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ORIGINAL ARTICLE

Plasma nitroproteome of kidney disease patients

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Abstract 3′-nitrotyrosine (3NT) is a post-translational modification (PTM) of body fluids and tissues that is sustained by chronic inflammation and oxidative stress, two main clinical traits of chronic kidney disease (CKD). Despite this background, protein targets and their differential susceptibility to in vivo nitration remain almost completely unexplored in CKD. This study reports a first investigation of plasma nitroproteome in these patients, carried out by both immunorecognition and LC-MS/MS techniques. Plasma proteins of chronic and end-stage KD patients showed a higher burden of nitration than in healthy controls, but main nitration targets appeared to be the same in these populations. Immunoblotting data showed that uremic albumin is largely represented in the uremic nitroproteome together with fibrinogen chains (A, B and C), transferrin, α1-antitrypsin, complement factor D, haptoglobin, and IgG light and heavy chains. However, immunopurification and affinity chromatography experiments demonstrated that the relative content of 3NT on the albumin molecule was very low when compared with that of the remaining plasma proteins. The uremic nitroproteome was investigated using also plasma proteins obtained by in vivo ultrafiltration from patients treated with protein leaking or standard high-flux hemodialyzers. The study of these samples revealed the possibility to selectively remove protein nitration products during hemodialysis. Identification of intramolecular sites of nitration was preliminarily obtained in IgG chains isolated by 2D PAGE and assessed by bidimensional tandem mass spectrometry after chemoselective tagging. Further studies are needed to confirm at the molecular level the presence of nitrated Tyr residues in other proteins tentatively identified as nitration targets in this study and to explore the biological meaning of such a selective modification of plasma proteins by reactive nitrogen species in uremia and dialysis patients.

Keywords 3′-nitrotyrosine · Protein nitration · Plasma proteins · Uremia · Hemodialysis · Chronic kidney disease · Uremic toxins · Proteomics · Nitroproteome · Mass spectrometry

Introduction

The covalent product of tyrosine nitration, 3′-Nitro-Tyrosine (3NT), has been measured in the free and protein-bound form in human plasma as biomarker of inflammation and cardiovascular risk (Ptolemy et al. 2007; Parastatidis et al. 2008; Shishehbor et al. 2003; Abello et al. 2009). Available evidence suggests that protein nitration is a pathway and target specific event with consequences on the structure–function relationship of tissue and body fluid proteins (reviewed in Abello et al. 2009; Souza et al. 2008; Ischiropoulos 2009; Peluffo and Radi 2007). Despite this, protein targets and biological consequences of nitration in

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plasma, i.e. the most investigated biological sample in clinical studies, remain poorly characterized in many conditions. Fibrinogen (Parastatidis et al. 2008; Heffron et al. 2009), ceruloplasmin, transferrin, α1-protease inhibitor, α1-antichymotrypsin, plasminogen (Gole et al. 2000; Pignatelli et al. 2001), and the apolipoproteins Apo B and Apo A1 (Zheng et al. 2004) are in vivo targets, and nitration has been proposed to produce immunogenic effects (Ohmori and Kanayama 2005; Thomson et al. 2007; Parastatidis et al. 2007). These pieces of evidence may suggest a role in coagulation, lipid metabolism, immune and vascular functions, but so far only early evidence of prothrombotic effects by fibrinogen nitration has been provided in literature (Parastatidis et al. 2008). Moreover, only few authors have been able to identify Tyr residues that are modified in vivo by reactive nitrogen species (RNS), which is in turn the conclusive proof of in vivo nitration in a given protein (Abello et al. 2009). In support of a specific and possibly regulated process, albumin as the most abundant serum protein (approx. 60% of total proteins) was not detected as target of nitration in vivo in normal subjects or smokers and lung cancer patients (Pignatelli et al. 2001), as well as in acute respiratory distress syndrome (ARDS) patients (Gole et al. 2000) investigated by immunoblotting methods. Recently, Wayenberg et al. (2009) reported of immunodetectable nitroalbumin in plasma of perinatal asphyxia patients and thanks to a newly developed ELISA they were able to confirm that the relative abundance (mol/mol) of modified and unmodified Tyr residues of albumin is in the order of 10^{-5}. This study pointed out also on the role that kidney damage may have to worsen protein damage by RNS.

This evidence was reported in several other studies that essentially investigated the free form of 3NT as well as other epitopes of protein damage by oxidative and carbonyl stress in uremic plasma (Agalou et al. 2005), while much less attention was paid to characterize protein bound forms. However, signs of protein damage by oxidative/nitrosative damage appear in plasma starting from earliest stages of chronic kidney disease (CKD) (Mitrogianni et al. 2009; Matsuyama et al. 2009) and return to normal after kidney transplantation (Galli 2007; Simmons et al. 2005), thus underlying the key role that the metabolic and depurative function of kidney play on protein metabolism. At the same time, oxidative damage to serum proteins has pathogenic relevance in kidney damage and may thus contribute to the progression of CKD (see Shi et al. 2008 and references therein).

Oxidative stress and protein damage reach their maximal expression in end-stage renal disease (ESRD) and particularly in patients on regular hemodialysis therapy (HD) in which inflammatory and vascular symptoms are also particularly exacerbated (reviewed in Galli 2007; Himmelfarb 2009). In recent years, these aspects have stimulated wide interest on large (or high MW) solutes produced by the abnormal chemistry of ROS/RNS in uremia and dialysis patients, which were increasingly considered for a role as uremic toxins (Vanholder et al. 2006) and for more effective treatment by dialysis therapy (Ward 2005).

Notwithstanding the available information on protein damage by nitration reactions in uremic plasma remains scarce. The extent and targets of nitration during disease progression from pre-dialysis (i.e. in CKD) to dialysis therapy (i.e. in HD patients) as well as the effect of dialysis methods and dialyzer membranes on protein nitration remains unexplored.

As in other clinical studies (recently reviewed by Peluffo and Radi 2007 and by Abello et al. 2009), identification of nitration targets was largely disregarded in uremic plasma. This actually remains an analytical challenge and early studies performed by Mitrogianni et al. (2004) by immunoblotting and immunoprecipitation methods and more recent studies by our group (Galli 2007; Piroddi et al. 2007) carried out by immunoblotting and LC-MS/MS analysis[1] tentatively identified only some of the most abundant targets already described in other inflammatory conditions. However, many other candidate nitration targets are expected to be present in plasma samples of these inflamed patients.

To explore these targets, in this work, we set up a proteomic platform that was applied to the systematic investigation of plasma nitroproteins (the plasma nitroproteome) (1) to preliminarily identifying a broad number of protein targets by 3NT immunodetection, (2) to isolate or enrich these targets for more accurate evaluation and identity assessment by LC-MS/MS, and finally (3) to confirm with selective analysis methods the presence of nitration epitopes within the structure of isolated proteins.

As a first explorative technique in this multi-step protocol, qualitative analysis of nitration targets in uremic and healthy control plasma was carried out by 2D PAGE and WB analyses, which were used also to fractionate immunoidentified nitrated forms to proceed with their identification by LC-MS/MS analysis. The uremic plasma nitroproteome was also investigated on protein samples obtained by in vivo ultrafiltration carried out during HD therapy of patients treated with different dialyzers membranes. Albumin depletion and immunopurification experiments followed by 1D WB analyses were used to selectively enrich 3NT-containing proteins in the uremic plasma. Immunoprecipitation and immunoblotting methods so far validated in previous studies (Gole et al. 2000; Pignatelli et al. 2001; Galli 2007; Mitrogianni et al. 2004) were also applied to further characterize the extent and targets of nitration in uremic plasma.

[1] Piroddi et al. unpublished data, manuscript in preparation. Preliminary data presented at the 11th ICAAP, Vienna 2009.

Unbiased identification of intramolecular targets of nitration was obtained for the first time for a series of selected protein spots corresponding to IgG chain regions on 2D PAGE. This identification was performed by bidimensional tandem MS after chemoselective tagging of nitration epitopes (Amoresano et al. 2007; Chiappetta et al. 2009).

Materials and methods

Patients and blood samples

This observational study has been performed with a cross-sectional design in order to compare levels and targets of protein nitration in pre-dialysis CKD, ESRD patients on hemodialysis therapy (HD), and healthy controls (Ctr). Essential characteristics of these groups are reported in Table 1. Plasma samples of HD patients (5 M/5 F) were obtained from the bank of plasma samples established in our laboratories (samples collected during the years 2007 and 2008 and maintained at −80°C).

