) sheep were analysed for copper content by inductively coupled plasma mass spectrometry (ICP-MS). The results are superimposed upon previously published values for normal copper levels in sheep [3].

Identification of proteins by peptide mass fingerprinting is dependent on high-quality MALDI-TOF mass spectra from which multiple monoisotopic peptide masses can be recovered. Further, the probability of an informative match is substantially raised if the unknown protein is present in a nonredundant peptide database. If this protein is absent from the database, a match is only possible if there is sufficient sequence similarity to other protein(s) present in the database. However, because relatively few peptides are required for successful matching, the search is tolerant to limited amino acid substitutions from one species to another. There are currently just over 1000 entries in the SWISS-PROT database for sheep proteins, and it was likely that we would be reliant on good cross-species sequence matching. Approximately half of the total spots submitted to MALDI-TOF-MS generated good spectra but database searching of the mass spectra of these proteins yielded no significant hits; these proteins are not identified in Fig. 2. Tandem mass spectrometry would yield partial sequence information and increase the likelihood of successful database identification, but this was beyond the scope of the present study.

We have been able to identify a number of proteins that were differentially expressed in the copper-challenged sheep liver. Table 1 summarizes those proteins that could be identified, details of cross-species matching and the confidence level of the identification in terms of a probability-based Mowse score and sequence coverage. Further, the direction of the response of these proteins is coded in the table (final column). From the 18 proteins listed in Table 1, only two proteins are present in the very

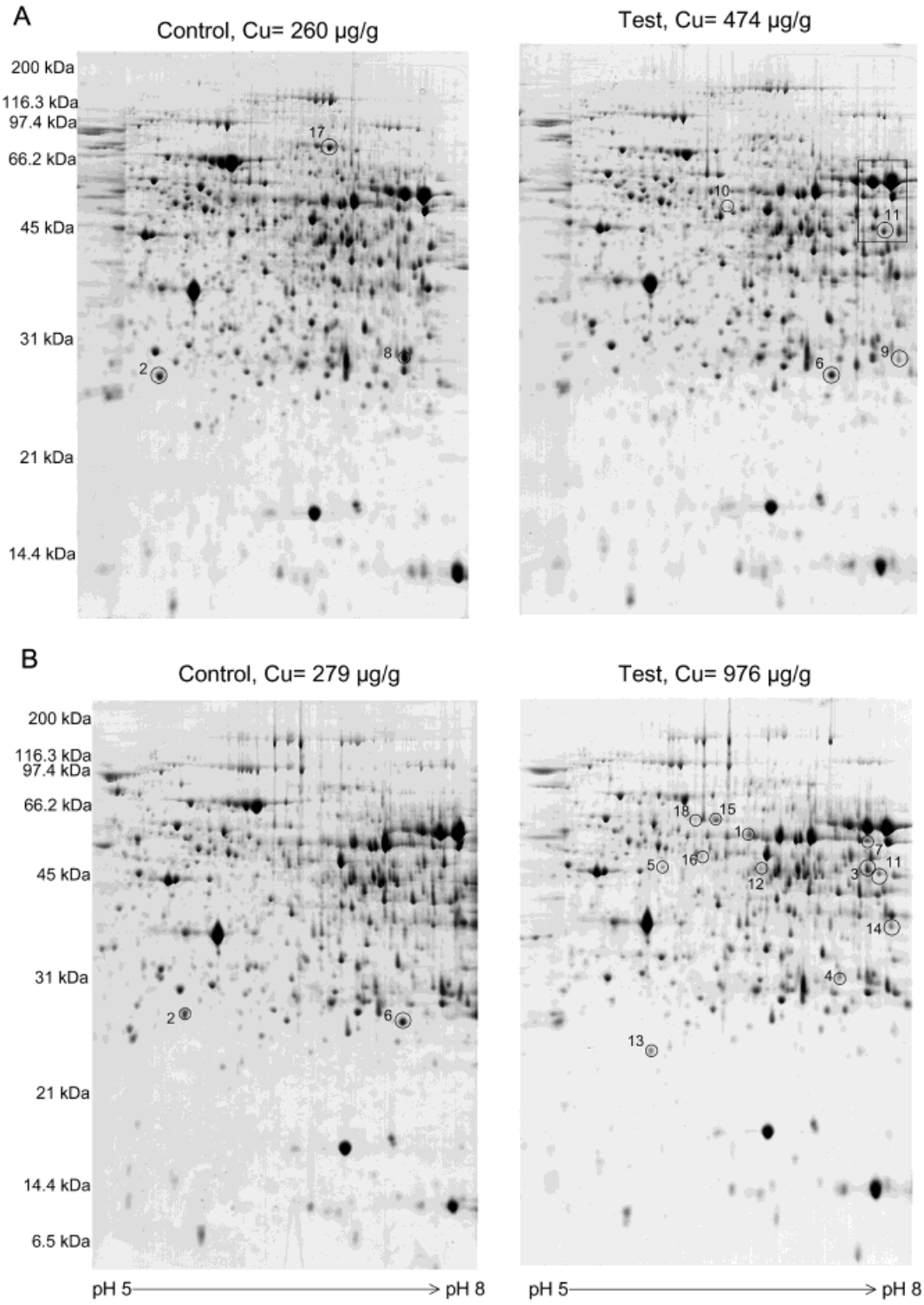


Figure 2. Two-dimensional gel electrophoresis of sheep liver cytosolic proteins following copper loading. Proteins were separated in the first dimension on pH 5–8 immobilized pH gradient strips, followed by SDS-PAGE on 12.5% gels. Gels were stained with Coomassie Brilliant Blue G-250. Differentially expressed protein whose levels differed by 2-fold or more between the control and copper-treated animals are circled, and the identities of these proteins are given in Table 1. The copper level in each individual liver (averaged across dorsal and ventral lobes, µg/g dry weight) is indicated on the top of each panel.

