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Original Contribution

Plasma protein thiolation index (PTI) as a biomarker of thiol-specific oxidative stress in haemodialyzed patients

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ABSTRACT

The role of oxidative stress in patients with end stage renal disease (ESRD), which occurs at significantly higher levels than in the general population, is often underestimated in clinical practice. Emerging evidence highlights the strong correlation of oxidative stress with chronic inflammation and cardiovascular disease, which are highly prevalent in most patients on maintenance haemodialysis (HD) and are a major risk factor for mortality in this population. In this study, total plasma thiols and plasma S-thiolated proteins were measured in patients with ESRD, before and after a regular HD session, and compared to age-matched healthy subjects. We found a significant decrease in the level of total plasma thiols and, conversely, a significant increase in the level of S-thiolated proteins in these patients. In most patients, post-HD plasma level of total thiols did not differ from the one in healthy subjects, whereas plasma level of S-thiolated proteins was lower in HD patients than in age-matched healthy controls. This suggests that a single HD session restores plasma thiol redox status and re-establishes the antioxidant capacity of plasma thiols. Additionally, we determined protein thiolation index (PTI), i.e., the molar ratio between the sum of all low molecular mass thiols bound to S-thiolated plasma proteins and protein free cysteinyl residues. Patients with ESRD had a significantly higher PTI compared to age-matched healthy subjects and HD was associated with a decrease in PTI to normal, or lower than normal, levels. Although this study is limited in size, our results suggest that PTI is a useful indicator of thiol-specific oxidative stress in patients with ESRD on maintenance HD. This study also emphasizes that PTI determination is a cheap and simple tool suitable for large-scale clinical studies that could be used for routine screening of thiol-specific oxidative stress.

1. Introduction

Patients with chronic kidney disease (CKD) and, more markedly, those with end stage renal disease (ESRD, i.e. CKD stage V), show a significantly increased oxidative stress, which may results from uraemia per se and inflammation [1–5]. Indeed, the strong association between renal dysfunction and different mediators/biomarkers of inflammation suggests that CKD is a low-grade inflammatory process in itself [6–10]. Other pro-oxidant conditions such as ageing, dyslipidemia, hypertension, diabetes mellitus, obesity and infectious complications, which are commonly present in patients with CKD stages I-IV (i.e., patients with different degrees of CKD but with residual renal function) and are even more dominant in patients with ESRD, can further aggravate the oxidative stress status [11–15]. Particularly in patients with ESRD on haemodialysis (HD), both acute-phase inflammation and elevated levels of oxidative stress, besides accelerated atherogenesis, dyslipidemia, and endothelial dysfunction, are associated with a high rate of cardiovascular morbidity and hospitalization [16–18]. These risk factors are associated with a 10- to 100-fold increase in

Abbreviations: AOPP, advanced oxidation protein products; CKD, chronic kidney disease; CVD, cardiovascular disease; CysGly, cysteinylglycine; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; ECL, enhanced chemiluminescence; ESRD, end stage renal disease; GSH, glutathione; GSSG, glutathione disulphide; Hcy, homocysteine; HD, haemodialysis; HRP, horseradish peroxidase; IAA, iodoacetic acid; LMM-SH, low molecular mass thiols; NEM, *N*-ethylmaleimide; PBS, potassium phosphate buffer; PSH, protein thiols; PSSX, S-thiolated proteins; PTI, protein thiolation index; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

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cardiovascular and all-cause mortality when compared to age-matched controls [10]. Furthermore, patients with CKD frequently present with lower levels of water-soluble antioxidant vitamins owing to their dietary restriction of fresh fruits and vegetables to avoid hyperkalaemia [2,19,20]. In particular, non-supplemented maintenance HD patients are at high risk for vitamin C deficiency [2,21,22] and a low plasma vitamin C level predicts fatal and major non-fatal adverse cardiovascular events among maintenance HD patients [21,22]. Moreover, the administration of intravenous iron to correct anaemia, a common finding in ESRD population, can further aggravate oxidative stress in these patients [23–25]. In addition, reactive oxygen species (ROS) released by peripheral polymorphonuclear leukocytes and monocytes during the recurrent contact of blood with dialysis membranes aggravates the already existing oxidative stress [2]. Taken together, these studies strongly suggest a prominent role of oxidative stress in haemodialyzed patients.