Essential criteria to identify CKD patients eligible for the inclusion in the study were a value of creatinine clearance ≤15 ml/min, that is grade IV → V of the KDOQI classification (National Kidney Foundation, Inc 2002; Eckardt et al. 2009). Exclusion criteria were age below to 30 years and higher than 70 years, the absence of clinical and laboratory signs of severe inflammation and protein-energy malnutrition (main laboratory indices considered were plasma levels of IL-6 >5 pg/ml and albumin levels of <3.5 g/dl; these were measured during the first visit for the inclusion in the study), the absence of diagnosis for diabetes, polycystic kidney disease, Lupus and other auto-immune diseases, malignancy and recurrent infections. A final group of 10 CKD patients (6 M/4 F) was identified to meet these criteria and, after providing informed consent, these were included in the study. After inclusion, during one of the morning visits at the ambulatory of Nephrology, 10 ml of heparinized blood were collected from the antecubital vein and plasma was obtained by centrifugation. This was divided in aliquots and stored at −20°C. Blood sampling was performed under overnight fasting conditions.

HD samples were obtained in the middle-week dialysis session from patients on regular three times/week HD. Exclusion criteria were the same than in CKD group plus a dialysis age of lower than 72 months and higher than 9 months. Moreover, only patients with good metabolic control and stabilized on HD therapy with high-flux membranes were considered. Two subgroups of HD patients were thus identified to investigate uremic proteins obtained by in vivo ultrafiltration according with the study design described before in (Galli 2007; Galli et al. 2005). These HD patients were on treatment with protein leaking dialyzers (PLD, $n = 5$) or standard non-protein leaking high-flux polysulfone dialyzers (NPLD, $n = 5$). These two types of high-flux dialyzers differ as regards hollow fiber composition (polymethylmethacrylate vs. polysulfone, respectively) and permeability characteristics (percentage of porosity and pore size result in nominal cut-off values of ≥70 KDa and ≤30 KDa, respectively), which in turn produce different protein patterns in ultrafiltrate samples (Galli 2007). Ultrafiltrate samples were obtained in the first and in the last 5 min of the dialysis treatment by applying a positive pressure on the blood side of the dialyzer to obtain by convection a plasmatic water rich of plasma proteins that was collected from the dialysate chamber by the outlet port in the dialyzer cartridge. Protein concentration in ultrafiltrate samples was checked using the Bradford method and the protein pattern was preliminarily verified by SDS-PAGE and densitometric analysis performed using the software Quantity One (Bio-Rad, CA, USA). Ultrafiltrates used in this study obtained from PLD- and NPLD-treated patients showed total protein levels of 2.4 ± 0.9 and 1.5 ± 0.5 mg/ml, respectively. Further details on the relative abundance and general characteristics of plasma proteins obtained during in vivo ultrafiltration, and dialyzer membrane permeability parameters (percent of porosity and pore size) can be found in (Galli 2007; Piroddi et al. 2007; Galli et al. 2003; Buoncristiani et al. 1999).

Table 1 Clinical characteristics of the CKD and HD subjects included in this study

	Ctr	CKD	HD (PLD)	HD (NPLD)
Age (years)	66 ± 13	72 ± 13	62 ± 12	70 ± 12
Sex (M/F)	5/5	6/4	5/0	3/2
Total proteins (g/dL)	7.0 ± 0.5	6.3 ± 0.5	6.9 ± 0.6	7.0 ± 0.2
Albumin (g/dL)	4.7 ± 0.2	4.6 ± 0.2	4.2 ± 0.3	4.0 ± 0.4
Transferrin (mg/dL)	272.4 ± 48.2	254.6 ± 99.0	291.6 ± 55.9	228.6 ± 157.2
Fibrinogen (mg/dL)	212 ± 26	236 ± 50	248 ± 25	245 ± 42
Creatinineclearance (mg/mL)	89 ± 22	14.9 ± 4.8	–	–
IL-6 (pg/mL)	2.03 ± 0.44	2.47 ± 0.24	5.0 ± 1.9	5.3 ± 2.15
hsCRP (mg/dL)	0.79 ± 3.6	0.77 ± 0.52	2.58 ± 28.6	2.27 ± 33.6

Laboratory determinations

Immunopurification of 3NT-containing proteins and albumin removal by affinity chromatography

Immunoprecipitation (IP) experiments were carried out in whole plasma and ultrafiltrate samples using protein G-Agarose immunoprecipitation magnetic beads (Invitrogen, Milan). The anti-3NT antiserum used was the mouse monoclonal IgG clone 39B6 from Abcam, Cambridge, UK.

Immunoaffinity (IA) of nitroproteins was performed using a Sepharose 4 fast-flow column conjugated with a rabbit polyclonal anti-nitrotyrosine IgG (Affiland, Liège, Belgium) that was used according with the specifications provided by the manufacturer. Nitrotyrosine-containing proteins obtained with this procedure were extensively dialyzed against water and dried down using a speed vacuum concentrator before analysis.

The nitroproteins obtained with IP or IA were resuspended alternatively in SB to run SDS-PAGE and WB experiments or in water to perform LC analyses.

In some experiments, albumin was removed from plasma samples using a blue-agarose gel (Affiland, Liège Belgium) according with the procedure suggested by the manufacturer.

Qualitative analysis of nitroproteins by 1D and 2D PAGE/WB and LC-MS/MS analysis

These analysis methods were used to screen and eventually to isolate potential nitration targets in plasma. Although 3NT immunorecognition has relatively low specificity, it remains an essential step of nitroprotein analysis since it allows to restrict the number of candidate targets in complex protein samples to be further investigated by higher levels of accuracy and precision for protein identity and nitration epitope confirmation (see chemoselective labeling and LC-MS/MS analysis protocols described below). For 1D WB analysis, whole plasma or ultrafiltrate proteins suspended in SB were incubated for 5 min at 100°C, and 15 µg of proteins were loaded in each lane of a monodimensional polyacrylamide gel (1D-PAGE; 10% of T) that was run using a mini Protean Cell System (Bio-Rad). After 1D-PAGE, proteins were transferred onto a nitrocellulose membrane using a trans-blot SD semi-dry transfer cell (Bio-Rad), and were incubated with the same mouse monoclonal IgG used for IP (clone 39B6 from Abcam, Cambridge, UK). Thus, a further incubation step was performed using a secondary goat anti-mouse peroxidase-conjugated antibody (Millipore, CA, USA). In some experiments nitroproteins were also immunodetected using a rabbit polyclonal anti-3NTprimary antibody (Sigma-Aldrich, Milan, Italy) and a goat anti-rabbit peroxidase-conjugated antibody (Millipore, CA, USA). The membrane was washed three times (5 min each) in TBS containing 0.1% vol/vol Tween 20 and the signal was developed on BioMax light films (KodaK, NY, USA) using an ECL solution containing 1 M Tris–HCl pH 8.5, 1.25 mM luminol in DMSO, 0.25 mM cumaric acid in DMSO, 0.015% vol/vol H_2O_2. Image analysis and densitometry were performed using Gel-Pro Analyzer 3.1 (MediaCybernetics, Inc., MD, USA).

Specificity of anti-3NT monoclonal antisera used in this study was tested by protein reduction with $Na_2S_2O_4$ according with the procedure previously reported in (Chiappetta et al. 2009). Other tests included the saturation of the incubation medium containing the first antibody with free 3NT and the omission of the primary antibody. These tests were performed with either low or high loads of plasma proteins during SDS-PAGE (7.5 or 15 µg/lane, respectively) and with different times of exposure of light films to ECL (from 10 to 60 s.).

For 2 PAGE and WB experiments, proteins were precipitated overnight in ice-cold acetone and the precipitate was centrifuged for 2 min at 14,000×*g*, 4°C. The final pellet (300 µg of proteins) was dissolved in rehydration buffer and then was diluted to the appropriate final protein concentration in 350 µl of DTT 60 mM, Tris 40 mM, urea 7 M, thiourea 2 mM, CHAPS 4%, Bio-Lyte ampholite (Bio-Rad) 0.7 µl, and bromophenol blue. The samples were loaded onto immobilized pH gradient gel strips (17 cm, pH 3–10 NL), and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad Labs). The second dimension was performed on 9 to 16% polyacrylamide linear gradient electrophoresis gels using a Protean II xi Cell (Bio-Rad). 2D WB was performed using the same procedure described above for 1D WB using a Trans-blot cell (Bio-Rad). 2D WB and PAGE image matching was performed by PD Quest software (Bio-Rad), selected spots were picked and in-gel trypsinization was carried out as described in (Sultana et al. 2006). Tryptic digest was evaluated by nanospray LC/MSMS using a an HCT nano-HPLC-Chip/MS system Agilent 1200 Series equipped with a tridimensional ion trap system (Agilent Technologies, Milan, Italy). A customized chip program for shotgun proteomics was applied according with the manufacturer. 2D map matching for protein identification was performed using the resources available in the SWISS-PROT database (http://www.expasy.org/cgi-bin/map2/def?PLASMA_HUMAN).