Table 1. Identification of copper-responsive proteins

Spot	Protein identification	Accession	Species	M_r	Mowse	Seq.	Change
1	Aldehyde dehydrogenase	gi/1706388	Sheep	55 417	390	62%	– – ↑↑
2	Apolipoprotein A-I	gi/245563	Cow	28 415	269	59%	↓↓→↓
3	Betaine homocysteine methyltransferase	gi/13540663	Rat	45 404	98	38%	– – ↑
4	Carbonic anhydrase II	gi/115460	Sheep	29 062	88	41%	– – ↑↑
5	Creatine kinase	gi/13096153	Cow	42 836	141	46%	– – →↑
6	Flavin reductase	gi/1706869	Cow	22 232	118	66%	↑↑→↓
7	Glutamate dehydrogenase	gi/13096299	Cow	55 868	154	29%	– – ↑↑
8	Glutathione *S*-transferase M5	gi/4504181	Human	25 847	87	29%	↓↓ – –
9	Glutathione *S*-transferase mu	gi/7582395	Cow	25 789	142	44%	↑↑ – –
10	Glutathione synthetase	gi/4504169	Human	52 523	99	28%	↑↑ – –
11	$NADP^+$-dependent isocitrate dehydrogenase	gi/4959708	Cow	47 098	385	63%	↑↑↑↑
12	Myotubularin-related protein 7	gi/11133658	Human	46 035	66	20%	– – ↑↑
13	Plasma retinol-binding protein	gi/132403	Cow	21 397	94	31%	– – ↑↑
14	Prostaglandin-F synthase 2	gi/1730509	Cow	37 118	81	21%	– – ↑↑
15	Protein disulphide isomerase A3	gi/729433	Cow	57 293	233	40%	– – ↑↑
16	RAB GDP-dissociation inhibitor β	gi/13638229	Mouse	50 993	98	29%	– – ↑↑
17	Serotransferrin precursor	gi/2501351	Cow	79 870	124	24%	↓↓ – –
18	T-complex protein 1 (α subunit)	gi/135536	Hamster	60 814	102	19%	– – ↑↑

Differentially expressed proteins whose levels differed by 2-fold or more between the control and copper-treated animals were identified by MALDI-MS and PMF. The direction of change is denoted by: – not detected, → unchanged, ↓ decreased, ↑ increased.

limited dataset of sheep protein sequences and the identification of most proteins is the result of matching to, and by implication, good sequence homology to bovine proteins. For example, a region of the 2-D gel in Fig. 2 (spot 11) has been enlarged to highlight the isoform of cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDH) that was strongly induced in all four copper-supplemented sheep (Fig. 3). This protein was identified through a match to the bovine homologue (Fig. 4). The high probability of this identification was attributable to a large number of matching peptides. The range over which data was acquired was between *m/z* 900 and *m/z* 4000. Of the 414 amino acids of the nascent polypeptide chain, 28 of the total of 30 peptides submitted to the Mascot search engine were matched to bovine cytosolic IDH, giving a sequence coverage of 63%. The Mowse score for this match means that the probability of this identification being incorrect is vanishingly low. Similarly high-quality matches were obtained for the other proteins described below.

Figure 3 also shows the densitometric quantification of the spot corresponding to this isoform of isocitrate dehydrogenase. In this example, the lack of a spot in the unchallenged animals makes a relative quantification, expressed as a fold-difference, meaningless. We therefore set the highest spot intensity to 100%, and express all other spots relative to this value. Each value (normalised spot quantity) is then expressed as the mean ± SEM for triplicate analyses of the liver tissue. Similar analyses have been applied to all other spots showing greater than a two-fold difference in protein expression level.

A number of other proteins showed changes in expression levels in the liver of copper-challenged sheep (Fig. 5). Some proteins, notably apolipoprotein A-1, and serotransferrin were downregulated. These proteins are destined for secretion from the cell, and the lower levels in tissue homogenates might reflect disruption of the protein synthesis and export machinery as much as the steady state level of protein in the cells. It is difficult to relate such changes to any specific mechanisms of response to copper challenge. Other proteins, such as prostaglandin-F synthase 2 and T-complex protein 1 showed a 2- to 3-fold increase in expression in the liver of copper challenged sheep (Fig. 5) but it is difficult to attach any mechanistic explanation of such changes. An increase in the levels of RAB GDP-dissociation inhibitor suggests a downregulation of vectorial trafficking mediated by RAB proteins in the liver of copper-treated animals and may reflect a general decline in hepatic function at this level of copper toxicity.

