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Plasma protein carbonylation in chronic uremia

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Abstract

Background: **Carbonylation is an irreversible modification caused by the introduction into proteins of carbonyl derivatives (aldehydes and ketones), which can alter protein structure and function and lead to cellular dysfunction and tissue damage. Chronic uremia may be associated with an increased carbonyl overload ("carbonyl stress"), though carbonyl formation has been proposed so far for major plasma proteins only. In this study we looked for evidence and for the targets of plasma protein carbonylation in patients on hemodialysis. We also examined the effect of in vitro carbonylated albumin on mRNA levels of endothelial cell adhesion molecules involved in early atherogenesis.**

Methods: **Carbonylated proteins in uremic plasma were detected by a covalent hydrazine bait strategy and identified by combining electrophoretic separation with mass spectrometry analysis of tryptic digests. Some plasma samples were first depleted of albumin and immunoglobulins to improve detection of lower abundance proteins. The functional impact of carbonylation was assessed in human vein endothelial cells by studying models of modified human serum albumin.**

Results: **Post-dialysis plasma carbonylated protein levels were significantly increased compared to predialysis levels. Susceptibility to carbonyl formation was described on a open-platform investigation for a number of plasma proteins, albumin being the main scavenger of carbonyl reactive species. Incubation of endothelial cells with low doses of carbonylated albumin caused a significant increase in intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 mRNA levels.**

Conclusions: **Chronic uremia appears as a state of "carbonyl stress" targeting several different plasma proteins. Carbonylated albumin displayed biological effects that may be relevant to uremic atherosclerosis.**

Key words: *Albumin, Atherogenesis, Carbonylation, Hemodialysis, Proteomics, Uremia*

Introduction

Protein carbonylation is an irreversible, non-enzymatic modification which is caused by the introduction into proteins of carbonyl derivatives (aldehydes and ketones) generated from direct oxidation processes or from secondary protein reactions with reactive carbonyl compounds (RCOs). RCOs such as glyoxal and methylglyoxal, derived from both carbohydrates and lipids by oxidative and non-oxidative chemistry (1-3), are biologically active species which can react with proteins inducing structural and functional alterations (4, 5) and eventually form AGEs (advanced glycation end products) and ALEs (advanced lipoxidation end products) (6). A role of RCOs has been proposed in the ultrafiltration failure of patients treated with peritoneal dialysis (7, 8). Carbonylation of proteins is usually associated with permanent loss of protein function, which may

be the cause of subsequent cellular dysfunction and tissue damage (9). Increased levels of protein carbonyls found in several human pathological states suggest they may have a potential causative role in disease onset and/or progression (9, 10).

Chronic uremia may be characterized not only by an increase in oxidative stress, but also by a more generalized increase in "carbonyl stress" (carbonyl overload) resulting in chemical modifications of proteins and in accumulation of AGEs and ALEs in plasma and tissue proteins (5). The levels of both total RCOs (11) and isolated RCOs (11-13) have been found to be raised in uremic plasma, and a general increase in plasma protein carbonyls has been documented in patients with end-stage renal disease (ESRD) (14-17). Moreover, in blood from uremic patients, carbonyl formation has been suggested to occur in major plasma proteins such as albumin, fibrinogen and immunoglobulins (18, 19). Whether other less abundant plasma proteins are specific targets for carbonylation in uremia is at the moment unknown.

The present study was undertaken to examine via an open-platform proteomic investigation the evidence for plasma protein carbonylation and the targets of carbonylation in chronic uremia. Moreover, since in ESRD endothelium may be a key target for the action of circulating elements, such as modified plasma factors that may facilitate inflammation and the vasculopathy associated with uremia (20), we investigated the in vitro effect of the main carbonylated protein species, human serum albumin, on endothelial cell mRNA levels of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), membrane proteins that play a crucial role in the early atherosclerotic process (21).

Subjects and methods

Patients

This is an observational study examining protein carbonylation in 14 hemodialysis (HD) patients (7 women and 7 men, mean age 72±10 years, dialysis vintage 50 ± 25 months). Only clinically stable patients older than 18 years were included in the study. Patients with diabetes (to avoid the influence of diabetes per se on protein carbonyl content [16]), acute infection or blood transfusion in the past 3 months, smoking, unstabilized erythropoietin dosage, and a history of malignancy were excluded.