Semiquantitative analysis of protein 3NT by immuno-dot blot analysis (DB)

Plasma samples were prepared for the analysis by appropriate dilution in Laemli buffer (Tris–HCl 0.12 M pH 6.8,

glycerol 21.7%, SDS 4.28%), and different amounts of total proteins were spotted onto a nitrocellulose sheet that was soaked in Tris–HCl 20 mM pH 7.5, NaCl 500 mM, and then mounted for the analysis onto a Bio-Dot microfiltration apparatus (Bio-rad Laboratories s.r.l., Milan). Immunoblot and image developing were obtained with the same procedure described above for 1D WB experiments.

The assay was calibrated using NO-BSA that was prepared according with (Amoresano et al. 2007). Briefly, BSA (10 mg/mL) in 200 mM Tris buffer (pH 8.0) was exposed to tetra-nitro-methane (Sigma-Aldrich) at the final concentration of 350 mM. The reaction mixture was stirred at room temperature for 30 min, and NO-BSA was rapidly desalted by size exclusion chromatography on a Sephadex G-25 M column (Sigma-Aldrich). Protein elution was monitored at 280 and 360 nm, and the protein-containing fraction was collected and stored at −20°C. The content of 3NT of this nitrated protein was checked by MALDI and nano-LC-MS/MS analysis according with the method reported in (Chiappetta et al. 2009). Assuming from MALDI data that approximately 30% of albumin exposed to TNM is nitrated, and that 4 Tyr residues per molecule can be converted to 3NT (Chiappetta et al. 2009), a value of 20 pmol of 3NT/μg of NO-BSA was considered to make calibration curves. Incremental concentrations of this reference protein were loaded in each DB experiment (in the range 0.4–160 pmol/spot that correspond to 0.02–8 μg/spot) and the assay response with these protein loads was verified by linear regression analysis within each analysis session. Linearity was also tested assessing three different times of exposure to ECL. With NO-BSA linearity ($R = 0.988$, $p < 0.01$) was achieved in the range 0.4–16 pmol of protein/spot with time of exposure to ECL of 15 s. Under these conditions, the observed detection limit was 0.2 pmol NO-BSA/spot. To further increase analysis precision, in the same session each DB experiment was run in duplicate. A first DB run was performed to calibrate protein load and ECL exposure (usually 0.5 min) and a second DB run was performed to confirm optimal conditions and to obtain final semi-quantitative data. In each of these two DB experiments, three incremental concentrations of plasma proteins were spotted in series. The amount of 3NT in plasma proteins was expressed in pmol/μg of proteins. An intra-assay CV % of ≤10% was observed for NO-BSA and of ≤19% for plasma proteins.

Reduction of nitrated plasma proteins to secondary amines by $Na_2S_2O_4$ (dithionite or sodium hydrosulfite) (Sigma-Aldrich) was used to confirm specificity of anti-3NT IgG used in these DB experiments by a stripping–reprobing procedure. Briefly, proteins transferred onto blotting membranes were stripped using the Restore™ stripping buffer (Thermo Scientific, IL, USA) and reduced incubating the membrane at room temperature in 20 mM dithionite dissolved in 50 mM borate pH 9.5 for 5 min. Reprobing was thus performed as described above using the same primary anti-3NT antibody.

Gel-free bidimensional tandem mass spectrometry analysis of nitroproteins after chemoselective tagging

Unbiased semiquantitative analysis of nitroproteins in human plasma was performed by chemoselective tagging and bidimensional nano-LC-MS/MS according with the procedure previously described in (Amoresano et al. 2007). Briefly, after 2D PAGE of plasma proteins, selected spots in the region corresponding to IgG lambda and heavy chains were isolated and processed for trypsinization. Reduction of nitro groups to amino groups in the trypsin digest was performed by $Na_2S_2O_4$ at a molar ratio of 100:1 with expected 3NT content as calculated in NO-BSA (Amoresano et al. 2007; Chiappetta et al. 2009). Then, 3-aminotyrosines were labeled with a solution of dansyl-chloride (DNS-Cl) 18.5 ng/μL in ACN (1,000-fold molar excess) and the peptide mixture was analyzed by LC-MS/MS analysis using a 4000Q-Trap machine (Applied Biosystems) coupled to an 1100 nano-HPLC system (Agilent Technologies). Total ion counts (TIC), precursor ion scan (PIS) and MS3 analyses were performed and resulting data were acquired using Analyst software (Applied Biosystems) and further processed by MASCOT in house software as described in (Amoresano et al. 2007).

Statistics

Results were expressed as the mean ± SD. Statistical analysis and data plotting were carried out using Microcal™ Origin 7.5 (Microcal Software Inc., Northampton, MA, USA) and Sigma Plot 9.0 (Systat Software Inc., San Jose, CA, USA). Probability values of <0.05 were considered as significant.

Results

Semi-quantitative analysis of nitroproteins by dot blot

Figure 1 shows a typical semiquantitative DB analysis carried out to measure nitroproteins in plasma of CKD, HD and Ctr groups. All the DB experiments were optimized as regards optimum of protein load, antibody performance and ECL exposure. The resulting photographic images (Panel A, left) were digitalized and analyzed by densitometry against a calibration curve of nitro-BSA (Panel B). Each DB experiment was tested also for specificity by reduction with dithionite (Panel A, right). Panel C shows semi-quantitative data obtained with this test. After correction of unspecific