3.2.1 Cytosolic IDH

As described above, cytosolic isocitrate dehydrogenase was dramatically induced in moderate or high copper challenge (Fig. 3). Both mitchondrial and cytosolic IDH

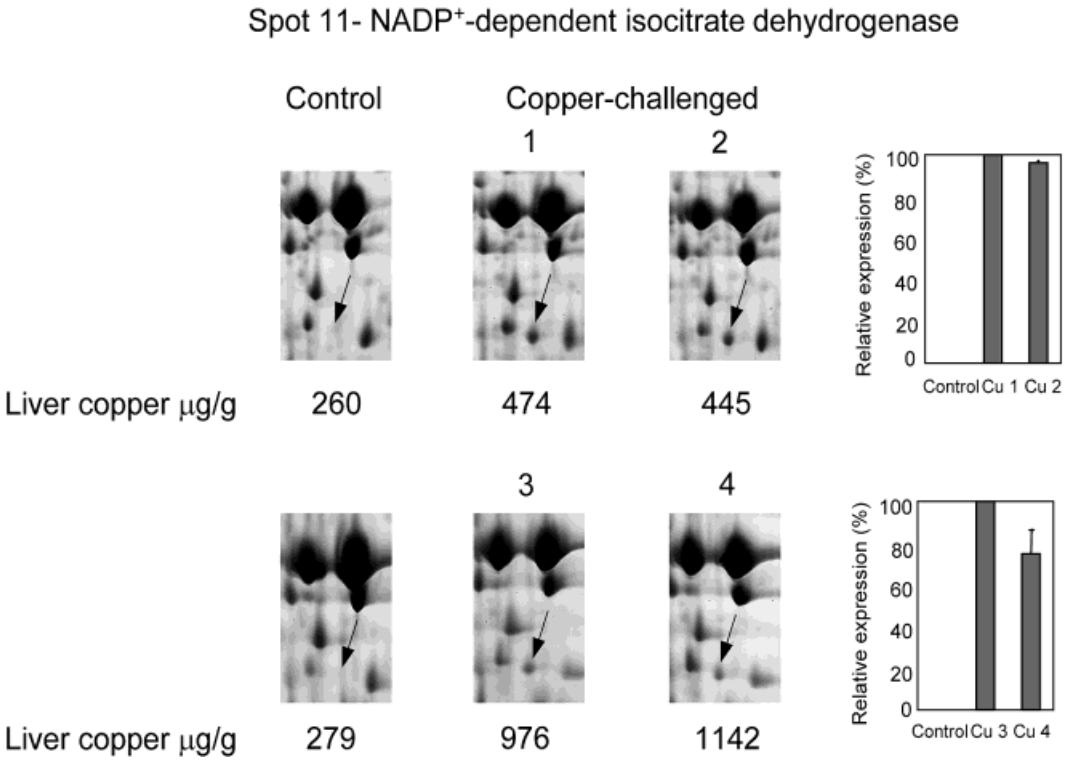


Figure 3. Differential expression of IDH in moderate and high copper-loaded sheep liver. The region of the gel encompassing a protein identified as cytosolic IDH by MALDI-TOF-MS and peptide mass fingerprinting is shown for two control animals, and four copper-challenged animals. The copper level in each individual liver (mean value of dorsal and ventral lobes), determined by ICP-MS is indicated.

are induced in response to oxidative stress [11, 12]. The *C*-terminal of cytosolic IDH includes a tripeptide peroxisome targeting sequence and the enzyme is predominantly present in the peroxisomes of rat hepatocytes [13]. IDH may play an important role in cellular defence against oxidative stress which is attributable to the increased levels of NADPH which is essential for efficient glutathione recycling, and the maintenance of the cellular redox state [11, 12]. It may also stimulate the production of 2-oxoglutarate, the precursor of glutamate and in turn, a component of glutathione.

3.2.2 Glutathione synthetase

Under conditions of moderate copper challenge, glutathione synthetase is substantially elevated from undetectable levels in control samples (Fig. 5). Deficiency of this enzyme leads to diminished levels of glutathione in tissues, and an impaired ability to resist oxidative stress [14]. The elevation of this enzyme in copper challenged hepatocytes is therefore unsurprising, and is consistent with the elevation of isocitrate dehydrogenase.

3.2.3 Glutathione *S*-transferases

Two glutathione *S*-transferases (GSTs) were affected by moderate copper challenge. Identification and resolution of the two isoforms was by observation of unique peptides in the MALDI-TOF mass spectra (Fig. 6). One isoform, identified by peptide mass fingerprinting as most like human isoform M5 was reduced to less than 50% of normal values (Fig. 5). A second isoform, identified as being most similar to bovine GST-mu was significantly upregulated, from undetectable levels in control liver samples. This reciprocal response of the two GST isoforms is quite remarkable. Multiple isoforms of GST are present in sheep liver and seven distinct cationic isozymes and five overlapping anionic transferases have been resolved by column chromatography [15]. The cationic GSTs are dimeric proteins comprising two 24 kDa subunits, the anionic isozymes are more hereogenous with respect to subunit composition [15]. A 25 kDa subunit of GST associated with the inner microsomal membrane (microsomal GST A1–1) has also recently been isolated from sheep liver. This microsomal GST A1-1 differs from the sheep liver cytosolic GSTs in substrate specific-