Patients were followed up for 3 consecutive months. Each month, blood was drawn before and after the first mid-week HD session on the occasion of the patient's routine monthly blood checks. After completion of the 3-month period, the plasma aliquots (1 per month) of each patient were pooled and analyzed. Patients were carefully informed about the use of drawn blood and gave written informed consent. The protocol was in conformity with the ethical guidelines of our institution, and the research was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Identification of carbonylated proteins

Sample preparation

Whole blood samples from uremic patients were collected in K_3EDTA vials (3 mL) and centrifuged at 4000 rpm for 20 minutes at 4°C. Plasma samples were stored at -80°C until analysis. The Sigma-Aldrich ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit was used for depletion of highly abundant plasma proteins, albumin and IgGs, to improve the detection of lower abundance proteins (22). Plasma depletion was carried out according to the manufacturer's instructions.

Protein quantification

Total protein quantification was done by the bicinchoninic acid assay (SIGMA). Samples were incubated at 60°C and using bovine serum albumin (BSA, SIGMA) standard for the calibration curve according to a protocol supplied by the vendor.

2,4-dinitrophenylhydrazine (DNPH) derivatization

Protein carbonyl groups in 10 µg of total proteins for each sample were derivatized with 10 mM DNPH in 2 N hydrochloric acid (1:4). The mixture was incubated at room temperature for 30 minutes in the dark with occasional mixing. Before derivatization, proteins were precipitated using 30% TCA in ice for 30 minutes followed by a centrifugation step at 13,000 rpm for 20 minutes to remove interfering substances. The pellet was then washed 3 times with an ice-cold solution containing 50% ethanol, 25% methanol, and 25% acetone and dried at room temperature (*c.* 15 min).

SDS-PAGE

Proteins were separated by SDS-PAGE in triplicate gels and then stained with silver nitrate (protein stain) or used for Western blot analysis (carbonyl signal).

Western blotting

After SDS-PAGE separation, proteins were electroblotted onto nitrocellulose membranes (0.22 µm, Protran; Whatman Schleicher & Schuell) in 25 mM Tris, 192 mM glycine and 20% v/v methanol transfer buffer according to Towbin (23). Protein transfer was carried out in an Amersham Bioscience transblot semidry transfer cell at 0.8 mA/cm^2 constant current for 2 hours. Immunodetection was performed as previously described (24), using anti-2,4-dinitrophenyl-keyhole limpet hemocyanin rabbit immunoglobulin G (Invitrogen, Molecular Probes; dilution 1:1000). The immunoreactive bands were detected using goat peroxidase-conjugated antirabbit immunoglobulin G (Santa Cruz Biotechnology; dilution 1:20,000) and a chemiluminescence detection method (Santa Cruz Biotechnology). The density of each lane was analyzed with the Gel Doc 2000 system (Bio-Rad) using Quantity One software (version 4.2.1, Bio-Rad). Total carbonylated protein levels were detected as optical density normalized by background subtraction.

Mass spectrometry (MS) analysis

Proteins resolved by SDS-PAGE analysis, as previously described, were stained with silver nitrate, a method performed without glutaraldehyde (25). Protein bands were excised from the gel, in-gel reduced, thiol-alkylated, and digested with sequence-grade porcine trypsin (SIGMA) (26) in 50 mM ammonium bicarbonate (SIGMA) at 37°C for 16-18 hours; the reaction was stopped by addition of 0.1% trifluoroacetic acid (Fluka). A control piece of gel was cut from a blank region of the gel and processed in parallel with the samples.

Protein identification was carried out by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/MS mass fingerprinting. A microcrystalline matrix surface was made by spotting a saturated solution of α-cyano-4-hydroxycinnamic acid in ethanol (SIGMA) onto a ground steel MALDI plate. Tryptic peptides were extracted by ZipTip C18 (Millipore) reverse-phase material and directly eluted and crystallized in an acetonitrile/water 1:1 (v/v) saturated solution of α-cyano-4-hydroxycinnamic acid, onto the first matrix surface. The solvent was allowed to evaporate at room temperature.

MALDI mass spectra were recorded in the positive ion mode with delayed extraction on a Reflex IV time-of-flight instrument equipped with an MTP multiprobe inlet and a 337-nm nitrogen laser. A 50 pmol/µL standard peptide mix solution of angiotensin I (1296.68 Da), ACTH 18-39 (2465.19 Da), bradykinin (1060.57 Da), [Glu1]-fibrinopeptide B (1570.68 Da) and renin substrate (1758.93 Da) was used for external calibration. Internal spectrum calibration was performed by a 3-point linear fit using the autolysis products of trypsin at m/z 842.50, m/z 1045.56 and m/z 2211.10.