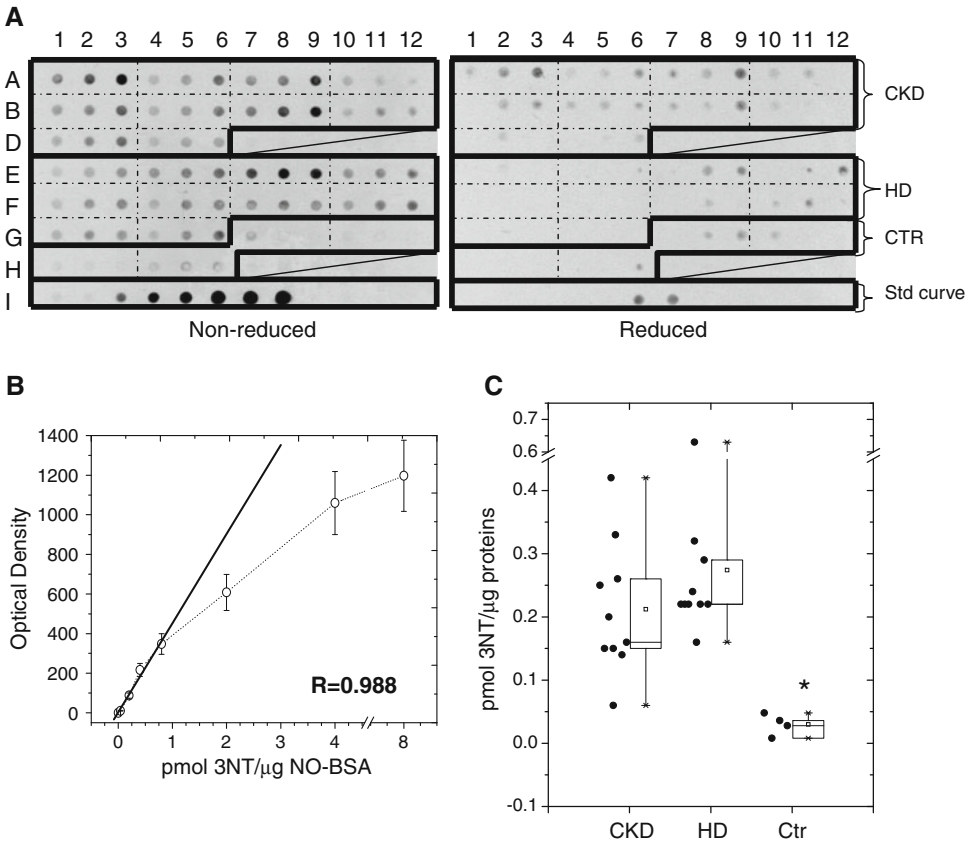

Fig. 1 Immuno-dot blot analysis (DB) of 3′-nitro-Tyr (3NT)-containing proteins in chronic kidney disease (CKD), hemodialysis (HD) and healthy control (Ctr) plasma. This test was carried out using an improved version of the procedure described by Pignatelli et al. (2001). 3NT immunodetection was performed with the method described in detail in the section "Materials and methods". DB tests (**a**) were performed by loading for each plasma sample three incremental amounts of total proteins (from 5 to 20 µg) that were selected in order to achieve a linear response during film exposure to ECL. Specificity in the immunorecognition of 3NT was checked by reduction with sodium dithionite (**a**, right). After densitometry, 3NT concentrations were calculated using a calibration curve of nitro-bovine serum albumin (NO-BSA; **b**). The linear portion of the calibration curve shown by the *straight fitting line* was used to calibrate the assay. Mean levels of 3NT in CKD, HD and Ctr groups are shown in **c** (as pmol 3NT/µg of total proteins). $*p < 0.01$ versus CKD and HD

recognition revealed by dithionite reduction (this was particularly marked in CKD group), the levels of immunodetectable 3NT in CKD and HD plasma proteins were determined as 0.212 ± 0.104 and 0.274 ± 0.132 pmol 3NT/µg total proteins, respectively (p = NS). A high inter-individual variability was observed in these two groups of patients (Panel C). In Ctr, a four-time higher protein load per spot than in uremic patients was needed to measure ECL signals that linearly corresponded to the amount of proteins assessed in the DB test (not shown), and a value of 0.030 ± 0.017 pmol 3NT/µg total proteins was calculated ($p < 0.05$ vs. both CKD and HD groups).

Qualitative analysis of nitrated proteins

Plasma samples from uremic patient and healthy controls were first analyzed by 1D PAGE and WB experiments carried out under saturating conditions (obtained with a load of 10 µg of total proteins per lane) to explore potential 3NT-containing proteins (shown in the supplemental material as Fig. 1a) that will be further investigated to confirm protein identity and nitration sites by LC-MS/MS (see below). The specificity of the monoclonal antisera used in this study was confirmed by applying different analytical conditions as shown in Fig. 1b (see the supplemental files).

Although this analysis provides very limited resolution, abundant immunodetectable material was recognized in both the uremic (only HD patients were assessed) and healthy control plasma. Main nitrated species could be identified in the region between 50 and 80 KDa, while another series of bands with lower intensity were present in the 100–260 KDa region. Finally, an intense band was observed just below 30 KDa. The conditions used to obtain this qualitative image (protein load and exposure time to ECL were maximized) interfered with the possibility to obtain semiquantitative information at least for most abundant nitrated species.

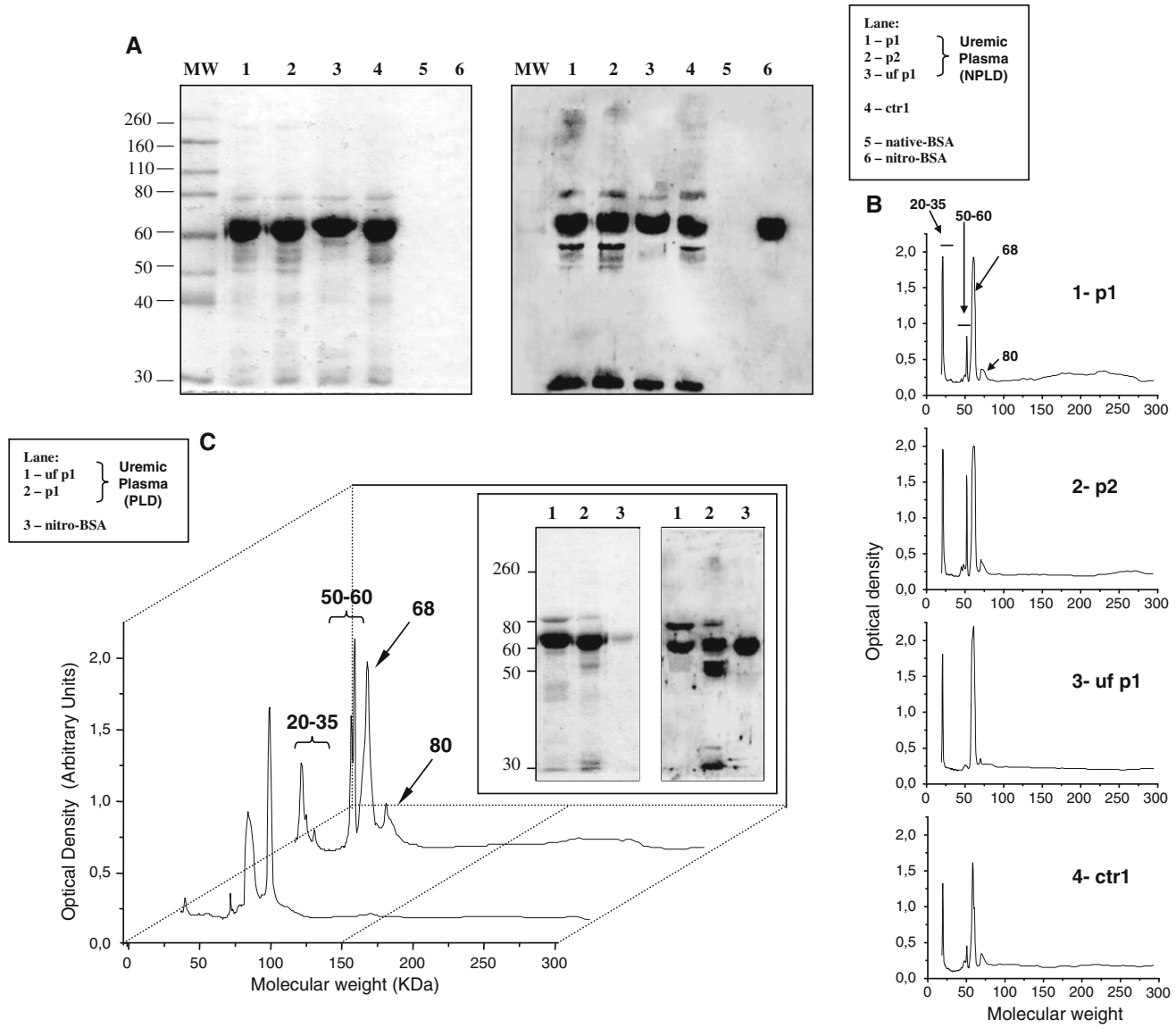

Fig. 2 Qualitative analysis of 3NT-containing proteins in HD plasma and ultrafiltrate samples. 1D-WB analyses were carried out in PLD and NPLD plasma proteins under non-saturating conditions (7.5 μg of proteins/lane, ECL exposure 0.5 min.). **a** The SDS-PAGE and WB analysis carried out in NPLD plasma and ultrafiltrate samples, representative of the entire set of samples investigated in this study. Corresponding densitometric data are shown in **b** and **c**. Densitometry profiles of WB analyses carried out on PLD plasma and ultrafiltrate are shown in detail (**c**) to highlight the enrichment of the protein band with apparent MW = 80 KDa. NPLD ultrafiltrate (**b**, Panel 3) shows a marked increase of the band in the region between 25 and 30 KDa. Abbreviations are as in Fig. 1

Figure 2a shows the 1D WB analysis carried out on whole plasma and ultrafiltrate samples from patients treated with PLD and NPLD dialyzers. Analyses were performed under the same conditions described in Fig. 1, but with lower protein loads (7.5 μg of total proteins per lane) to avoid signal saturation and thus to tentatively increase resolution in the region between 50 and 80 KDa, i.e. the region in which the most abundant nitrated proteins were observed.

Densitometry scanning of 1D WB images showed very similar patterns of nitrated proteins in uremic and healthy control plasma (Fig. 2b, panels 1, 2 and 4). Although semiquantitative analysis of immunorecognized species by this test has limited reliability, all the assessed samples of uremic plasma showed a higher area under the curve than in the healthy controls, thus confirming DB results shown in the previous paragraph.