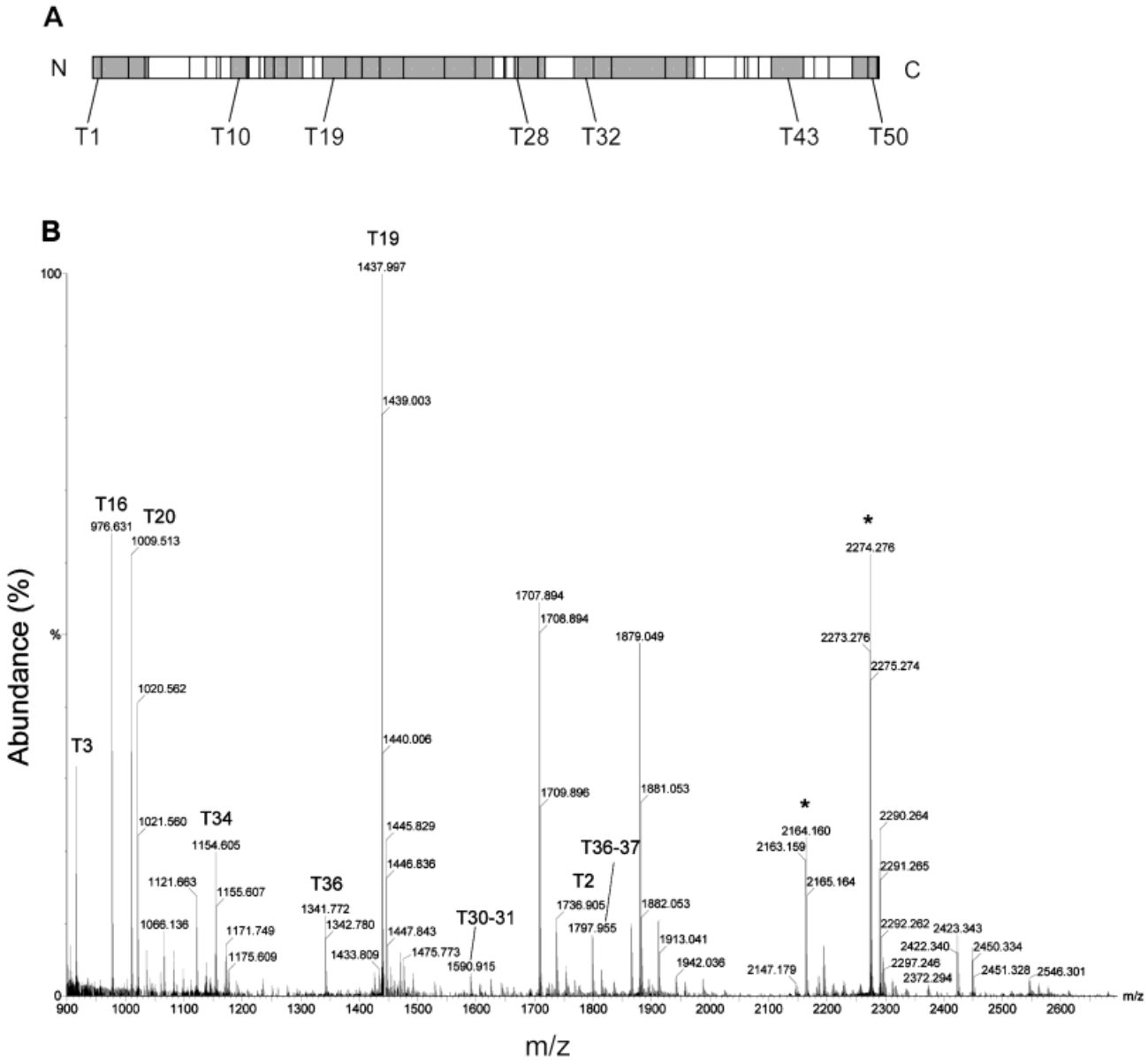
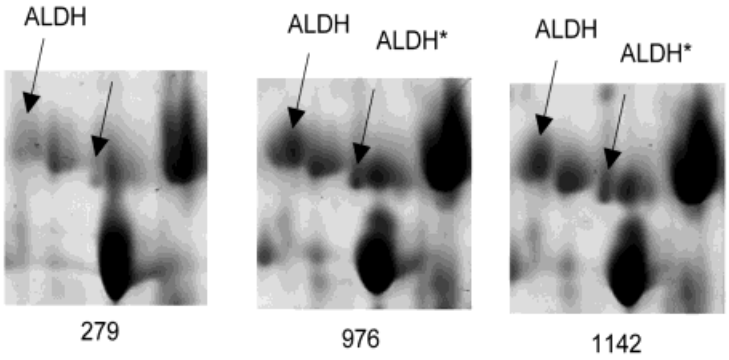


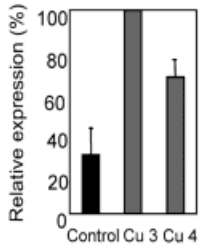
Figure 4. Identification of ovine IDH by MALDI-TOF-MS and peptide mass fingerprinting. (A) Theoretical tryptic digest map of bovine cytosolic IDH. Each horizontal bar is one tryptic peptide. The shaded bars indicate the peptides present in the MALDI-TOF spectrum of the tryptic digest of the spot corresponding to the ovine protein. (B) MALDI-TOF mass spectrum of the ovine IDH. From the 28 peptide masses submitted to the Mascot search engine, 63% sequence coverage of the bovine protein was obtained. Asterisks denote the presence of tryptic autolysis peaks.

ity profile and molecular mass and might have an important role in the protection of biological membranes against oxidative damage [16]. Multiple forms of GST are also present in the mitochondria and are upregulated in response to oxidative stress [17]. GST isoforms GSTA1, A4, Pi and mu are upregulated during mouse liver regeneration [18]. Variant GSTA4 is induced by the pro-inflammatory cytokines tumor necrosis factor α, interleukin-6 and epidermal growth factor in cultured hepatocytes. This protein has been identified in both mitochondria and cytosol being preferentially increased in cytosol during liver regeneration [18].

