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Proteomic analysis of UVC irradiation-induced damage of plasma proteins: Serum amyloid P component as a major target of photolysis

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Abstract Ultraviolet-C (UVC) irradiation is a pathogen inactivation method used for disinfection of pharmaceutical products derived from human blood. Previous studies have shown that UVC can potentially damage proteins through photolysis or can generate reactive species resulting in protein thiol oxidation. In this study, two fluorescence-based quantitative proteomic approaches were used to assess the effects of a novel UVC-disinfection strategy on human plasma fractions. We show minimal changes in protein content, but gross alterations in protein thiol reactivity, indicative of oxidative damage. We identify a number of the damaged proteins by mass spectrometry, including serum amyloid P component, and further demonstrate UVC-induced photolysis of its disulphide bond.

Keywords: Proteomics; Two-dimensional difference gel electrophoresis; Cy dyes; Cysteine labelling; Mass spectrometry; Ultraviolet-C; Plasma proteins; Photochemical damage

1. Introduction

Transmission of viruses present in human- or animal-derived plasma and sera represents a considerable risk for recipients, particularly when used as raw materials for the production of biopharmaceutical products such as purified plasma proteins and blood clotting agents. Therefore, procedures for inactivating pathogenic viruses in these products are mandatory. Conventional techniques used for pathogen inactivation include pasteurization, dry and vapour heat and solvent-detergent treatments [1–3]. Ultraviolet irradiation was one of the first methods investigated in the pioneering years of biopharmaceutical plasma fractionation, but soon proved ineffective at preventing transmission of "serum hepatitis" [4]. Recently, however, it has received renewed attention for its capability to inactivate physically resistant non-enveloped single-stranded viruses such as Hepatitis A and parvovirus B19 [5,6]. Flow-through reactors have been proposed and viral reduction has been demonstrated using pathogenic viruses and bacteriophages [1,7,8]. Although these disinfection strategies can effectively inactivate virus infectivity, some studies have shown that UV treatment causes photo-damage to proteins through disulphide photolysis and oxygen radical oxidation [9–11]. While dose-dependent damage could be shown with purified proteins [12], such effects were not distinctly apparent in protein mixtures such as prothrombin complex concentrates. Such results raise concerns about the effect of UV-induced protein modifications on the biological activity of these products, but more importantly, on their potential toxicity.

Two-dimensional gel electrophoresis (2-DE) is one of the most widely used proteomic separation methods and is often employed for differential protein expression analysis of biological samples [13]. Despite this, 2-DE is inherently variable and the protein visualisation methods often used have narrow linear dynamic ranges of detection. A significant improvement came with the introduction of 2D-difference gel electrophoresis (2D-DIGE), where co-detection of multiple fluorescently labelled samples is possible on the same 2-DE gels, alleviating the problems of gel-to-gel variation and improving the dynamic range of detection [14–16]. Conventional 2D-DIGE methodology uses three mass and charge-matched *N*-hydroxysuccinimidyl (NHS) ester-derivatives of the cyanine dyes 3-(4-carboxymethyl)phenylmethyl-3′-ethyloxacarbocyanine halide (Cy2), 1-(5-carboxypentyl)-1′-propylindocarbocyanine halide (Cy3) and 1-(5-carboxypentyl)-1′-methylindocarbocyanine halide (Cy5) for labelling the ε-amine groups of lysine residues in samples prior to mixing, 2-DE separation and multi-wavelength

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Abbreviations: UVC, Ultraviolet-C; 2-DE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional difference gel electrophoresis; Cy2, 3-(4-carboxymethyl)phenylmethyl-3′-ethyloxacarbocyanine halide; Cy3, 1-(5-carboxypentyl)-1′-propylindocarbocyanine halide; Cy5, 1-(5-carboxypentyl)-1′-methylindocarbocyanine halide; NHS, *N*-hydroxysuccinimidyl; ICy, iodoacetylated cyanine dye; MS, mass spectrometry; DTT, dithiothreitol; Chaps, (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate); CCB, colloidal Coomassie blue; ddH_2O, double deionised water; AmBic, ammonium bicarbonate; MALDI, matrix-assisted laser desorption/ionisation; TOF, time of flight; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SAP, serum amyloid P component; ALB, albumin; RBP, retinol binding protein; DTNB, 5,5′-dithiobis-2-nitrobenzoate; CRP, C-reactive protein

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fluorescence detection. Alternative strategies have also been developed whereby alkylating forms of the Cy-dyes are used to label cysteine thiol groups at higher stoichiometry [17]. Indeed, our laboratory recently reported a pair of iodoacetyl cyanine (ICy) dyes and used them to monitor redox-dependent thiol modifications and expression changes in a model cell system exposed to hydrogen peroxide [18].