On the basis of apparent MW, qualitative results obtained by 1D WB experiments were consistent with the tentative identification of albumin as main target of nitration in uremic and healthy control plasma (Fig. 2b, peak at 68 KDa). The peaks with apparent MW between 50 and 60 KDa were tentatively assigned to the already reported nitration targets fibrinogen chains, α1-antitrypsin and α1-antichymotrypsin (Parastatidis et al. 2008; Heffron et al.

2009; Gole et al. 2000; Pignatelli et al. 2001). Finally, the nitrated peak with MW of 80 KDa could be compatible with transferrin, while the peaks between 20 and 35 KDa were consistent with the reported nitration of Apo-A1 (Zheng et al. 2004), but also with a series of other proteins including the IgG light or λ chain, one of the most abundant protein fractions in plasma.

When examined by 1D WB, ultrafiltrate samples from patients treated with NPLD (Fig. 2b, panel 3) and PLD (Fig. 2c) showed the presence of a less complex pattern of nitrated proteins with respect to whole plasma. In particular, NPLD ultrafiltrates essentially showed the presence of peaks at 68 KDa and in the region 20–35 KDa, while PLD ultrafiltrate contained peaks with MW of 68 and 80 KDa. In both ultrafiltrate samples, the bands corresponding to nitrated proteins occurring in the 50–60 KDa region were markedly reduced with respect to whole plasma.

2D PAGE and LC-MS/MS identification of nitrated proteins

Figure 3 shows typical 2D PAGE analyses and the corresponding WB images obtained with anti-3NT antisera of uremic proteins in plasma (Fig. 3a), and in PLD and NPLD ultrafiltrate samples (Fig. 3b) that were previously examined by 1D WB (see above and Fig. 2). After matching WB and coomassie stained 2D PAGE patterns (Fig. 3a), immunorecognized nitrated proteins were isolated by cutting gel spots, and these candidate nitroproteins were submitted to molecular identification by in situ digested with trypsin and LC-MS/MS analysis of the resulting peptide digests. Spectral data were used to search for a non-redundant protein database using the Mascot software and identified proteins are shown in Table 2. Most abundant plasma proteins such as albumin, IgG light and heavy chains, antithrombin and α1-antitrypsin were tentatively identified with this procedure as nitration targets. However, also some low abundant proteins like the complement factor D could be identified. Other candidate targets of nitration were assigned by direct matching of WB images with existing 2D PAGE images of human plasma available for consultation and analysis at: http://www.expasy.org/cgi-bin/map2/def?PLASMA_HUMAN). These proteins included fibrinogen A, B and C chains that showed their characteristics sequel of spots in the 2D MW/PI region, serotransferrin, ceruloplasmin and haptoglobin (Fig. 3a; Table 2). The same analyses carried out on selected spots from 2D-PAGE gels of PLD and NPLD ultrafiltrate samples (Fig. 3b), produced essentially comparable results.

This LC-MS/MS identification of nitrated proteins fractionated by 2D-PAGE was useful to further increase the level of information obtained in the preliminary 1D WB experiments performed on whole plasma and ultrafiltrate samples shown in Fig. 2. Thus, the peak at 68 KDa (Fig. 2b, c) very likely corresponds to albumin and the peak region of 20–35 KDa should include IgG light chains together with other minor components as complement factor D. In the same way, the peak at 80 KDa essentially consisted of transferrin, which is highly enriched in PLD ultrafiltrate. The findings described for the 1D WB analysis of ultrafiltrate samples were also confirmed by this analysis. Main nitrated forms of the region 50–60 KDa are absent in these samples (essentially fibrinogen A and B chains, and IgG heavy chain) while α1-antitrypsin was detected together with other minor components in the PI region of albumin that was not identified.

Immunopurification and albumin depletion

In an attempt to selectively enrich the relative concentration of plasma nitroproteins, immunoaffinity (IA) and immunoprecipitation (IP) experiments (Fig. 4a, b, respectively) were performed using the same antisera used in the WB and DB experiments of above. The IA procedure produced similar protein recoveries in uremic and healthy control subjects suggesting that the experiment was performed under saturating conditions with respect to the binding capability of anti-3NT antisera. Accordingly, the fractions not retained during the immunopurification process contained abundant anti-3NT positive material (Fig. 4c). The IP experiment was then carried out under non-saturating conditions providing a higher purification yield than IA (Fig. 4b). Indeed, nitroproteins were not detectable in the washing buffer and in the supernatants after IP (Fig. 4c), and the amount of 3NT-containing proteins was lower in healthy controls than in HD patients (mean difference $= 22\%$; $n = 4$ each).

IA and IP samples prepared from both whole plasma and ultrafiltrate samples were then analyzed by 1D PAGE and WB showing that most abundant nitrated components have an apparent MW between 50 and 60 KDa. This finding confirms the elective nitration of proteins such as fibrinogen chains that were described in the 2D WB analysis of Fig. 3 and in literature (see Parastatidis et al. 2008; other references are reported in the "Discussion" section). In this MW region, nitration of α1-antitrypsin and IgG heavy chains had also been tentatively inferred by 2D WB (Fig. 3). At the same time, albumin does not appear to be recovered during IA and IP experiments. This was particularly obvious in the IA experiment of Fig. 4a. This finding was confirmed by albumin depletion of plasma samples carried out with blu-agarose affinity chromatography and subsequent SDS-PAGE and DB analyses (shown in the supplemental files as Fig. 2a). In both uremic or healthy control plasma, the large majority of the immunodetectable 3NT (approx. 95% of the signal detected following dithionite reduction) was observed in albumin-depleted