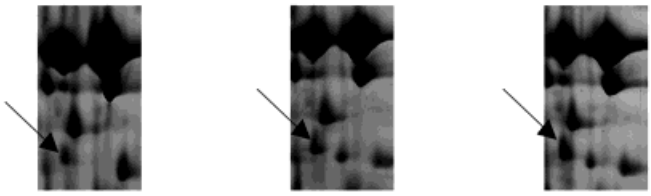
In contrast to moderate copper loading where glutathione synthetase and glutathione transferase were detected in gels prepared from the livers of the copper-treated animals but not the control sheep, at the high copper loading (approx. 1000 μg/g copper) these proteins could not be

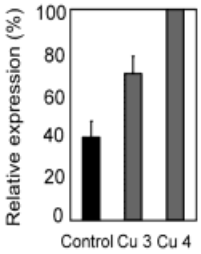
Spot 1 Aldehyde dehydrogenase (ALDH)



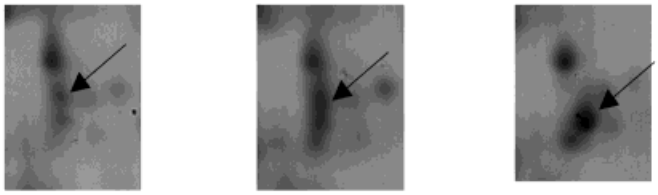


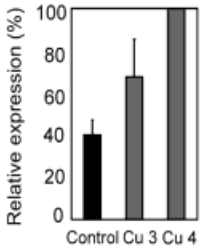
Spot 3 Betaine-homocysteine methyltransferase