In the present study, the effects of a novel Ultraviolet-C (UVC)-irradiation strategy for treatment of the crude human plasma fraction containing proteins of the prothrombin complex are assessed [19]. It is possible that such treatment could damage the protein components of this blood product affecting its potency or immunogenicity. A combination of lysine- and cysteine-labelling 2D-DIGE and mass spectrometry (MS) was thus used to determine if protein abundance and thiol reactivity are altered in the plasma fraction following exposure to different UVC doses, which plasma proteins are targets of such UVC-induced damage and what is the nature of this damage.

2. Materials and methods

2.1. UVC treatment of plasma protein samples

UVC irradiation experiments were performed according to a validatable method for determining a photochemically effective dose sufficient to inactive pathogens in a biological sample and enabling batch irradiation (Patent application WO2004075931) [20]. Briefly, 120 mL of plasma fraction at a protein concentration of 15.5 g protein/L ($a_{253.7} = 7\ cm^{-1}$) in a 4 cm diameter quartz glass test tube with a magnetic stirrer was irradiated in a laboratory-scale batch reactor, illuminated with two individually switchable, water-thermostated, low-pressure, mercury vapour lamps (253.7 nm). Irradiation is dependent on the optical density of the test sample, average irradiance emitted by the lamps and residence time in the irradiation chamber. In the UVC irradiation experiments, a dose rate effective in the solution of 3.186 mJ/cm^2/min was achieved with irradiation times of 6 min 17 s, 12 min 34 s and 25 min 8 s for 20 mJ/cm^2, 40 mJ/cm^2 and 80 mJ/cm^2, respectively. Unirradiated fraction was used as a control. Purified serum amyloid P component (SAP) and C-reactive protein (CRP) [21] were irradiated at the indicated doses in a CL-1000 UV cross-linker (Ultra-Violet Products) fitted with 5×8 W 254 nm dual bipin discharge type tubes.

2.2. Labelling of plasma proteins

NHS-Cy2 was purchased from GE Healthcare. NHS-Cy3/Cy5 and ICy3/ICy5 were synthesised "in-house" and stored as described [18]. For NHS-Cy dye labelling, UV-irradiated and control plasma fractions at ~15 mg/mL were diluted to 1 mg/mL in denaturing 2D lysis buffer (8 M urea, 2 M thiourea, 4% (w/v) (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (Chaps), 0.5% NP-40 (w/v), 10 mM Tris–HCl, pH 8.3). Triplicate samples of 100 μg were minimally labelled with 400 pmol of dye. Control samples and 20 mJ/cm^2 UVC-treated samples were labelled with Cy3 and Cy5, respectively. An equal pool of all samples was also prepared and labelled with Cy2 as a standard run on all gels to aid in spot matching and cross-gel quantitative analysis. Labelling was performed on ice in the dark for 1 h, reactions were quenched with a 20-fold molar excess of free lysine to dye for 10 min, and then dithiothreitol (DTT) added to 65 mM. For ICy labelling, plasma fractions were diluted to 1.5 mg/mL in ICy labelling buffer (8 M urea, 4% (w/v) Chaps, 10 mM Tris–HCl, pH 8.3) and 250 μg labelled with 20 nmol of ICy3/5 on ice in the dark for 1 h. Reactions were quenched with DTT (to 65 mM). Labelled samples were mixed appropriately and carrier Ampholines/Pharmalytes, pH 3–10 (50:50 (v/v)) (GE Healthcare) added to a final concentration of 2%.

2.3. 2-DE, protein detection and image analysis

2-DE was performed using 18 cm, non-linear, pH 3–10, immobilised pH gradient (IPG) strips (GE Healthcare) and 12% bonded gels exactly as described [18]. Gels were scanned between low-fluorescence glass plates using a Typhoon 9400 multiwavelength fluorescence imager and ImageQuant software (GE Healthcare) and gels post-stained with colloidal Coomassie Blue G-250 (CCB). Images were curated, matched and quantitative analysis performed using DeCyder™ software (GE Healthcare) essentially as described [18]. Comparison of test spot volumes with corresponding standard spot volumes gave a standardised abundance for each matched spot and values were averaged across triplicates for each experimental condition. Statistical analysis was performed to pick spots matching across all images, displaying a $\geqslant 1.5$ average-fold increase or decrease in abundance between conditions, and with P values <0.05 (Student's t-test).