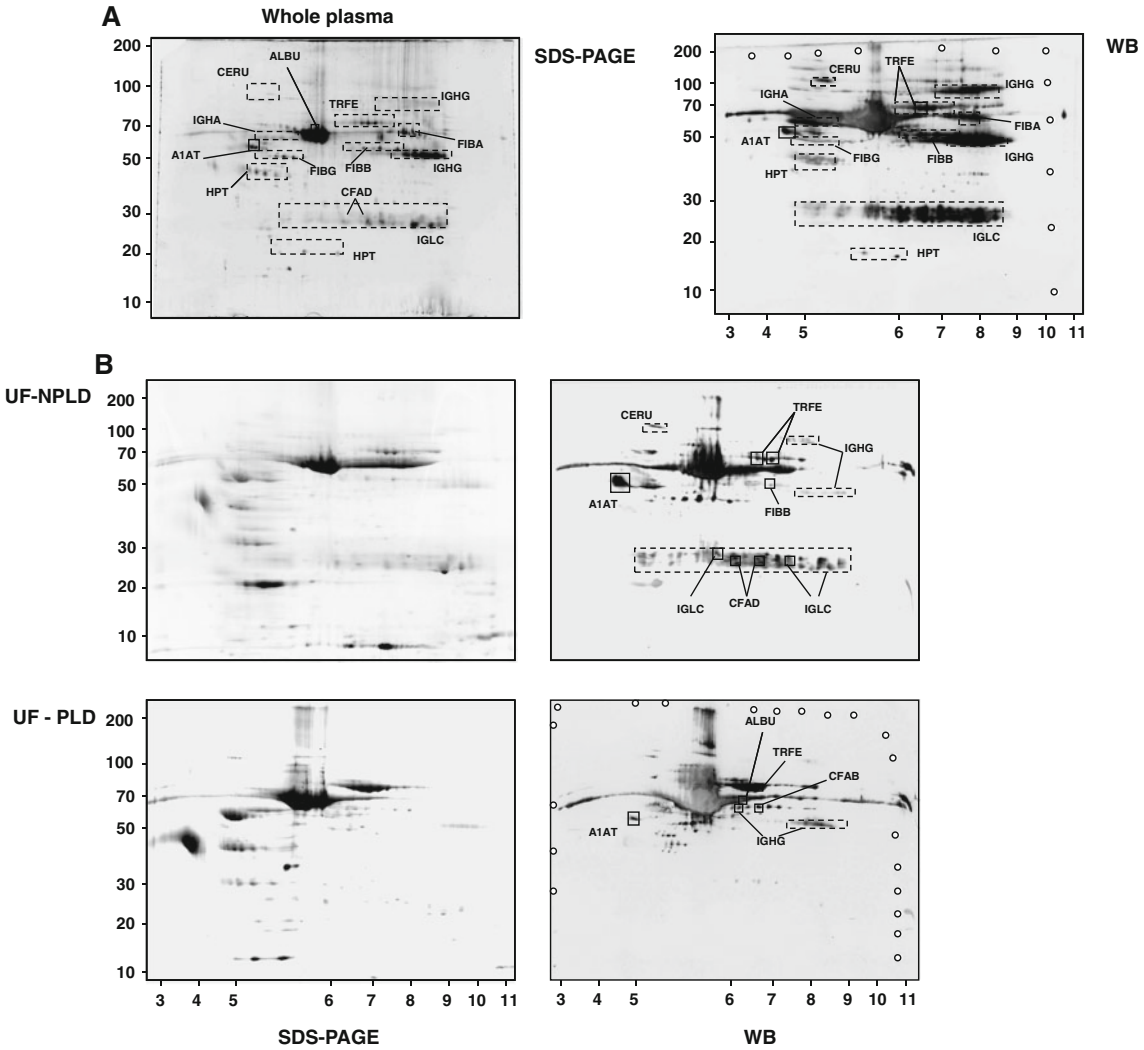

Fig. 3 2D-PAGE and WB analysis for the qualitative evaluation of 3NT-containing proteins in HD plasma and ultrafiltrate samples. Qualitative identification of nitrated proteins in HD plasma and ultrafiltrate samples was carried out by 2D-PAGE (**a** top panel, **b** left panels) and WB (**a** bottom panel, **b** right panels) according to the procedure previously described in (Galli 2007; Piroddi et al. 2007). Protein identification by LC-MS/MS analysis was carried out in selected protein spots (highlighted by *squared straight line*). Other proteins were identified by gel matching (spots highlighted by *squared dashed line*) using as template the human plasma 2D map available for consultation at the Expasy website (http://www.expasy.org/cgi-bin/map2/def?PLASMA_HUMAN). The list of proteins identified with this procedure is reported in Table 2. *White dots* shown in WB films indicate reference points used during image matching with the corresponding 2D PAGE by PD Quest software (Bio-Rad)

samples. As calculated by densitometric analysis of SDS-PAGE, these samples contained approximately 45% of the total proteins (shown as supplemental Fig. 2b). These data demonstrate that albumin is much less extensively nitrated than fibrinogen and other less abundant species in plasma.

Bidimensional MS analysis of nitroproteins after chemoselective labeling and identification of 3NT residues

Direct identification of nitrated proteins and accurate definition of nitration sites in uremic plasma proteins was inferred by bidimensional tandem mass spectrometry (Amoresano et al. 2007) after trypsinization and dansyl-chloride labeling of nitropeptides. Selected spots of 2D PAGE maps and immunoresponsive to anti-3NT antisera during 2D WB experiments were assessed with this procedure. After selective labeling of 3NT residues by reduction to 3-aminotyrosine and specific dansylation, the exact location of original nitro-groups within the amino acid sequence of nitropeptides was successfully identified by bidimensional tandem MS analysis in some components of the IgG chain region (Table 3). This MS analysis was performed by PIS and MS3 scan mode combination.

Table 2 Tentative identification of nitroprotein targets in uremic plasma as inferred from 2D maps and WB analyses

Spot Nr	Protein	SP code	MW	Nr peptides
LC-MS/MS analysis				
A1AT	Alpha 1-antitrypsin	P01009	46878	37
ANT3	Antithrombin-III	P01008	53025	4
ALBU	Serum Albumin	P02768	71317	39
CFAD	Complement Factor D	P00746	27529	3
IGKC	Ig K chain C region	P01834	11773	9
–	Ig K chain V–III region WOL	P01623	11853	3
Gel-matching procedure				
IGHG	Ig gamma chain C region	P01860		
IGLC	Ig lambda chain C region	P01842		
FIBA	Fibrinogen alpha chain	P02671		
FIBB	Fibrinogen beta chain	P02675		
FIBG	Fibrinogen gamma chain	P02679		
CERU	Ceruloplasmin	P00450		
HPT	Haptoglobin	P00738		
TRFE	Serotransferrin	P02787		
IGHA	Ig alpha-1 chain C region	P01876		

In PIS mode, the MS detector can only detect peptide precursor ions producing the *m/z* 170 fragment. The selected precursor ions were then subjected to a combined MS2 and MS3 experiment to specifically detect only those ions originating the dansyl-specific *m/z* transition 234 → 170 in MS3 mode. Figure 5 shows a typical profile obtained from this analysis carried out in some of the main spots corresponding to the IgG chain regions in the 2D map shown in Fig. 3a. As expected, the TIC chromatogram (Fig. 5, upper panel) exhibited a larger number of signals most of which were very likely unrelated to 3NT-containing peptides. In fact, the corresponding precursor ion TIC profile shown in the lower panel, exhibited a much simpler ion pattern in which the MS2 spectra showed only the diagnostic fragment ion at *m/z* 170. Further selection based on MS3 scan recording the *m/z* transition 234 → 170 (Amoresano et al. 2007) removed the remaining false-positives leading to the identification of truly nitrated peptides. Finally, MS/MS analysis of the labeled peptides produced fragmentation spectra that were used to determine peptide sequences for unbiased evaluation of protein identity and unambiguous location of the nitration site within its structure. The results of this PIS and MS3 procedure are reported in Table 3 that shows modified Tyr residues in chemoselective-labeled nitropeptide and the SwissProt accession code for protein identification of seven different peptides all belonging to immunoglobulin species.

Discussion

NO-mediated protein damage may contribute to sustain events of uremic toxicity and the cardio-inflammatory comorbidity of CKD patients. Actually, protein nitration is increased in uremia as well as in other inflammatory conditions and may produce functional consequences that are compatible with an increased cardiovascular risk (Ischiropoulos 2009). Notwithstanding, protein nitration in uremic plasma remains poorly characterized as levels, targets and clinical importance are concerned. The main reason for this lack of information is the complexity and poor reliability of methods and techniques currently available to perform these studies. Extensive characterization of 3NT-containing proteins in complex biological samples, such as plasma, remains in fact an analytical challenge and even most updated and sophisticated shotgun proteomics techniques fail to provide suitable (high-throughput and selective) analytical platforms (Abello et al. 2009). The proteomic platform developed and applied in this study to investigate qualitative and semi-quantitative aspects of uremic plasma nitroproteome includes low-specificity exploration tools such as 2D-PAGE, immuno-detection/isolation techniques, and also specific tools for the unbiased identification of 3NT-containing species by bidimensional MS after chemoselective labeling.

The immuno-dot blot analysis method originally proposed by Pignatelli et al. (2001) was improved and used to obtain a semi-quantitative assessment of total nitroproteins in plasma before to proceed with qualitative assessments. Although this analysis method overestimates the actual levels of 3NT in plasma proteins (Ryberg and Caidahl 2007), it has been useful to demonstrate an increased burden of nitration in uremic plasma than in healthy control plasma. Indeed, CKD and HD plasma contained 0.212 ± 0.104 and 0.274 ± 0.132 pmol 3NT/μg total plasma proteins, respectively, while healthy control plasma contained 0.030 ± 0.017 pmol 3NT/μg total proteins ($p < 0.01$ vs. both the two groups of patients).

In the exploration of uremic nitroproteins (qualitative analysis) carried out by 1D and 2D WB, albumin appeared the most abundant plasma nitroprotein; however, immunopurification experiments and selective removal of this protein showed that the relative contribution of the nitrated form of albumin to total protein 3NT levels is very low. These findings are consistent with the results obtained in earlier studies carried out by some of us on uremic proteins obtained by in vivo ultrafiltration (Galli 2007; Piroddi et al. 2007) and also with the results obtained by other Authors that studied whole plasma and also anti-3NT immunoprecipitated uremic proteins (Mitrogianni et al. 2004).