A database search with the monoisotopic peptide masses from mass fingerprinting peptide experiments was performed against the National Center for Biotechnology Information (NCBI) nonredundant database using the peptide search algorithm MASCOT (Matrix Science, http://www.matrixscience.com). The parameters employed in the database search were: mass tolerance of 100 ppm, single miss cleavage site per peptide fragment, carboamidomethyl modification of cysteine residues and optional presence of methionine oxidation. Masses corresponding to keratin tryptic fragments or evaluated as environmental contaminants by specific blank controls were excluded.

Protein identification by liquid chromatography (LC)-peptide fragmentation analysis was performed on extracted peptide containing injected solutions (6 µL) using a CapLC system (Micromass, Waters) coupled online with a nano-ESI-Q-TOF instrument (Micromass, Waters). Data were analyzed using a MASCOT MS/MS ion search engine (Matrix Science) with the NCBInr database. The query was restricted to human proteins, the maximal tolerance for peptide masses was 50 ppm and the maximal tolerance for MS/MS data was 0.2 Da, searching peptide charges of 2+ and 3+. Peptide modifications were defined as previously reported for mass fingerprint analysis.

Endothelial cell studies

Preparation of carbonylated HSA

Human serum albumin (HSA, 1.5 mmol/L, Sigma) was carbonylated as previously reported (27). The supernatant was removed and protein concentrated with extensive washes in a centrifugal filter with a 3 kDa cutoff (Microcon YM-3, Millipore). The HSA control (noncarbonylated HSA) was subjected to the same procedure, except for the presence of glucose in the incubating solution. To verify carbonylation of HSA, SDS-PAGE and Western blot experiments were carried out on control HSA and carbonylated HSA after incubation. Samples were derivatized with DNPH and immunodetected with anti-DNPH antibody. The concentration of carbonylated HSA and control HSA was determined by Bradford's protein measurement method.

Preparation of endothelial cells

Umbilical cords were obtained from randomly selected healthy mothers giving birth at Chieti University Hospital. Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained as previously described (28). After perfusion of umbilical cords with 0.1% collagenase at 37°C, HUVECs were grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium supplemented with 20% fetal calf serum, 10 µg/mL heparin, and 50 µg/mL endothelial cell growth factor (complete medium). In all experiments cells were used between the third and sixth passage in vitro.

Experimental protocol

For real-time PCR experiments, HUVECs were plated in T75 flasks (75 cm^2) (1.75 x 10^5 cells mL^{-1}) in complete M199 endothelial growth medium, and grown to confluence. After 72 hours, the HUVEC monolayer was washed twice with phosphate-buffered saline to remove traces of serum, and incubated for 20 hours with DMEM/F12 with 1% fetal calf serum (basal) for starvation. Then HUVECs were stimulated for 6 hours with fresh HSA (1-25 mg/L), carbonylated HSA (1-25 mg/L), noncarbonylated HSA (1-25 mg/L), or TNF-α (1 ng/mL) as control in basal medium.

VCAM-1, ICAM-1, and endothelial nitric oxide synthase (eNOS) mRNA quantification by real-time PCR

Total RNA was extracted from the HUVECs using ABI Prism 6100 Nucleic Acid PrepStation (PE Applied Biosystems, Foster City, CA, USA) according to manufacturer recommendations and quantified by spectrophotometric reading at 260 nm. One microgram total RNA was used for the synthesis of first-strand cDNA (High-Capacity cDNA Archive Kit, PE Applied Biosystems). The real-time PCR reactions were optimized for VCAM-1, ICAM-1, and eNOS mRNA quantitation with specific primers and probes using TaqMan Technology (PE Applied Biosystems), relative to 18s rRNA level, the validated endogenous control gene. The real-time PCR experiments were run on Sequence Detector ABI PRISM 7900HT and data were recorded using Sequence Detector Software (SDS version 2.1, PE Applied Biosystems).

Statistical analyses

Data were normally distributed and were analyzed by *t*-test. P values <0.05 were considered statistically significant. The results are presented as mean ± SD.

Results

Detection and identification of carbonylated proteins in uremic plasma

Carbonylation of plasma proteins was detected by the reaction of carbonyl groups with DNPH to form hydrazones, which are detected by the use of antibodies against DNP (2,4-dinitrohydrazones). Immunoblotting analysis with anti-DNP was performed on plasma samples obtained from 14 chronic HD patients both before and after the dialytic procedure, each patient being evaluated independently in triplicate.

Figure 1A shows representative Western blots from an HD patient: it can be appreciated that there is considerably more carbonyl detected after the dialysis procedure. Figure 1B analyzes the extent of plasma protein carbonyl formation before and after the HD procedure in the whole group of 14 ESRD patients. In post-dialysis samples, levels of total carbonylated plasma proteins proved to be significantly increased as compared to pre-dialysis values ($p<0.02$).