2.4. Spot picking, in-gel digestion and protein identification by MS

Post-stained CCB images were matched with Cy dye images using DeCyder software and a pick list of coordinates created for spots of interest relative to a pair of reference markers fixed to the glass plates. Spots were excised using an Ettan spot picker (GE Healthcare). In-gel tryptic digestion was performed exactly as described [22] and extracted peptides resuspended in 6 μL of water. For peptide mass fingerprinting, 0.5 μL of tryptic digest was mixed with 1 μL of matrix (saturated aqueous 2,5-dihydroxybenzoic acid) and spotted onto a sample target plate and dried. Matrix-assisted laser desorption/ionisation; time-of-flight (MALDI-TOF) mass spectra were acquired using an externally calibrated Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics) in the reflector mode. After internal calibration using trypsin autolysis peaks, prominent peaks in the mass range m/z 500–5000 were used to generate a peptide mass fingerprint which was searched against the updated NCBI database using Mascot version 2.0.02 (Matrix Sciences). Identifications were accepted when a minimum of six peptide masses matched a particular protein (mass error of ±50 ppm allowing 1 missed cleavage), sequence coverage was >30%, MOWSE scores were higher than the threshold value ($P = 0.05$), and the predicted protein mass agreed with the gel-based mass. Nano-HPLC-electrospray ionisation-collision-induced dissociation MS/MS was performed on an Ultimate HPLC with a PepMap C18 75-μm inner diameter column (both LC Packings) at a flow rate of 300 nL/min, coupled to a Q-Tof (Micromass) mass spectrometer. Spectra were processed using MassLynx (Waters) software and submitted to Mascot database search routines. Positive identifications were made when at least two peptide sequences matched an entry and MOWSE scores were above the significance threshold value ($P = 0.05$).

2.5. Immunoblotting

Immunoblotting was used to validate expression of SAP in 20 μg plasma fractions using an anti-SAP polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:1000. Both unlabelled and ICy3 labelled fractions were analysed. Enhanced chemiluminescence (Perkin–Elmer Life Sciences Inc.) was used for detection.

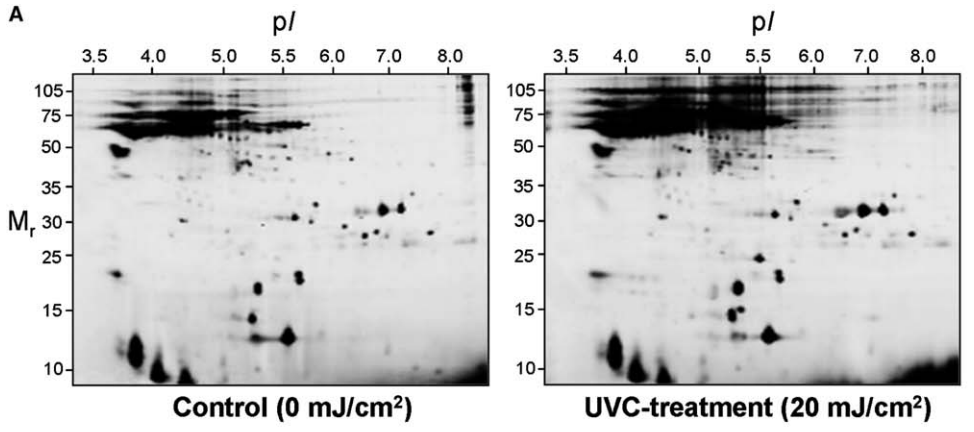
2.6. Quantitative determination of free thiols (Ellman's test)

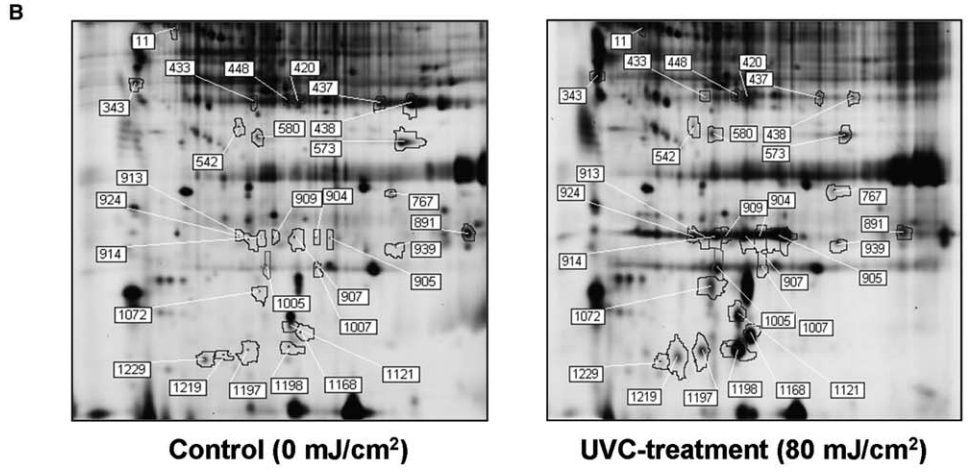
Measurement of free thiols was determined using Ellman's reagent [23]. Briefly, 3.3 μL of 10 mM 5,5′-dithiobis-2-nitrobenzoate (DTNB) in 0.1 M sodium phosphate buffer (pH 8.0) was added to the indicated amounts of control or UVC-irradiated purified human SAP diluted in 96.7 μL of the same buffer. The mixture was left to stand at room temperature for 15 min in the dark. The absorbance was measured at 405 nm against a no protein reference and the concentration of sulphydryl groups was calculated using the equation: [SH] = [A412 (sample) − A412 (reference)]/13 650.