Albumin was not detected as nitration target by WB even after immunoprecipitation of 3NT-containing plasma

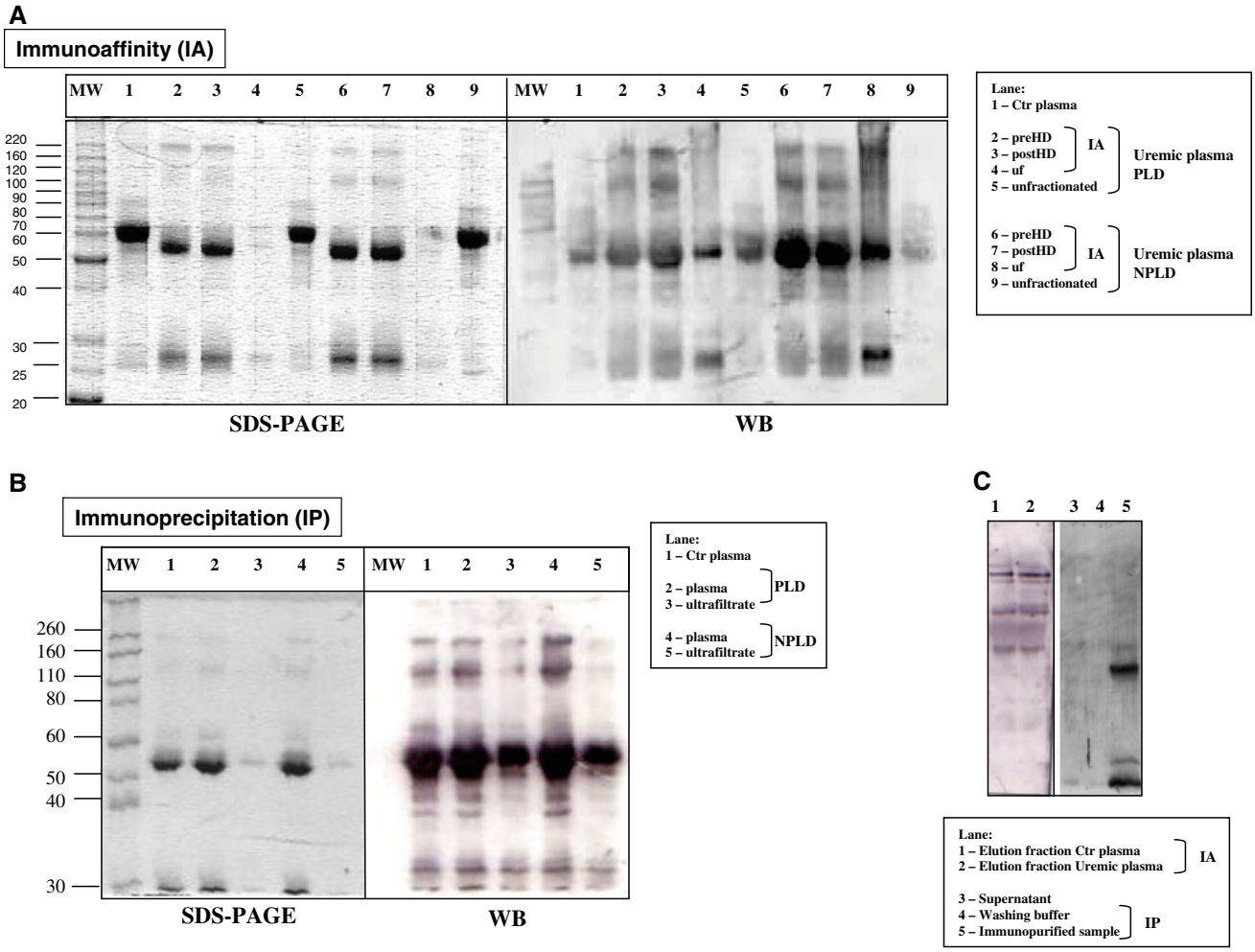

Fig. 4 Partial purification of nitrated species in uremic plasma by immunoaffinity and immunoprecipitation. Pooled samples of HD and healthy control plasma were treated for IA or IP analysis as described in the text and the resulting protein samples were assessed by 1D PAGE (15 μg proteins/lane) and WB analysis (**a** and **b**, respectively). WB analysis of the nitrated proteins recovered in the elution fractions of IA experiments and the supernatant and washing buffer obtained from IP experiments are shown in **c**. Further details are reported in the text. Abbreviations are as in Fig. 1

proteins in the other observational studies of Gole et al. [2000] and Pignatelli et al. [2001]. Accordingly, no identification of intramolecular targets was reported for the in vivo nitrated form of albumin that after in vitro exposure to peroxynitrite was identified to contain modified Tyr residues in position 138 and 411 (Jiao et al. 2001). On the contrary, nitration sites have been identified in the in vivo nitrated form of fibrinogen β chain (Parastatidis et al. 2008), which is proved to be one of the most nitrated species in human plasma in spite of a relative abundance on total proteins of 3%. Thus, albumin appears to sustain the unspecific immunorecognition of 3NT in plasma proteins, and this may explain also why in the absence of nitroprotein separation, immunodetection methods largely overestimate total nitroprotein levels in plasma.

Immunodetectable nitroalbumin has been recently reported and quantitated with a newly developed ELISA test, in plasma of neonates with asphyxia-induced encephalopathy by Wayenberg et al. (2009). These authors observed in patients with neurological symptoms median levels of nitroalbumin of 14.4 ng/ml that decreased to 7.3 ng/ml in asymptomatic neonates, which correspond to approximately 3.7 and 1.8×10^{-5} mol nitroalbumin/mol human serum albumin (HSA), respectively. These values increased to approximately 5.7×10^{-5} mol nitroalbumin/mol HSA (i.e. 22.6 ng/ml) in patients that in combination with neurological symptoms developed severe renal injury, but not hepatic or cardiac complications. These results confirm the key role that the kidney plays to control plasma protein damage and also the substoichiometric character of albumin nitration that in turn reflects the low levels of protein 3NT and the selectivity of nitration pathways in human plasma (Abello et al. 2009; Souza et al. 2008; Ischiropoulos 2009; Ryberg and Caidahl 2007).

Table 3 Bidimensional tandem mass spectrometry identification of nitration sites in protein spots corresponding to main IgG regions of 2D PAGE (Fig. 3)

Protein	SP code	Nitration site
Ig lambda chain V–I region BL2	P06316	VTISCSGSSSNIGNDY*V
Ig lambda chain V–I region EPS	P06888	VSISCSGSSSNIGKNYVDWY*QQLPGTAPK
Ig heavy chain V–II region ARH-77	P06331	YYY*GMDVWG
Ig heavy chain V–III region BUR	P01773	TYY*ADSVRGR
Ig heavy chain V–III region VH26	P01764	DNSKNTLY*LQMNSLR
		STYY*ADSVKGRFTISRDNSK
		GLEWVSAISGSGGSTYY*ADSVK

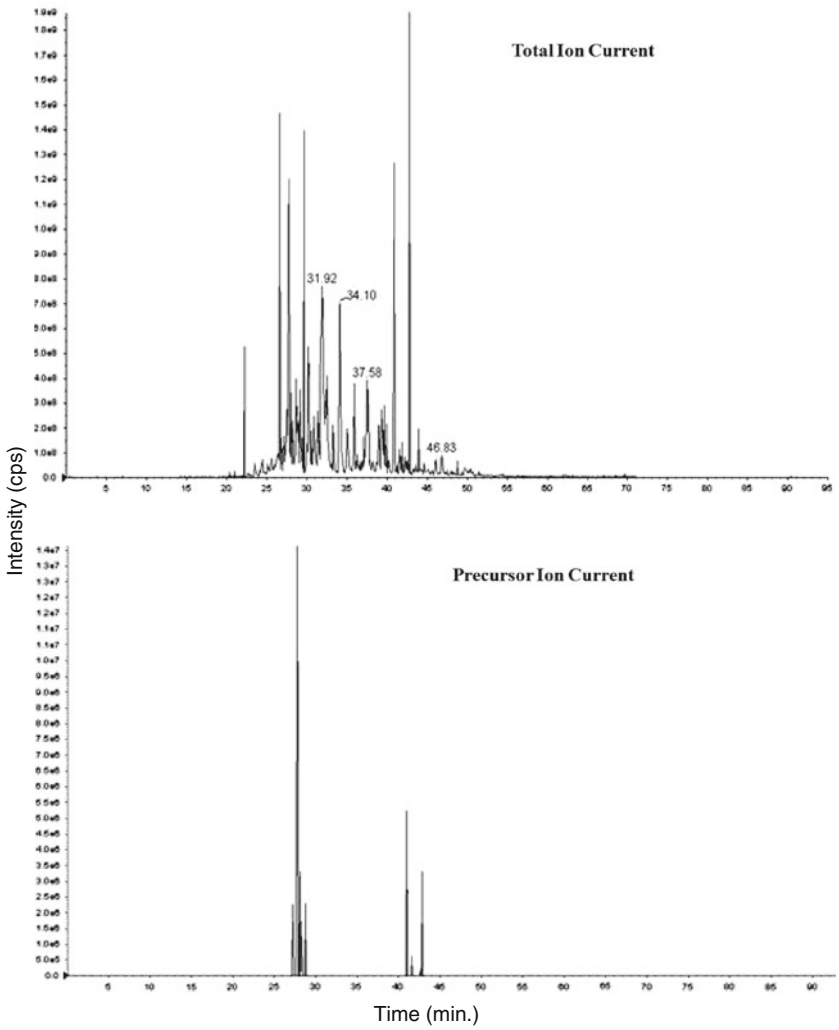

Fig. 5 Bidimensional LC-MS/MS analysis of IgG nitropeptides after chemoselective labeling. Tryptic peptide mixtures from selected spots occurring in the IgG regions of the 2D map and immunoresponsive to anti-3NT IgG were reduced and the resulting aminotyrosine epitopes of nitropeptides were labeled by dansyl-chloride and processed for 2D-LC-MS/MS analysis according to (Amoresano et al. 2007). The *upper* and *lower* panels show the total ion current (TIC) chromatogram and the precursor ion scan monitoring of the dansyl-chloride fragment ion with *m/z* 170, respectively. These ion chromatograms are representative of the entire set of 2D-LC-MS/MS analyses performed to identify nitropeptides of IgG chains reported in Table 3