Excised bands from silver-stained SDS-PAGE were used to identify uremic plasma protein targets of carbonylation, detected by Western blot before (Fig. 2, panel A) and after (Fig. 2, panel B) depletion of albumin and immunoglobulins. The representative Western blot image shows, in all lanes, some bands of carbonylated proteins in the range of high molecular weight between 200 kDa and 40 kDa. In particular, the intensity of 3 bands appearing at about 40-80 kDa was strong and discernible in both uremic plasma samples. In the low molecular weight range (about 30 kDa) there was 1 band only.

Tables I and II show the carbonylated proteins identified by MALDI-TOF/MS mass fingerprinting and nanoLC-MS/MS analysis. The latter was coupled to an additional separation strategy by nano high performance LC in order to further reduce the molecular complexity of the samples before fragmentation sequencing. The slice number corresponds to the band number in Figure 2. The database search with the monoisotopic peptide masses was performed against the NCBI nonredundant database using the peptide search algorithm MASCOT (Matrix Science) with an ions score of -10*Log(P), where P is the probability that the observed match is a random event, and protein scores greater than 64 were considered significant ($p<0.05$) for peptide mass fingerprinting analysis while individual ions scores >39 were considered significant

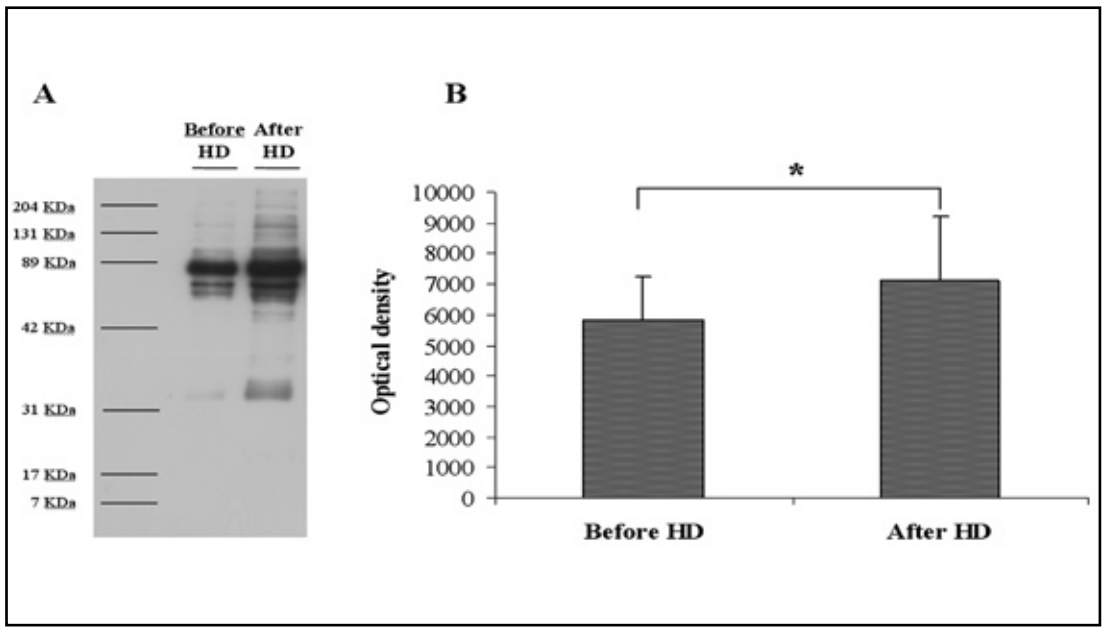

Fig. 1 - A) A representative Western blot analysis of DNP-modified carbonylated proteins in plasma samples obtained before and after hemodialysis in an ESRD patient. The standard protein molecular weights shown are those used in SDS-PAGE. B) Levels of total carbonylated proteins, expressed as optical density, in plasma obtained before and after hemodialysis in the whole group of 14 ESRD patients. *p<0.02 vs. before HD.