3. Results and discussion

3.1. Proteomic analysis of UVC-induced plasma protein damage

We assessed whether UVC-irradiation of a plasma fraction, prepared as a pharmaceutical clotting agent, would result in changes to the protein components of the sample, indicative of protein damage. Lysine-labelling 2D-DIGE expression profiling was first applied to assess changes in protein abundance and/or isoform distribution following UVC treatment. Untreated and UVC-treated (20 mJ/cm^2) fractions were differen-





C

Spot No.	20 vs. 0	*p*-value	40 vs. 0	*p*-value	80 vs. 0	*p*-value
11	1.61	0.0059	1.75	0.003	2.13	0.0013
343	2.21	0.00034	2.01	0.0011	2.32	0.00024
420	-1.33	0.038	-1.57	0.0012	-1.86	0.00026
433	-1.7	0.0059	-1.81	0.003	-1.78	0.0069
437	-3.99	0.0036	-3.47	0.0051	-3.55	0.0043
438	-7.11	0.00048	-5.65	0.00087	-6.22	0.00082
448	-1.2	0.0051	-1.49	0.0009	-1.88	0.0002
542	1.39	0.02	1.2	0.059	1.62	0.0054
573	-1.15	0.0047	-1.5	0.00075	-1.74	3.50E-05
580	1.52	0.0025	1.38	0.0024	1.67	0.0016
767	-1.42	0.019	-1.53	0.011	-1.79	0.0033
891	-1.13	0.34	-1.25	0.048	-1.57	0.0025
904	4.31	0.0053	8.26	0.0012	15.13	0.00048
905	7.47	1.2E-05	10.49	0.000041	15.24	1.80E-05
907	2.58	0.0058	3.86	0.0013	5.89	0.00049
909	4.4	0.018	6.58	0.0068	13.41	0.0025
913	1.67	0.007	2.02	0.021	2.75	0.0067
914	3.26	0.0047	5.96	0.00067	9.5	0.00031
924	3.87	0.0024	6.2	0.00075	9.8	0.00031
939	2.13	0.035	2.81	0.017	3.98	0.0056
1005	2.23	0.0092	3.35	0.0019	4.79	0.00063
1007	1.28	0.09	1.4	0.014	1.57	0.0047
1072	1.87	0.0015	2.26	0.0006	4.09	8.90E-05
1121	1.26	0.59	1.05	0.69	-1.63	0.0049
1168	14.33	0.0048	22.61	0.0028	25.56	0.0026
1197	8.43	0.0016	11.33	0.00015	14.39	0.00049
1198	15.89	8.8E-07	22.82	0.000034	23.82	4.00E-06
1219	2.03	0.011	2.74	0.04	9.08	0.0024
1229	-1.21	0.48	-1.96	0.0082	-2.03	0.023

D

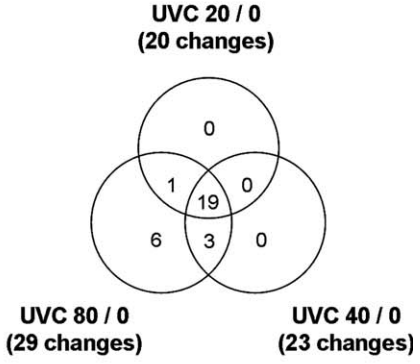


Fig. 1. Visualisation of differential labelling of UVC-treated versus untreated plasma fractions using iodoacetyl cysteine labelling ICy dyes. (A) Representative ICy dye images of UVC treated (right) and control (left) plasma samples. (B) Zoomed images of UVC-treated (right) and untreated (left) fractions with protein spots displaying altered ICy-labelling. (C) Table showing average fold-differences in labelling intensity with *P*-values between UVC-treated and untreated samples. (D) Venn diagram showing the number of spots and their altered labelling at different UVC doses.

tially labelled in triplicate and run on three 2D gels with a Cy2-labelled standard, comprising an equal mixture of both samples [14]. Image analysis revealed 11 significant changes (>1.5-fold up- or down-regulated; $P < 0.05$) with some protein spots present as trains of similar molecular weight, but different p*I*s indicative of post-translational modifications (data not