During preliminary immunorecognition tests (qualitative analysis by 2D PAGE and WB, Fig. 3) other nitration targets identified in uremic plasma corresponded to protein targets previously described in small observational trials on other inflamed patients, as ARDS and lung cancer patients (Gole et al. 2000; Pignatelli et al. 2001). These included transferrin, ceruloplasmin, fibrinogen, and α1-antitrypsin. At the same time, other targets were tentatively identified for the first time in this study by 2D PAGE/WB and LC-MS/MS analysis and included IgG light and heavy chains, complement factor D, and haptoglobin.

Fibrinogen β chain, the most characterized nitration target in human plasma (Parastatidis et al. 2008; Nowak et al. 2007), was confirmed as one of the main nitrated species also in uremic patients. However, this nitrated form of fibrinogen was present also in healthy control plasma and the present study suggests also that α and γ chains of fibrinogen are nitrated in uremic and healthy control

plasma. Thus, fibrinogen chains seem to represent constitutive targets of nitration in human plasma and future studies are required to quantify their extent of nitration in patients and healthy controls, since this could reveal a higher risk of thrombogenicity (Parastatidis et al. 2008; Peluffo and Radi 2007; Heffron et al. 2009; Nowak et al. 2007). Fibrinogen nitration was recently proposed to represent a surrogate marker of inflammation of possible relevance in clinical trials (Heffron et al. 2009) and thus could be adopted in laboratory protocols to assess sub-clinical inflammation in uremia and dialysis patients. Further studies should be addressed to establish whether nitration of coagulation proteins is part of the oxidative stress-mediated events that are believed to sustain a defective coagulation (increased fibrinolytic activity) of uremia and dialysis patients (Pawlak et al. 2006). Thus, the study of fibrinogen nitration and more in general of other nitration targets among coagulation proteins (these include for instance α1-antitrypsin, which is abundantly expressed in the nitroproteome of uremic plasma and NPLD ultrafiltrate), could be of relevance in uremia and dialysis patients since chronic inflammation and impaired coagulation are main uremic symptoms that cannot be corrected with present dialysis therapies.

The study of the uremic nitroproteome performed on plasma proteins separated by in vivo ultrafiltration revealed first information as regards the potential of extracorporeal treatments to correct the accumulation of large solutes containing RNS-derived post-translational modifications (PTMs). These particular plasma protein samples were obtained from patients on treatment with PLD and NPLD dialyzer membranes. These two types of high-flux dialyzers produce different protein patterns in ultrafiltrate samples due to percentage of porosity and pore size characteristics that result in nominal cut-off values of $\geq$70 KDa and $\leq$30 KDa, respectively (Galli 2007). In this study, we found that albumin is the most abundant component recognized by 3NT immunoblotting in both the PLD and NPLD ultrafiltrate samples, followed by IgG and fibrinogen chains, transferrin, α1-antitrypsin, ceruloplasmin and haptoglobin. Transferrin is one of the most abundant nitrated forms selectively removed by PLD. This is the most important pool of iron in the body with the highest turnover rate for this transition metal, and noteworthy dialysis patients show a characteristic tendency to develop disturbances of transferrinemia and impaired transferrin saturation (Canavese et al. 2004). Another target of nitration in uremic plasma associated with iron metabolism is ceruloplasmin, which was observed in ultrafiltrate samples from NPLD-treated patients, while only traces of this protein were observed in PLD ultrafiltrates. The nitration of this protein was already identified by immunoprecipitation and WB analysis of HD plasma in the pioneering study of Mitrogianni et al. (2004). These authors identified only this target as more extensively nitrated in HD patients than in healthy controls. Nitration of these two iron related proteins in uremic plasma might have implications for an abnormal metabolism of this transition metal, for uremic anemia and for a higher susceptibility to oxidative stress (Sezer et al. 2007). A defective iron metabolism increases also the susceptibility to infections and is a well recognized cause of impaired immune function in CKD and HD patients. These clinical traits are often associated with iron overload by massive iron therapy that is used to combat severe anemia in HD patients (Canavese et al. 2004).

NPLD were found to selectively remove α1-antitrypsin and IgG light chains, which are heavily represented in the nitroproteome of uremic plasma. On the contrary, IgG heavy chains, another main nitration target in uremic plasma, are not present in ultrafiltrate samples. Key components of complement pathways as the B and D factors have been observed in traces in the nitroproteome of NPLD ultrafiltrates. Complement factor B and D are part of the alternate pathway of the complement system that, together with complement factor 3b, generate the C3 or C5 convertase. Complement activation during HD reflects the scarce biocompatibility of the extracorporeal treatment and may have severe clinical consequences for these patients (Koller et al. 2004). Changes in IgG and complement factors in uremic plasma induced by HD therapy have been proposed to sustain leucopenia and neutrophil activation with mechanisms that involve oxidative stress (Koller et al. 2004; Kormoczi et al. 2001). Nitration of both IgG and complement factors has been investigated in vitro. IgG nitration results in a defective binding of C1q subcomponent of human complement (McCall and Easterbrook-Smith 1989), while nitration of C5a results in a lowered biological activity by reduced binding to granulocyte receptor (Johnson and Chenoweth 1985).

Noteworthy, IgG light chains, that are the second most represented nitroproteins in NPLD ultrafiltrates after albumin, are markedly increased in CKD serum being excreted and metabolized primarily by the kidney (Cohen and Horl 2009). These interfere with respiratory burst and apoptotic cell death mechanisms of neutrophils thus leading to defective cell-mediated immunity and impaired resolution of inflammation. The contribution of IgG nitration to IgG light chain accumulation and uremic toxicity remains unexplored.

As a key finding in this study, for the first time we have been able confirm by an unbiased approach that IgG chains are nitrated in vivo in uremic plasma. Using a chemoselective labeling procedure previously set up by some of us (Amoresano et al. 2007), nitropeptides corresponding to the lambda and heavy chains of uremic IgG were identified by bidimensional tandem mass spectrometry analysis and

nitration sites were specifically assigned. Further analyses are in progress to identify by means of this unbiased approach other nitrated proteins in plasma and a recent evolution of this procedure (Chiappetta et al. 2009) is expected to translate present qualitative data into quali-quantitative experiments.

In conclusion, with this study we provide the first extensive analysis of nitration targets in uremic plasma proteins carried out by immunorecognition and LC-MS/MS analysis. Since specificity of this qualitative investigation needs to be assessed for each protein target, in this study we developed a procedure of chemoselective labeling to obtain the unbiased identification of nitroproteins during bidimensional MS/MS of plasma proteins that are preliminarily screened and isolated by 2D PAGE and WB. This approach has been successfully applied for the first time to the identification of nitration sites in the human IgG molecule exposed to RNS in vivo, and we expect that it could be used in future large-scale proteomic studies to identify other plasma nitroproteins.

Moreover, the nitroproteome analysis of this study has revealed that uremic plasma shows an abnormal burden of nitration and the tentatively identified nitration targets suggest pathway and disease-specific events that deserve further investigation to be confirmed and to ascertain their clinical relevance with respect to immuno-inflammatory and cardiovascular symptoms of ESRD. Disease-specific differences in the relative abundance of nitrated forms cannot be excluded at present and will be further investigated using the chemoselective labeling and MS/MS analysis approach proposed in this study.

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