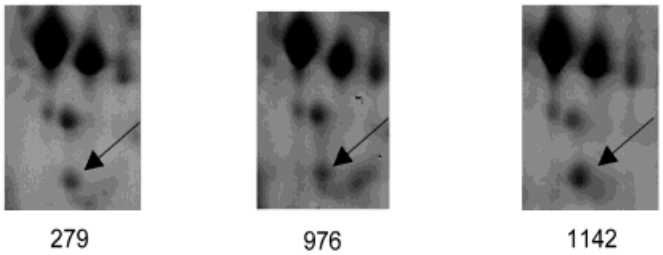


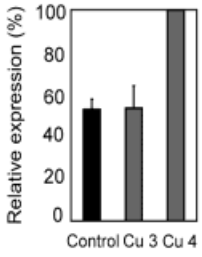
Spot 4 Carbonic anhydrase II



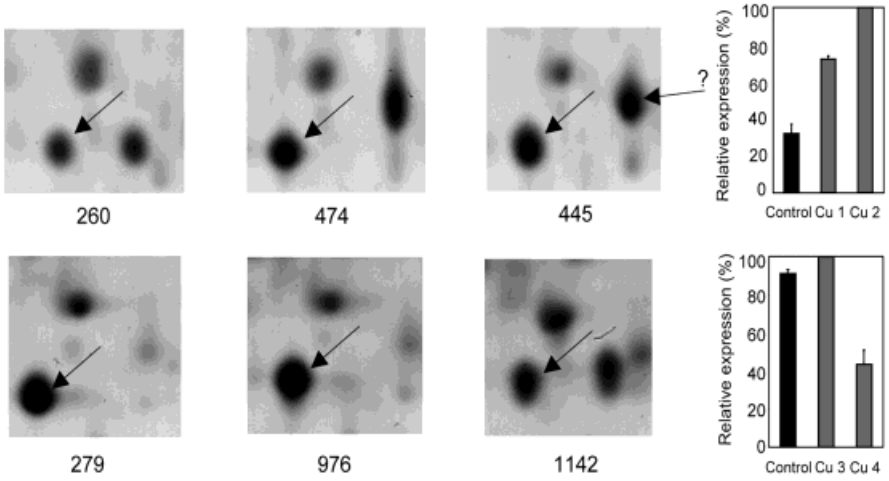


Spot 5 Creatine kinase

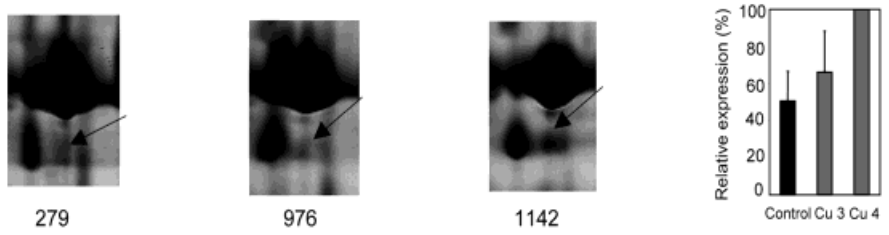




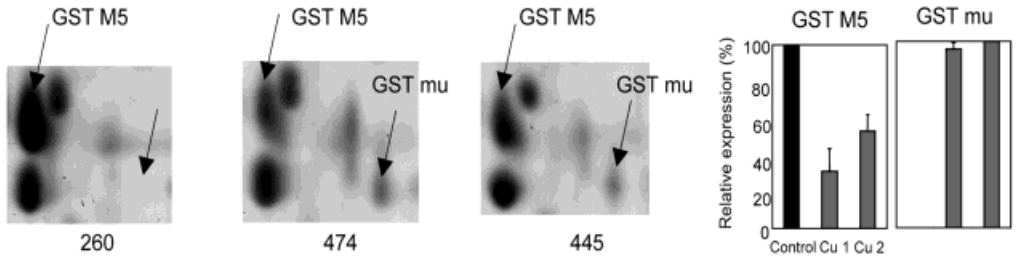
Spot 6 Flavin reductase



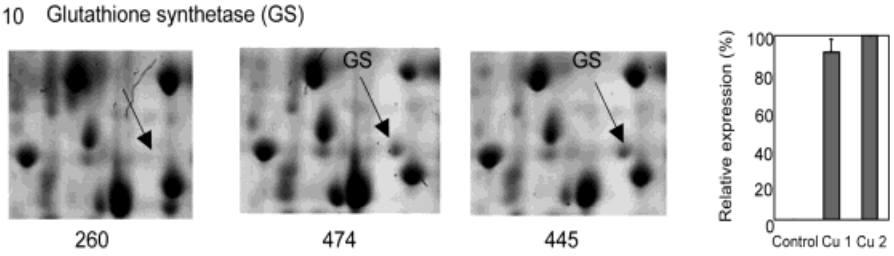
Spot 7 Glutamate dehydrogenase



Spots 8 & 9 Glutathione S-transferase (GST M5 & mu)



Spot 10 Glutathione synthetase (GS)



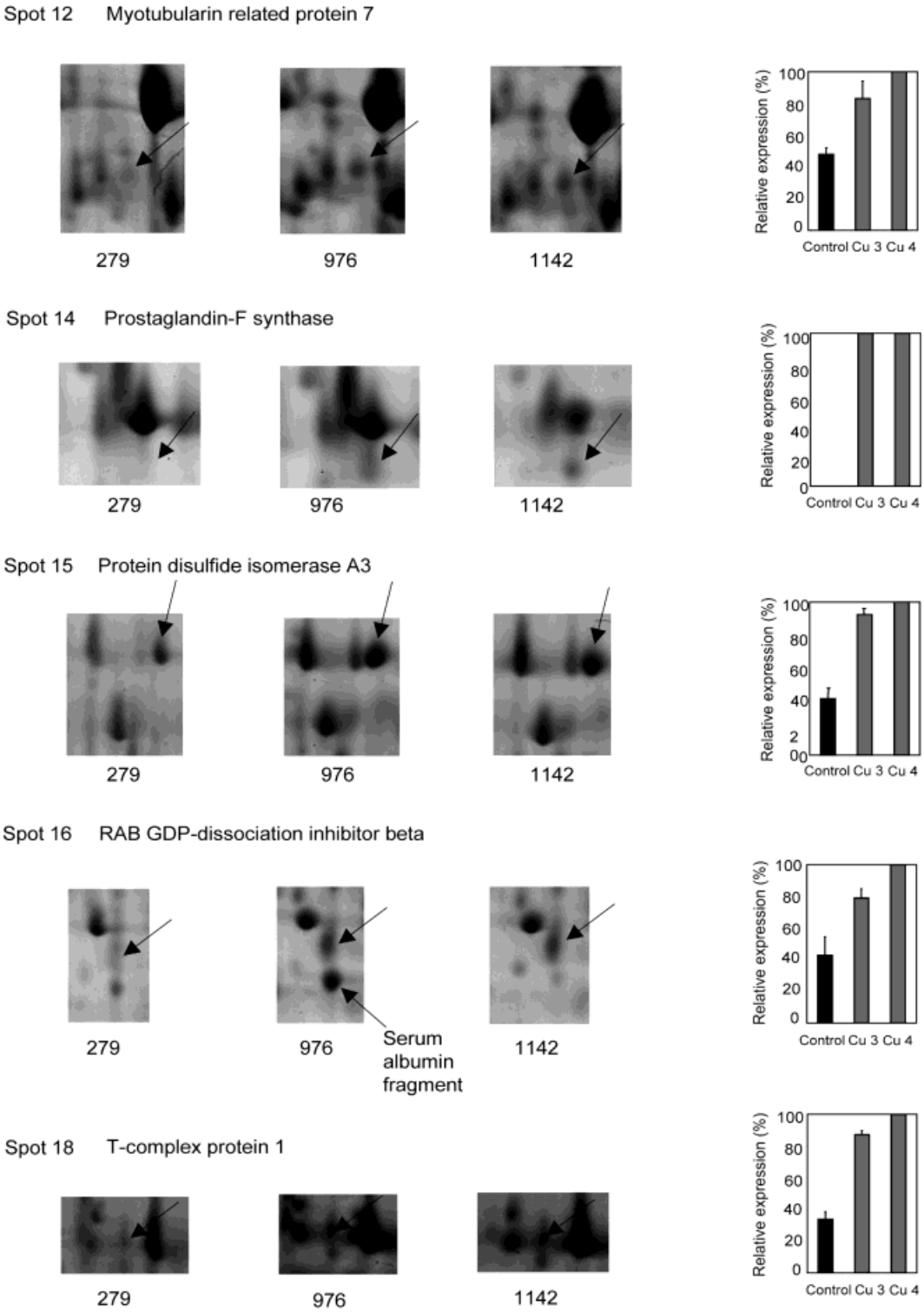


Figure 5. Differential expression of proteins following moderate and high copper load. The regions of gels encompassing proteins identified by densitometry as being differentially expressed by more than 2-fold is shown for many of the proteins listed in Table 1. For each tissue sample, gels were run in triplicate. The most abundant protein in each set was normalized to 100% and all other samples are expressed relative to that 100% value. Thus, the histograms reflect relative rather than absolute expression, which is the more appropriate parameter in a comparative study. The spot marked '?' gave a good mass spectrum but could not be identified by peptide mass fingerprinting.