Table 1
Plasma fraction proteins displaying UVC-induced thiol reactivity changes

Spot no.	Protein name	% Cov	MOWSE Score	Est. mass	Est. pl	Pred. mass	Pred. pl	Accession number	20 mJ/cm2 UVC vs. control		40 mJ/cm^2 UVC vs. control		80 mJ/cm: 2 UVC vs. control		Function
									Av. ratio	*P*-value	Av. ratio	*P*-value	Av. ratio	*P*-value	
11	Kininogen precursor	35	81/64	70000	5.5	71945	6.3	125507	*1.61*	0.0059	*1.75*	0.003	*2.13*	0.0013	Blood coagulation
343	Thrombin precursor[a;b]	40	97/64	69000	5.3	71475	5.6	135807	*2.21*	0.00034	*2.01*	0.0011	*2.32*	0.00024	Blood coagulation
420	ALB protein[b]	53	181/64	45000	6	47361	6	27692693	−1.33	0.038	*−1.57*	0.0012	*−1.86*	0.00026	Carrier protein
433	Actin-β	31	67/64	43000	5.5	42188	5.4	15277503	*−1.7*	0.0059	*−1.81*	0.003	*−1.78*	0.0069	Cytoskeletal protein
437	Complement component C4	35	90/64	46000	7	48037	5.8	40737308	*−3.99*	0.0036	*−3.47*	0.0051	*−3.55*	0.0043	Complement activation; immunity
438	Complement component C4	39	113/64	46000	7.5	48037	5.8	40737308	*−7.11*	0.00048	*−5.65*	0.00087	*−6.22*	0.00082	Complement activation; immunity
448	ALB protein[b]	31	90/64	45000	5.9	48664	6	27692693	−1.2	0.0051	−1.49	0.0009	*−1.88*	0.0002	Carrier protein
573	Thrombin (fragment?)[a]	46	94/64	35000	7.5	34245	8.3	809450	−1.15	0.0047	*−1.5*	0.00075	*−1.74*	0.000035	Blood coagulation
907	SAP[a]	39	90/64	24000	6	23358	6.1	576259	*2.58*	0.0058	*3.86*	0.0013	*5.89*	0.00049	Binding protein; innate immunity
909	SAP[b]	39	107/64	24000	5.8	23358	6.1	576259	*4.4*	0.018	*6.58*	0.0068	*13.41*	0.00025	Binding protein; innate immunity
913	SAP	39	112/64	24000	5.6	23358	6.1	576259	*1.67*	0.007	*2.02*	0.021	*2.75*	0.0067	Binding protein; innate immunity
914	SAP[a]	33	70/64	24000	5.7	23358	6.1	576259	*3.26*	0.0047	*5.96*	0.00067	*9.5*	0.00031	Binding protein; innate immunity
924	SAP[a]	39	78/64	24000	5.5	23358	6.1	576259	*3.87*	0.0024	*6.2*	0.00075	*9.8*	0.00031	Binding protein; innate immunity
1072	Retinol binding protein[a,b]	75	103/64	21000	5.5	20959	5.3	230284	*1.87*	0.0015	*2.26*	0.0006	*4.09*	0.000089	Vitamin A transport

Differentially labelled proteins form 2D-DIGE analysis were identified by MALDI-TOF peptide mass fingerprinting and LC-MS/MS. The % coverage of analysed peptides, the MOWSE score from Mascot searches and significance threshold score, the estimated mass and p*I* (from gels), the predicted mass and p*I* (from the database) and the Accession number (NCBI gene identifier) are shown for each protein. Proteins displaying an average fold-difference of $\geqslant$ 1.5-fold up (+) or down (−) regulation between pairs of conditions where $P < 0.05$ and where spots matched across all images are italicised.

[a]Proteins identified in two or more independent experiments.

[b]Protein identified by MALDI-TOF MS and LC-MS/MS.

shown). Repeat experiments using freshly prepared and irradiated fractions also showed subtle changes in isoform distribution, although only two isoforms changed consistently between experiments. It was noted that all changing spots were of relatively low fluorescence intensity and few could be aligned with co-migrating CCB-stained spots. Multiple attempts to identify these proteins by MALDI-TOF MS failed, likely reflecting the low abundance of these isoforms. Thus, although UVC-irradiation altered the abundance/distribution of several protein isoforms, these changes were subtle.

UVC radiation is known to modify protein cysteinyl thiol groups [9–11]. We therefore applied a recently developed 2D-DIGE methodology utilising iodoacetyl ICy dyes [18] to assess UVC-induced changes in the thiol reactivity of the plasma proteins. Experiments were conducted on UVC-irradiated samples at doses of 0, 20, 40 and 80 mJ/cm^2, in triplicate. Individual ICy3-labelled samples were run on 2D gels against an equal load of ICy5-labelled standard pool consisting of an equal mixture of all samples to aid in spot matching and improve the accuracy of quantitation. Around 1400 features were detected with particularly strong labelling of a set of high molecular weight acidic proteins (Fig. 1A). Twenty nine of these features displayed significant UVC-induced changes in labelling and with a high degree of overlap at the different doses