Information on the occurrence of oxidative stress in humans derives, in most cases, from measurements carried out on blood or plasma with the assumption that any alteration of the hematic biomarkers should reflect the one occurring in other less accessible tissues. A series of biomarkers of oxidative stress were measured in HD patients, including the increased plasma level of carbonylated proteins [26] and advanced oxidation protein products (AOPP) [27,28]. Blood levels of both high molecular mass thiols (i.e., protein thiols, PSH) and low molecular mass thiols (LMM-SH), namely homocysteine (Hcy), cysteine (Cys), cysteinylglycine (CysGly), and glutathione (GSH), are frequently measured as biomarkers of oxidative stress [29]. PSH are also present as mixed disulphides with LMM-SH, as a whole referred to as S-thiolated proteins (PSSX) [30,31]. S-thiolation plays both a regulatory and an antioxidant role, because it protects PSH against irreversible oxidation [32,33]. Alteration in plasma levels of LMM-SH [34,35], decreased plasma PSH [26,36], increased S-thiolated and homocysteinylated plasma proteins [37–39], and cysteinylated albumin [40,41] highlighted an oxidative shift in the plasma thiol redox status in patients with ESRD. The precise and accurate measurement of these thiol-specific oxidative stress biomarkers requires HPLC and/or mass spectrometry methods, as well as time-consuming procedures and skilled personnel. Therefore, they are difficult to apply in large-scale clinical studies. A recently published spectrophotometric method suggests that it is possible to rapidly assess oxidative perturbations in the thiol redox status measuring simultaneously PSH and PSSX, obtaining the Protein Thiolation Index (PTI) [42]. Specifically, PTI=$[\text{tLMM-SH}]_{PSSX}/[PSH]$, where $[\text{tLMM-SH}]_{PSSX}$ is the concentration of all LMM-SH (i.e., chiefly GSH, Cys, Hcy, and CysGly) released by all types of PSSX under reducing conditions and [PSH] is the concentration of protein free cysteinyl residues. The PTI was validated and applied to the plasma of both healthy humans and subjects affected by pathologies associated with increased oxidative stress. The PTI showed an age dependency with a near linear increase during ageing in healthy humans and was significantly higher in patients suffering from alkaptonuria (a genetically recessive metabolic disease associated with oxidative stress) [42].

The purpose of the present study was to determine the plasma thiol-specific oxidative stress in patients with ESRD, before and after HD, compared to age-matched healthy subjects. Thiol-specific oxidative stress was measured as total plasma thiols, S-thiolated plasma proteins, and PTI.

Table 1
Characteristics of study group. Data are expressed as mean ± SD.

	Haemodialyzed patients (n=20)
Age (years)	70.0 ± 11.7
Sex	12M, 8F (1.5:1)
Dialysis Vintage (years)	4.4 ± 1.9
CRP[a] (mg/dL)	0.50 ± 0.39
Albumin (g/dL)	3.47 ± 0.36
Fibrinogen (mg/dL)	375.2 ± 77.8
White Blood Cells (cells/mm^3)	7286.7 ± 1476.3
Haemoglobin (g/dL)	10.7 ± 1.0
Urea (mg/dL)	158.0 ± 43.9
Creatinine (mg/dL)	8.9 ± 2.0
Sodium (mmol/L)	137.1 ± 2.76
Potassium (mmol/L)	5.2 ± 0.7
Calcium (mmol/L)	2.2 ± 0.2
Phosphorus (mmol/L)	1.6 ± 0.4
Ferritin (ng/mL)	240.1 ± 139.9
TIBC[b] (g/L)	180.8 ± 24.5

[a] CRP=C-reactive protein
[b] TIBC=total iron-binding capacity.

2. Materials and methods

2.1. Chemicals

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB; product code D8130), biotin-maleimide (N-biotinoyl-N′-(6-maleimidohexanoyl) hydrazide; product code B1267), dithiothreitol (DTT; product code D0632), *N*-ethylmaleimide (NEM; product code 04260), iodoacetic acid (IAA; product code I4386), ninhydrin (product code 33437) and hydrindantin (product code H17309) were purchased from Sigma-Aldrich (Milan, Italy). Horseradish peroxidase (HRP)-conjugated streptavidin (product code RPN1051) was purchased from GE Healthcare (Milan, Italy). All other reagents were of analytical grade (Sigma-Aldrich, Milan, Italy).

2.2. Study participants

All the patients enrolled in the study belong to stage V of CKD and are referred to as ESRD patients. These patients don't have a residual renal function and thus require renal replacement therapy. Blood samples were collected after informed written consent was obtained from ESRD patients on maintenance HD at the Nephrology Unit of the Humanitas Clinical and Research Center (Rozzano, Milan, Italy). The presence of a clinically overt infectious process was the only exclusion criteria. For every patient an anamnestic record was collected. A de-identification of the samples was performed before any additional data processing. Twenty haemodialyzed patients were recruited in the study (Table 1). Control blood samples were collected from 20 age-matched voluntary healthy donors at the Analysis Laboratory of the University of Milan (Laboratorio Analisi Università di Milano), after obtaining informed verbal consent. Criteria included no known history of CKD or other diseases that could influence the analysis. In particular, healthy subjects were tested for serum creatinine in order to exclude CKD.

2.3. Sample collection

From HD patients, venous blood samples of 10 ml were collected before HD and 5 ml were obtained after the same session. All samples were collected on the long inter-dialytic interval, i.e. two days apart from the previous HD session. Blood was withdrawn from the arteriovenous fistula or central venous catheter. K_3EDTA was used as anticoagulant in all the blood samples. From healthy donors, 10 ml of venous blood was collected from the antecubital vein and treated with K_3EDTA. All the samples were processed within the first hour from blood withdrawal through centrifugation for 10 min at 1000 *g*, obtaining pre- and post-dialysis plasma aliquots from haemodialyzed patients and plasma