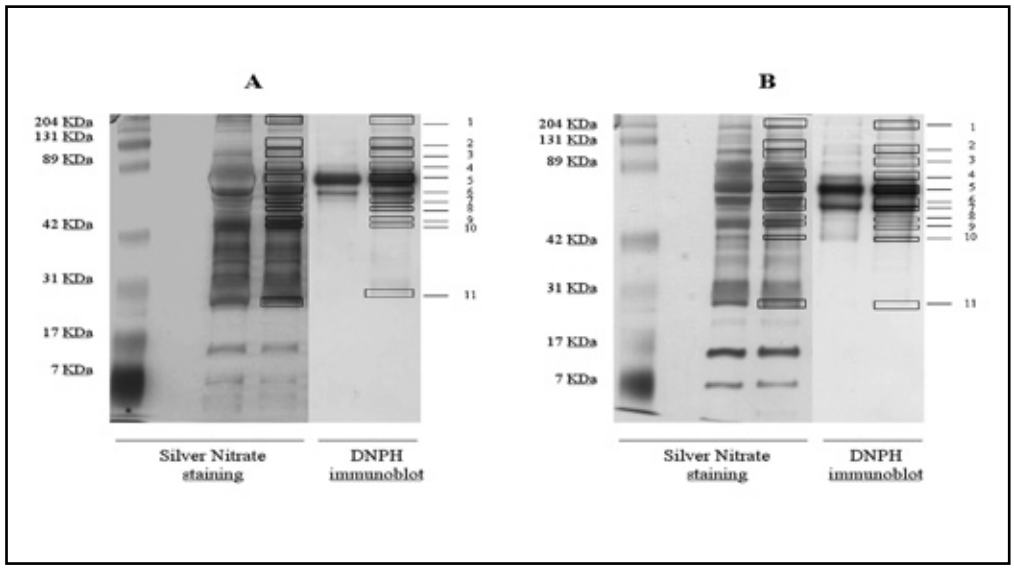

Fig. 2 - Excised bands from a representative silver stained 1-dimensional SDS-polyacrylamide gel used to identify carbonylated proteins detected by Western blots in DNPH-derivatized whole uremic plasma A) and in albumin- and immunoglobulin-depleted uremic plasma B).

(p<0.05) for peptide fragmentation analysis.

In Table I, peptide mass fingerprinting (A) and peptide fragmentation (B) analyses show that the bands present in 1-dimensional SDS-PAGE, obtained from uremic plasma without albumin and immunoglobulin depletion, contained polypeptide species from the following gene products: α2-macroglobulin; transferrin; serum albumin; 1 mixture of α1-antitrypsin-serum albumin; immunoglobulin gamma 1; fibrinogen gamma; 1 hypothetical albumin fragment and proapolipoprotein. Moreover, in these experiments serum albumin was identified in multiple bands that were DNPH-positive in Western blot analysis. Bands 2 and 3 were not identified in these experiments while they were identified, after albumin and immunoglobulin depletion, as a ceruloplasmin precursor (Tab.IIA, B) and plasminogen (Tab. IIA), respectively.

MALDI-TOF/MS mass fingerprinting and nanoLC-ESI-Q-TOF-MS/MS identification of carbonylated proteins in depleted plasma confirmed the presence of polypeptides having an extensive sequence overlap with α2-macroglobulin, transferrin, serum albumin and proapolipoprotein (Tab. IIA), or apolipoprotein A-I (Tab. IIB). Band 10 shown in Figure 2, panel A, identified as a probable serum albumin fragment (Tab. IA, B), was not revealed

TABLE I
(A) MALDI-TOF/MS MASS FINGERPRINTING AND (B) nanoLC-nano-ESI-Q-TOF MS/MS IDENTIFICATION OF CARBONYLATED PROTEINS FROM UREMIC PLASMA

A.

Excised slices	Score	Matched peptides	Sequence coverage	# NBCI	Name	MW (Da)
1	159	25	29%	224053	α2-Macroglobulin	162072
2					Not identified	
3					Not identified	
4	78	14	23%	37747855	Transferrin	79310
5	168	18	34%	23307793	Serum albumin	71344
6	100	16	46%	157831596	Chain A α1-antitrypsin	44280
	193	23	41%	23307793	Serum albumin	71344
7	71	12	25%	23307793	Serum albumin	71344
8	96	11	33%	49065860	Immunoglobulin heavy constant gamma 1-like protein	52544
9	94	12	49%	182439	Fibrinogen gamma chain	50077
10	79	13	29%	23307793	Serum albumin	71344

B.