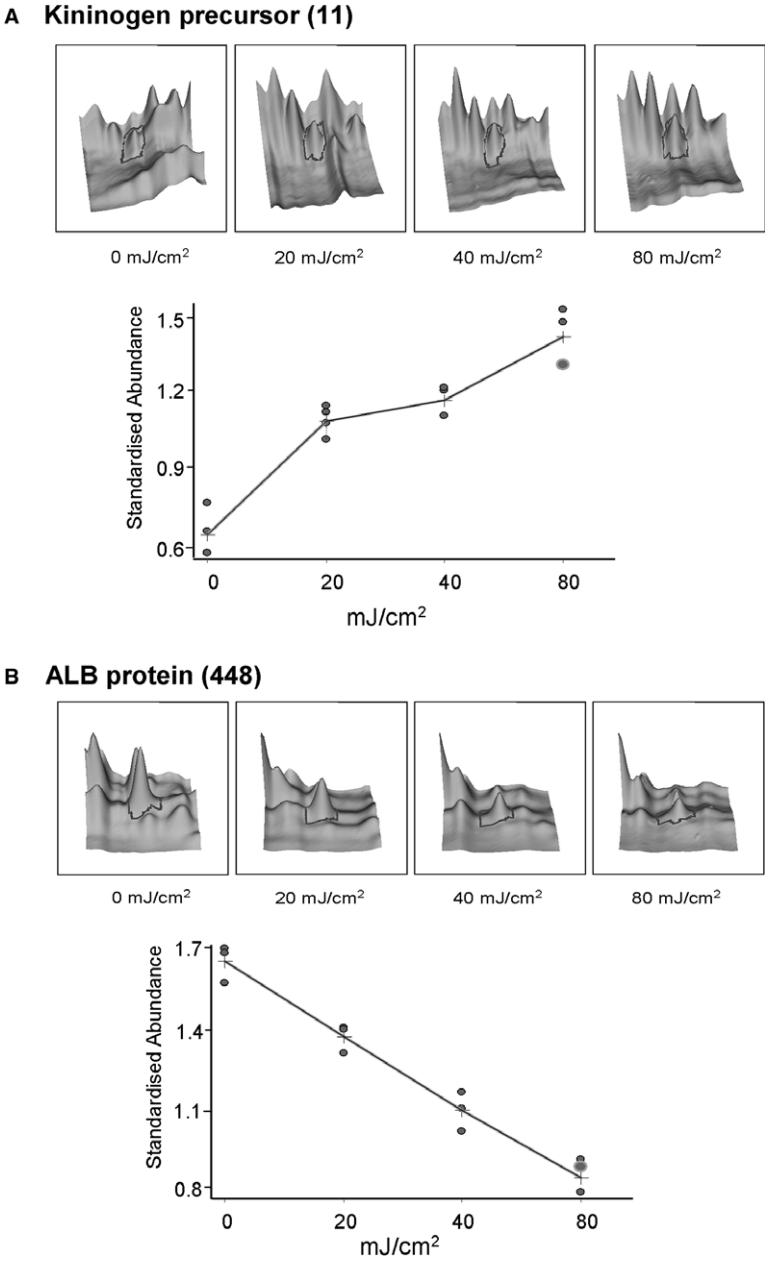


Fig. 2. Examples of protein isoforms identified by MS displaying UVC-induced changes in labelling. Data for (A) kininogen; (B) ALB protein; (C) serum amyloid P component and (D) retinol binding protein are shown. 3D-images of gel features are shown for untreated (0 mJ/cm^2) and UVC-treated (20, 40 and 80 mJ/cm^2) samples. Graphs show changes in the standardised abundance of protein spots (ratio of the volume of a test gel feature versus the corresponding standard gel feature). Triplicate data points are shown with lines joining the average values.