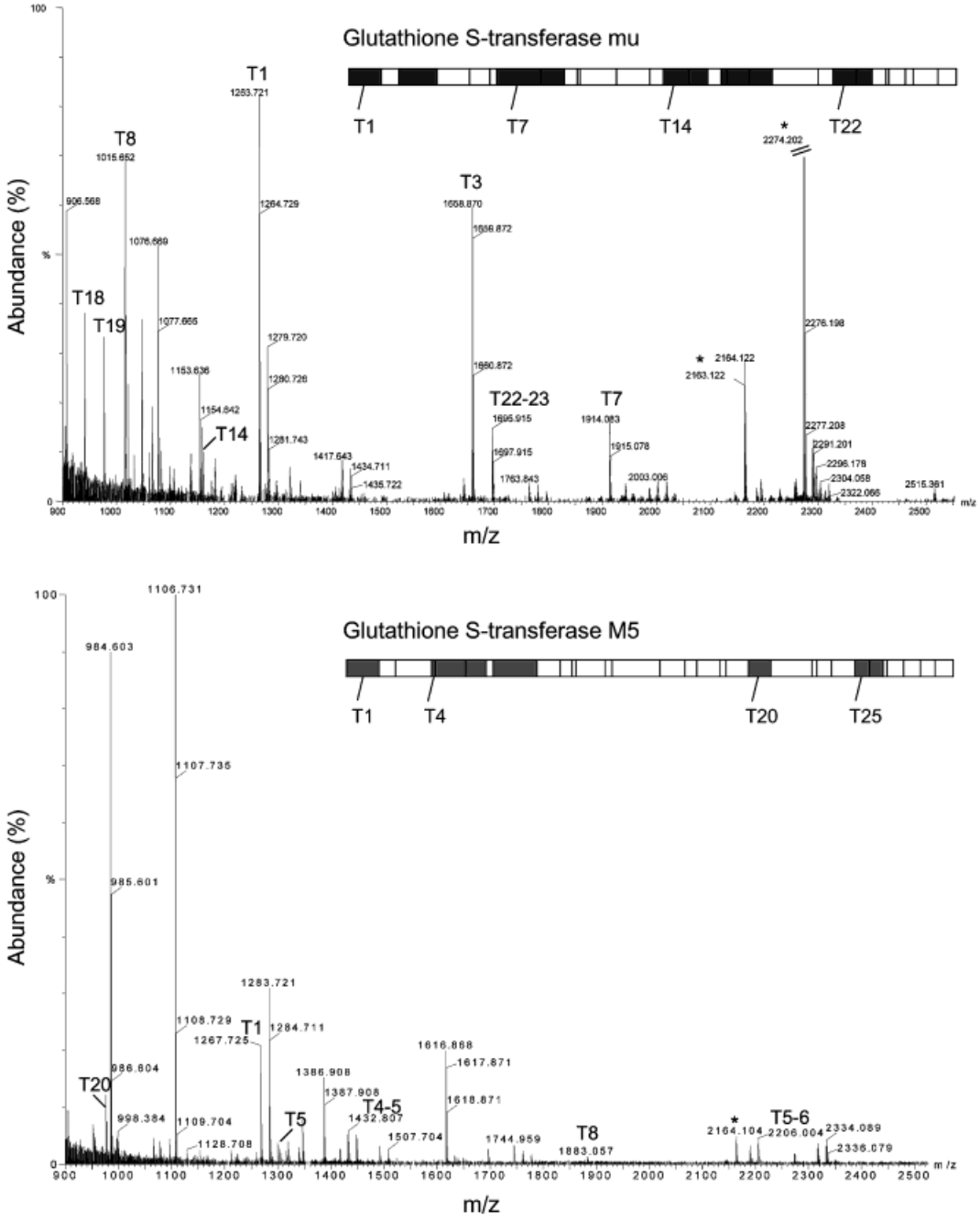


Figure 6. Identification of two isoforms of GST by MALDI-TOF-MS and peptide mass fingerprinting. Tryptic digest maps and mass spectra of (A) GST mu and (B) GST M4. The shaded regions indicate the peptides present in the MALDI-TOF spectrum. Asterisks denote the presence of tryptic autolysis peaks.

detected. The liver pathology of the copper-challenged sheep at moderate copper load indicates that the livers were able to compartmentalize excess copper in the lysosomes, there being an absence of the hepatocellular changes associated with liver damage (unpublished observations). Since the morphological changes were minimal it is unlikely that the induced GST isoform is associated with tissue regeneration. Expression of glutathione synthetase and GST mu might therefore be viewed as sensitive biochemical markers of the adaptive measures of the liver to copper challenge in order to combat and maintain homeostasis. At the higher copper load, exten-

sive degeneration was observed by electron microscopy at which stage a concomitant loss of homeostatic control might be anticipated.

3.2.4 Protein disulphide isomerase

At high levels of copper challenge, protein disulphide isomerase was upregulated between 2- and 3-fold in response to copper-challenge (Fig. 5). Protein disulphide isomerase plays a role in protein folding and has recently been localized to the endoplasmic reticulum as part of a multiprotein complex [19]. The upregulation of protein disulphide isomerase as a result of copper challenge is likely to be a stress response to the oxidative challenge elicited by copper.