Excised slices	Score	Matched peptides	Sequence coverage	# NBCI	Name	MW (Da)
1	330	10	6%	224053	α2-Macroglobulin	162072
2					Not identified	
3					Not identified	
4	420	11	15%	37747855	Transferrin	79310
5	130	4	5%	23307793	Serum albumin	71344
6	359	10	28%	157831596	Chain A α1-antitrypsin	44280
	429	13	19%	23307793	Serum albumin	71344
7	182	5	10%	2765425	Immunoglobulin lambda heavy chain	53379
8	142	3	8%	49065860	Immunoglobulin heavy constant gamma 1-like protein	52544
9	170	6	12%	182439	Fibrinogen gamma chain	50077
10	132	4	6%	28592	Serum albumin	71316
11	317	8	30%	178775	Proapolipoprotein	28944

TABLE II
(A) MALDI-TOF/MS MASS FINGERPRINTING AND (B) nanoLC-nano-ESI-Q-TOF MS/MS IDENTIFICATION OF CARBONYLATED PROTEINS FROM ALBUMIN- AND IMMUNOGLOBULIN-DEPLETED UREMIC PLASMA

A.

Excised slices	Score	Matched peptides	Sequence coverage	# NBCI	Name	MW (Da)
1	174	26	28%	224053	α2-Macroglobulin	162072
2	91	16	23%	4557485	Ceruloplasmin precursor	122983
3	72	13	27%	4505881	Plasminogen	93247
4	81	11	17%	37747855	Transferrin	79310
5	135	18	33%	23307793	Serum albumin	71344
6	159	14	39%	157831596	Chain A α1-antitrypsin	44280
7	79	11	50%	47124562	HP protein	31647
8	78	9	34%	49065860	Immunoglobulin heavy constant gamma 1-like protein	52544
9					Not identified	
10d					Not identified	
11	127	12	49%	178775	Proapolipoprotein	28944

B.

Excised slices	Score	Matched peptides	Sequence coverage	# NBCI	Name	MW (Da)
1	937	23	17%	46812315	α2-Macroglobulin	167505
2	443	11	11%	4557485	Ceruloplasmin precursor	122983
3					Not identified	
4	637	16	26%	37747855	Transferrin	79310
5	228	10	14%	23307793	Serum albumin	71344
6	44	1	2%	157831596	Chain A α1-antitrypsin	44280
7	94	2	7%	47124562	HP protein	31647
8	204	4	9%	49065860	Immunoglobulin heavy constant gamma 1-like protein	52544
9					Not identified	
10d					Not identified	
11	363	11	49%	90108664	Chain A apolipoprotein A-I	28061

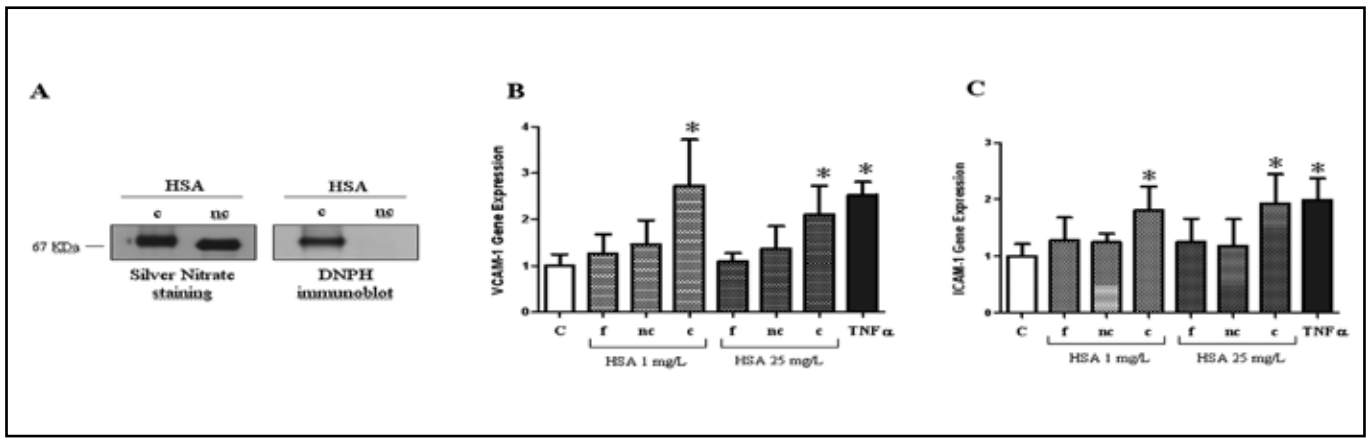

Fig. 3 - A) Silver-stained SDS-PAGE and Western blot of carbonylated HSA (c-HSA) and noncarbonylated HSA (nc-HSA) showing carbonylation of HSA. B-C) Real-time PCR evaluation of VCAM-1 and ICAM-1 gene expression. Real-time PCR analysis of VCAM-1 (B) and ICAM-1 (C) mRNA levels, relative to 18s mRNA levels, in HUVECs stimulated for 6 hours with fresh HSA (f-HSA), nc-HSA or c-HSA at 1 or 25 mg/L concentration, and expressed vs. basal (unstimulated). TNF-α (1 ng/mL) was used as a positive control. Bar plots represent the mean ± SD of 3 independent real-time PCR experiments (*p<0.05 vs. C).