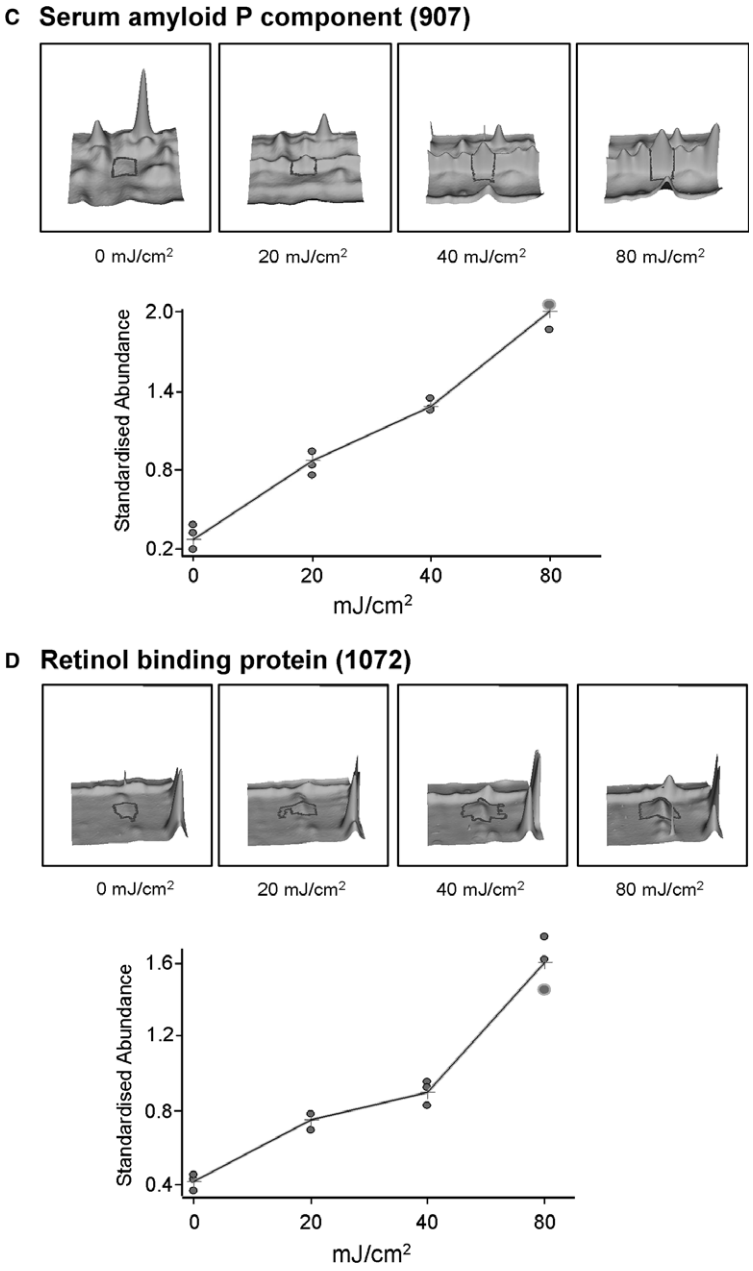


Fig. 2 (*continued*)

(Fig. 1B–D). CCB post-staining and matching with fluorescence images allowed confident picking of 15 gel features, with unpicked spots representing low-abundance isoforms.

3.2. Identification of UVC-damaged plasma proteins

Fourteen of these proteins (representing seven gene products) were identified by MALDI-TOF peptide mass fingerprinting and liquid chromatography-tandem mass spectrometry (LC-MS/MS), with several displaying UVC dose-dependency in ICy labelling (Table 1 and Figs. 1 and 2). All of the identified proteins contain at least one cysteine, and since the ICy dyes target reduced cysteinyl thiols, these results suggest that UVC must alter the oxidative status of some of these thiol groups. The differentially labelled proteins fell into several functional groups including the blood coagulation and complement proteins kininogen, thrombin and complement component C4. Kininogen is a thiol protease inhibitor which complexes with prekallikrein and factor XI, which after cleavage by kallikrein or factor XIIa, forms a disulphide-linked two subunit protein with procoagulant activity. Kininogen displayed a dose-responsive increase in ICy labelling following UVC irradiation (Fig. 2A). Thus, its disulphide bridge must be broken to generate new thiol groups for ICy labelling. Two isoforms of the disulphide-containing protease prothrombin were also found in two spots, one at the expected mass of full-length prothrombin (~70-kDa) and the other a putative fragment (35-kDa) which may correspond to the activated form. The two forms displayed opposite ICy labelling responses following UVC treatment (Table 1).

Albumin (ALB), a 48-kDa fragment of albumin, the major binding/transport protein in serum, displayed a dose-dependent reduction in cysteine labelling (Fig. 2B), suggesting that

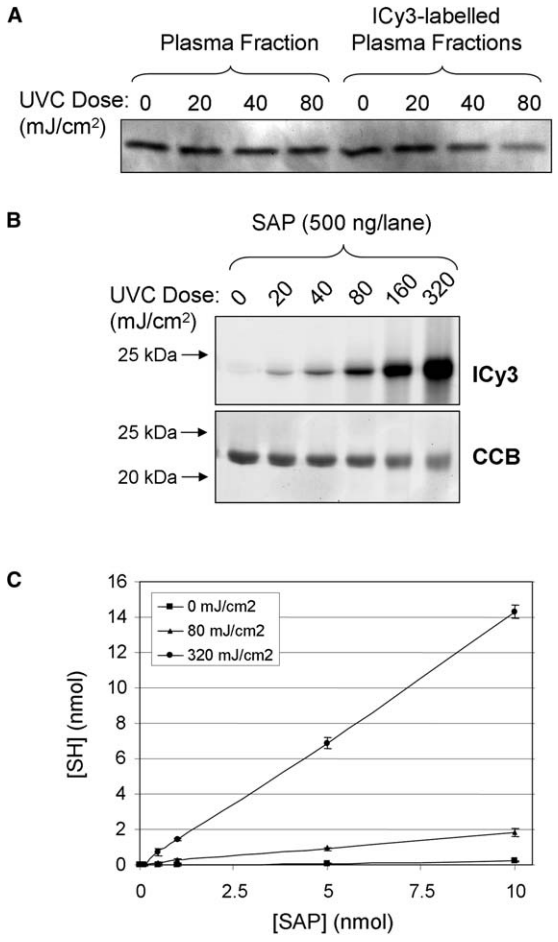


Fig. 3. Validation of UVC-induced disulphide photolysis of SAP. (A) The level of SAP in 20 μg of unlabelled and ICy3 labelled plasma fraction in response to UVC-irradiation was assessed by immunoblotting with an anti-SAP antibody. (B) Increased ICy labelling of purified SAP after UVC irradiation. 500 ng of purified human SAP was irradiated at the indicated doses of UVC and immediately labelled with 40 pmol of ICy3 prior to 15% SDS–PAGE. The fluorescent image (upper panel) and the corresponding CCB-stained image (lower panel) are shown. (C) Quantitation of free thiol content of irradiated SAP using Ellman's reagent (see Section 2).