3.2.5 Aldehyde dehydrogenase and retinol binding protein

The expression of aldehyde dehydrogenase was markedly enhanced during high copper loading. Aldehyde dehydrogenase binds free retinal and retinol-binding protein bound to retinal. Interestingly, plasma retinol-binding protein was also upregulated on copper challenge between 1.5- and 2-fold (results not shown). The upregulation of aldehyde dehydrogenase may ameliorate the effects of copper-induced oxidative stress by binding the retinol-binding protein:retinal complex. Conversion of retinal to retinoic acid generates NADH which would be rapidly consumed in the oxidative environment produced by copper. A second protein spot, also identified as an isoform of aldehyde dehydrogenase was seen exclusively in gels from copper challenged sheep. This may be a post-translationally modified product of the primary spot, but the MALDI-TOF data does not permit the identification of the site of the modification. The relationship between these two spots remains unclear.

3.2.6 Flavin reductase

Flavin reductase was substantially upregulated in three of the four copper challenged animals. It converts bilirubin, a hydrophobic antioxidant to soluble biliverdin which is subsequently excreted in the bile, a reaction that generates NADPH. This enzyme also plays a role in protecting cells from oxidative damage [20].

3.2.7 Betaine homocysteine methyltransferase

This is a key liver enzyme that catalyses the synthesis of methionine from betaine and homocysteine and is upregulated in the liver of copper-challenged sheep. This protein is also upregulated in ethanol-fed rats [21]. Ethanol administration increases lipid peroxidation and the formation of reactive oxygen species, leading to the downregulation of methionine synthetase with a compensatory increase in upregulation of betaine homocysteine methyltransferase to maintain *S*-adenosylmethionine at normal levels [21].

3.2.8 Glutamate dehydrogenase

Glutamate dehydrogenase was also upregulated in one of the livers containing a high level of copper (Fig. 5). The function of this enzyme might be in the reductive deamination of glutamate to generate protective reducing equivalents in the form of NADPH. However, it could also play a role in the generation of a direct precursor of glutathione. At present, it is impossible to discriminate between these two possibilities, as this would require careful measurement of metabolic flux through this reaction.

3.2.9 Carbonic anhydrase II

This enzyme is significantly upregulated under conditions of heavy copper challenge (Fig. 5). Carbonic anhydrase III is the liver isoform which can be rapidly glutathionylated in hepatocytes under conditions of oxidative stress [22] and this particular isoform has also been reported to protect cells from hydrogen peroxide induced apoptosis [23]. The sequence of the sheep carbonic anhydrase III is not available, and the match to carbonic anhydrase II is limited, with only nine of 39 peptides matching. It is possible that a better match would have been obtained to the sheep isoform III, but this must wait until the full sequence is available. However, the enhancement of this spot is consistent, once again, with an oxidative stress response.

3.2.10 Creatine kinase

Creatine kinase is well established as an enzyme that is particularly sensitive to oxidative stress. The appearance of an enhanced spot might reflect the formation of a thiolated or oxidatively damaged variant, or it might reflect the only spot corresponding to this protein. This serves to emphasize one of the difficulties of a selective proteomics analysis – by selecting those proteins that show consistent and marked changes, it is not possible to ascertain the position of unmodified creatine kinase. This would require a comprehensive and global analysis of all spots on the gels that are within the p*I* and molecular weight range corresponding to this protein. Such an approach is highly inefficient, and inappropriate for a study such as this.

4 Concluding remarks

When the liver pathology (unpublished observations) is correlated with protein expression, a greatly enhanced understanding of the pathophysiology of copper overload begins to emerge. At only very moderate levels of copper loading, despite hepatocellular compartmentalization of copper with no sign of injury, there is unequivocal biochemical evidence of an oxidative challenge provoking an adaptive response to maintain cellular homeostasis. At high levels of copper loading (copper overload) these protective mechanisms are subdued and oxidative stress-induced injury becomes apparent. The fine balance between oxidative tolerance and oxidative stress in the liver may explain certain well-attested though puzzling observations in copper-induced disease in both people and animals. It has long been recognized that liver copper accumulation may sometimes be poorly related to pathological change and that disease progression can be activated by potential physiological or pathological stressors such as parturition or intercurrent disease. Under either of these conditions the finely poised adaptive equilibrium breaks down and oxidative stress is precipitated in what would appear to be only a relatively modest liver copper burden.

Conversely, there is an explanation for the established therapeutic effect of zinc salts in Wilson disease patients and others, in which stabilization of copper and regression of pathological change is effected, rather than down-loading of copper from the liver. Zinc has long been regarded as having an antioxidant effect, a supposition that would receive support from our studies. A rational consequence from this would be that the value of antioxidants, in addition to chelation therapy, is something that should be vigorously explored in copper-associated liver disease.

Finally the findings from this proteomics study clearly illustrate the biochemical adaptive interface which can be activated to maintain intracellular copper homeostasis consequent to copper challenge and which prevents the onset of injury and subsequent disease. The Cambridge sheep can be regarded as a generic model of copper-induced disease in this fundamental respect. A comparable study of North Ronaldsay sheep would explore the differences in protein expression that underlie the distinctive pathology peculiar to this primitive breed and perhaps give new insights into childhood copper toxicosis.

This work was supported by a research grant from the Wellcome Trust: 063730/Z/01/Z/GM/AC/AF. Grants from the BBSRC supported the purchase of instrumentation.

Received June 4, 2003
Revised June 27, 2003
Accepted July 2, 2003

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