after albumin and immunoglobulin depletion (Fig. 2, panel B). Furthermore, in both peptide mass fingerprinting and nanoLC-MS/MS analysis (Tab. IIA, B), α1-antitrypsin and haptoglobin (HP) were assigned without any albumin and immunoglobulin contamination and bands 9 and 10d were not identified, probably due to a low protein concentration. The presence of immunoglobulin chains after depletion might be due to the partial efficacy of the affinity-capturing material, as already reported (22), or to a loss of protein structure due to the high degree of carbonyl damage present in the polypeptide structure.

Effect of carbonylated albumin on endothelial adhesion molecule mRNA levels

Figure 3 panel A shows SDS-PAGE and Western blotting of carbonylated HSA and noncarbonylated HSA samples, which demonstrate carbonylation of HSA. Figure 3 panels B and C show that carbonylated HSA (1 and 25 mg/L) significantly increased VCAM-1 and ICAM-1 gene expression levels ($p<0.05$, respectively). By contrast, in the same experimental conditions, eNOS gene expression was not modified (data not shown). As expected, the positive control TNFα (1 ng/mL) significantly increased both VCAM-1 and ICAM-1 gene expression levels ($p<0.05$ for each, Fig. 3) and decreased eNOS expression ($p<0.05$, data not shown). No significant effect on VCAM-1 and ICAM-1 mRNA levels was observed when HUVECs were cultured with noncarbonylated HSA (Fig. 3, panels B and C).

Discussion

The presented data provide some novel features regarding the process of protein carbonylation in uremia. The experimental techniques performed (SDS-PAGE, Western blotting, peptide mass fingerprinting, and peptide nanoLC-MS/MS analysis) allowed us to detect carbonyl compounds in plasma from stabilized nondiabetic HD patients and to identify the main protein targets of carbonyl stress. We also used a molecular reduction strategy in order to reduce sample complexity by first applying a depletion strategy of the main carbonyl scavengers and then coupling an additional high performance separation by reverse-phase nanoLC. Our results are the first to show that several different plasma proteins may be targets for carbonylation in uremia. Furthermore, the in vitro finding that even low concentrations of carbonylated albumin may have proatherogenic properties indicates a potential role for protein carbonylation in the complex pathophysiology of the uremic syndrome.

Carbonyl stress in chronic uremia, as documented by several studies (5, 14-19) including the present one, may be the consequence of 2 competing but not mutually exclusive mechanisms: increased generation or decreased removal (clearance or detoxification) of RCOs. Uremia-associated oxidative stress (29) could modify proteins either directly by reactive oxygen species or indirectly by RCOs generated by the autoxidation of a variety of sources (5). The buildup of RCOs in uremia might also be accounted for by a decrease in their renal clearance (30). In addition, since RCOs react reversibly with the thiol group of glutathione and are

subsequently detoxified, the significant decrease in both erythrocyte glutathione concentration and serum activity of glutathione-dependent enzymes found in uremia (31, 32) could result in increased levels of RCOs.

While it appears that carbonyl stress may result from uremia per se (17, 18), the present study supports the concept (14-16, 30, 33) that HD treatment may adversely affect the carbonyl balance and exacerbate carbonyl stress. Our data show a significant increase in plasma total carbonylated protein levels after the dialysis procedure. During hemodialysis there are several conditions that may all contribute, singly or in concert, to increase carbonyl stress: the absence of complete correction of uremic toxicity, bioincompatible reactions following the contact of blood with the dialyzer membrane leading, among others, to increased oxygen radical production (34, 35), reduced activities of antioxidants (16), and possible increased generation of RCOs (36). Identification of the exact biological mechanisms involved in HD-induced carbonylation of some plasma proteins was, however, outside the scope of the present investigation and requires further studies. Such studies might help in identifying those dialysis strategies that best antagonize the carbonyl overload in this patient population.

Previous studies in uremic patients have demonstrated the susceptibility of major plasma proteins to carbonyl formation (18, 19). Our data are therefore confirmatory of those findings, providing molecular detection by peptide MS experiments. In particular, albumin acts as a key scavenger of carbonyl reactive species, as attested by the fact that multiple isoforms of HSA were found to be reactive to the DNPH bait. Such a buffering role of HSA has already been reported for the redox stress response in plasma from patients suffering from primary nephritic syndromes (37), although the functional impact of the chemically injured HSA isoform has not yet been elucidated.