its free thiol is photo-oxidised or forms new disulphide cross-links which may result in protein aggregation, as previously observed [5]. Other serum transport proteins identified were SAP and retinol binding protein (RBP), which displayed the largest increases in ICy labelling (Table 1). SAP belongs to the pentraxin family of Ca^{2+}-dependent ligand binding proteins, plays roles in host-defence by binding bacterial lipopolysaccharide, in amyloidogenesis by binding amyloid fibrils and is the major DNA- and chromatin-binding protein in plasma [24]. In this study, several SAP isoforms were identified in a charge train with all isoforms displaying increased labelling with UVC dose (Table 1 and Fig. 2C). SAP contains a single intramolecular disulphide bond [25], implying that UVC treatment causes photolysis of this bond, creating sulphydryl groups for ICy labelling. Although the multiple isoforms were poorly focused, ICy labelling alone would not generate more than two p*I*-shifted isoforms, indicating that formation of the charge train is contributed by other modifications, possibly differential glycosylation. RBP is the major retinol transporter in plasma and its synthesis regulates retinol release from the liver and uptake by target cells [26]. RBP contains six cysteines forming three disulphide bonds, and like kininogen and SAP, showed increased ICy labelling with UVC dose consistent with photolysis and the generation of new thiol groups (Fig. 2D).

3.3. Validation of UVC-induced disulphide photolysis

SAP showed the greatest increase in ICy labelling following UVC-irradiation and was therefore selected for further characterisation. Immunoblotting of plasma fractions showed that SAP protein levels were unaffected by UVC treatment, although ICy3 labelling itself slightly reduced the protein level at higher UV doses (Fig. 3A). Whilst this suggests that ICy3 labelling may affect the solubility of SAP in plasma fractions or reactivity to the antibody, it shows that the increased ICy labelling observed in the 2D-DIGE experiment was not due to a change in protein level, rather an increase in the thiol content of SAP. We next examined ICy labelling of irradiated purified human SAP [21]. As with the plasma fractions, UVC caused a dose-responsive increase in ICy labelling, consistent with photolysis of the SAP disulphide bond (Fig. 3B). Notably, a similar response was observed for purified human CRP, a pentraxin family member closely related to SAP (data not shown). To further confirm disulphide photolysis and generation of new thiol groups, SAP samples were mixed with Ellman's reagent and assayed for free thiol content. The assay showed that 0.02, 0.18 and 1.43 mol/mol thiol/SAP were present after 0, 80 and 320 mJ/cm^2 UVC exposure, respectively (Fig. 3C). Thus, UVC irradiation of SAP results in a significant proportion of disulphide photolysis, corroborating the findings of the 2D-DIGE analysis. These data also suggest that members of the pentraxin family have unique molecular structures/sequences that promote UVC-sensitivity.

3.4. Conclusions

The ICy labelling data presented here, together with biochemical, structural and photochemical data, support the hypothesis that UVC induces the formation of free thiols in certain proteins through disruption of disulphide bonds. For SAP, RBP and kininogen, this leads to a dose-responsive increase in ICy labelling. The photochemical mechanism by which this is likely to occur is through photolysis of disulphide bonds, which in some cases might be favoured by absorption of UVC light with the generation of solvated electrons through adjacent tryptophan residues [27]. Photolysis may also promote the formation of new disulphide cross-links and possibly protein aggregation which may explain the observed loss in ICy labelling of other proteins, such as complement component C4 and ALB. Alternatively, UVC-induced reactive oxygen species (ROS) or protein-derived peroxides may directly oxidise thiol groups to form the sulfenic (–SOH), sulfinic (–SO_2H) or sulfonic acid (–SO_3H) forms of cysteine, which would not react with the ICy dyes. Although previous work has shown that flavonoid antioxidants are able to protect plasma proteins from UVC irradiation damage [5,6], these compounds are also effective as scavengers of solvated electrons as well as ROS. Thus, the contribution of either pathway to the mechanism of photochemical damage remains to be elucidated.

It is important to note that none of the affected ICy-reactive proteins showed changes in lysine labelling, indicating that

there were no gross changes in protein abundance, for example due to peptide backbone photolysis or aggregation and precipitation. Despite the possible molecular damage to the coagulation factors thrombin and kininogen, our previous work has shown that UVC irradiation does not significantly alter the clotting activity of our plasma fractions or, in contrast to earlier reports, the overall free thiol level (data not shown) [28]. Apart from SAP, our own observations may suggest that the photolytic and oxidative damage occurs at low-stoichiometry, supporting the only modest effects on protein abundance. However, even limited modifications may be of concern as the breakage or formation of disulphide bonds may adversely affect the structure and biological activity of these proteins. More important than loss of bioactivity is whether these modifications are toxic, and as such it remains to be proven if they represent a danger to patients receiving UVC-disinfected coagulation factor concentrates.

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