However, since the relative amount of a protein is not a factor determining the degree of carbonylation (38), we also examined whether less abundant plasma proteins are targets for carbonylation in uremia. To address this issue, plasma samples obtained from HD patients were depleted of albumin and immunoglobulins, and then analyzed again by the same proteomic approach. Our results indicate that several different plasma proteins may be carbonylated in chronic uremia (Tab. II), which extends previous information. Our study suggests that in uremia carbonylation could be a selective process involving also proteins present in blood in small amounts. The susceptibility of proteins to oxidation might be determined by their metal-binding sites or structural characteristics (39). Target proteins of carbonylation in uremia such as transferrin, albumin and ceruloplasmin contain 8 iron ion binding sites, and 1 and 12 copper ion binding sites, respectively (39). Different sensitivity to carbonylation might also depend on pathogenetic factors that make proteins more susceptible to oxidation in certain microenvironments (40).

Carbonylation can cause several different protein modifications, every one of which may produce (or not) a specific effect on the biological activity of different proteins (9). Carbonylation of haptoglobin and ceruloplasmin, as we detected in uremic blood, can impair the antioxidant protective properties of those proteins (41, 42). It has been shown that carbonyl stress (AGE)-modified proteins may exhibit several biological activities initiating a range of inflammatory responses (reviewed in 5). Furthermore, the interaction of reactive carbonyl compounds (either free or protein bound) with cell surface membrane proteins may induce intracellular responses (43, 44), triggered by signaling pathways involving $P21^{RAS}$, mitogen-activated protein kinases, and nuclear factor-κB (43).

Although the pathophysiological significance of protein carbonylation in uremia remains to be definitively established, carbonyl stress may be relevant to various complications of chronic renal failure (6, 45). Dialysis-associated amyloidosis, in particular, has been related to carbonyl stress (5, 6). It is thought that carbonylation of fibrinogen may contribute to the impaired clotting activity observed in HD patients (19). Accumulation of AGEs may be an important pathogenetic factor for low bone turnover in dialysis patients (46). Moreover, several studies have demonstrated that oxidative alteration of albumin may adversely affect its vasculoprotective effects on ESRD patients (18, 47). In addition, there is circumstantial support for the hypothesis that carbonyl stress is involved in the pathogenesis of alterations in left ventricular geometry and function in these patients (45).

Atherosclerosis is another potential target of carbonylation in uremia (6). Patients suffering from ESRD experience accelerated atherosclerosis (48), and atherosclerotic cardiovascular events account for a large proportion of the morbidity and mortality in these patients (49). It is commonly accepted that endothelial cell injury including increased expression of the endothelial adhesion molecules ICAM-1 and VCAM-1, which support firm leukocyte adhesion and transmigration into stimulated endothelium, is an initial event in atherosclerosis (21). The blood concentrations of VCAM-1 and ICAM-1, which may be shed from activated endothelial cells into the circulation, are increased in patients with renal failure (50), and experimental models have demonstrated the involvement of these molecules in uremic atherosclerosis (51). However, just how uremia induces a proinflammatory state leading to increased endothelial

adhesion molecule expression and hence accelerated atherosclerosis has not been fully elucidated.

Our study supports the notion that carbonylated albumin may directly damage the endothelium, contributing to the loss of endothelial function. The results obtained in cultured HUVECs demonstrated that low doses of carbonylated albumin significantly increased the mRNA levels of the endothelial adhesion molecules in vitro, indicating that protein carbonylation processes may play a major role in the early atherogenic events of chronic uremia. On the other hand, in our cell model eNOS mRNA levels were not influenced by exposure to carbonylated albumin, indicating that the observed effect of carbonylated albumin on endothelial adhesion molecule expression may be independent of nitric oxide availability (20).

There are many possible applications for proteomic approaches to issues related to nephrology. A recent study using proteomics revealed significant differences in the platelet expression of some proteins between dysfunctional uremic platelets and uremic platelets with normal functionality (52). The data presented here, obtained by means of proteomic techniques, provide further evidence of protein damage in uremia (53) by showing carbonyl formation in several plasma proteins, and support the view that carbonyl stress may be a contributor to uremic toxicity (6), particularly uremic accelerated atherosclerosis. Further research is required to identify carbonylating mechanisms and develop effective therapeutic strategies to reduce the carbonyl overload in uremia. Interventions targeting protein carbonylation may have the potential to prevent vascular lesions under uremic conditions, a concept which requires further investigation.

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