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OXIDATIVE MODIFICATIONS OF PLASMA PROTEINS IN DIFFERENT STAGES OF CHRONIC RENAL FAILURE

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Summary. *In this study we investigated oxidative modifications of plasma proteins in non-dialyzed patients with varying degrees of chronic renal failure (CRF), and in patients on regular haemodialysis treatment. The results presented here show that level of 2,4 dinitrophenylhydrazone-reactive carbonyl derivatives (CRD), as a marker of oxidative modification of proteins, increased in plasma of all chronic renal failure patients compared to healthy subjects. Statistically significant positive correlation between the serum creatinine level and plasma CRD content in CRF was observed. The concentration of protein sulphydryl groups (P-SH) in plasma, which are important chain breaking "sacrificial" antioxidant, were markedly reduced in individuals with CRF compared to healthy subjects. However, the degree of renal impairment does not appear to influence plasma P-SH in our data. The results presented support the statement that in chronic renal failure accumulation of free radical species and an unscheduled oxidation of susceptible protein injury occurs. Thus, chronic renal failure appears to a state of "carbonyl stress" with potentially damaging proteins. It was suggested that carbonyl overload in CRF might contribute to the development of long-term uraemic complications, such as atherosclerosis, aging and amyloidosis.*

Key words: *Chronic renal failure, reactive carbonyl derivatives, thiol groups, oxidative stress, free radicals*

Introduction

There now exists a vast array of biological processes in which free radicals have been implicated (1). The implication of free radicals in biological macromolecules damage is more and more focused as an important phenomena. These modifications take place in a lot of chronic diseases such as diabetes mellitus, cancer or chronic renal failure (1). Exposure of lipids to free radicals and the subsequent lipid peroxidation products, leads to vascular lesions or accelerated aging and inflammation (2,3). Besides, the protein molecules may also be attacked whenever free radicals are generated (4, 5). All the constituent amino acid side chains in proteins are susceptible to attack by oxidants and free radicals, and carbonyl groups (aldehydes and ketones) may be introduced into proteins by any of these reactions (4-6). Thus, appearance of such carbonyl groups is taken as presumptive evidence of oxidative modification, and assay of carbonyl groups in proteins provides a convenient technique for detecting and quantifying oxidative modification of proteins (7).

Chronic renal failure is accompanied by very complex long-term manifestations such as accelerated aging, atherosclerosis, heart disease, polyneuropathies and amyloidosis (8), which may be related to the hyperproduction of free radicals. That reactive oxygen species are involved in progressive renal injury, is supported by several lines of evidence. In a number of studies, an increased production of reactive oxygen species (9,10) and lipid peroxidation (11-14) in plasma and red blood cells of haemodialyzed chronic renal failure (CRF) patients has been reported. An imbalance in the activity of extracellular and intracellular antioxidant enzymes in chronic renal failure patients has been reported (14,15). Also, we found that the degree of intracellular and extracellular oxidative stress is related to the severity of renal failure (14,15). According to these results it seems reasonable to assume that the accumulation of free radical species in chronic renal failure may results in unscheduled oxidation of protein substituents.

It is generally believed that blood plasma is exposed to more severe oxidative stress than intracellular fluids. The level of antioxidant enzymes is much lower than in the intracellular space (1). Sulfhydryl groups of serum proteins, including serum albumin, have been suggested to be a "sacrificial" antioxidant in plasma and extravascular spaces (1). Recently, in the search for a more accurate marker of oxidative stress in end-stage renal disease (ESRD) patients, Witko et al. (16) have demonstrated the presence of oxidatively damaged protein products in plasma, designated as "advanced oxidation protein products". These products were found at very high concentrations in plasma of dialysis patients as well as in patients in preterminal renal failure, not yet on dialysis (17). However, despite considerable interest in this area, major questions still remain regarding evolution of protein oxidation at different steps of the evolution of chronic renal failure.

Thus, the present study was undertaken to investigate plasma reactive carbonyl derivatives (CRD, carbonyl groups) and protein thiol (P-SH) groups level, as markers of oxidative modification of proteins, in patients with different stages of chronic renal failure not requiring dialysis and in patients on maintenance haemodialysis.

Patients and methods

Patients and controls

Forty-six (46) chronic renal failure (CRF) patients (ages 57±13.5 years, 28 female and 18 males) were included in this study. Smoking, diabetes mellitus, inflammatory diseases and alcohol abuse were the exclusion criteria. Of the 46 patients, ten patients were dialysis-dependent and 36 dialysis-independent with various degrees of renal insufficiency. According to their creatinine clearance (Ccr), nondialyzed CRF patients were divided into three subgroups: Ccr < 20 ml/min, Ccr = 21-50 ml/min, and Ccr = 51-90 ml/min. Dialysis patients (age 46.7±10.7 years, 6 female and 4 male)

were dialyzed with cuprophan membrane dialyzers for 5 h three times a week under the same conditions. A control group of 13 healthy volunteers comparable with respect to sex and ages of the renal patients was selected.

Laboratory methods

Blood sampling. Five ml of peripheral venous blood for analysis was collected in tubes over trace-element free heparin in the morning after an overnight fast, and immediately before dialysis in patients who were receiving haemodialysis. Plasma was separated by low speed centrifugation at 4°C. Determination of carbonyl and protein-thiol groups content was done immediately after blood collection and plasma separation.

Determination of carbonyl groups in oxidized plasma proteins. The protein oxidation level was monitored by determination of carbonyl content by the method of Levine (18) using 2,4-dinitrophenylhydrazine (DNPH) as a classic carbonyl reagent. Spectrophotometric measurement of plasma reactive carbonyl derivatives (RCD) values was performed and calculated using the extinction coefficient of DNPH-reactive carbonyl derivatives at 370 nm = 22×10^3 L $mol^{-1}cm^{-1}$ and expresses as μmol/g proteins. Protein concentration was determined by the Lowry et al. method (19).

Determination of protein thiol groups. The amount of plasma thiol (P-SH) groups was determined according to the method of Jocelyn et al (20), and expressed as mmol/L.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was evaluated by Student's *t* test. Differences were considered significant at $p<0.05$. Correlation between the parameters tested was studied by a regression analysis.

Results

Table 1 summarizes data on total protein and albumin levels in plasma of controls, nondialyzed patients with chronic renal insufficiency of various degree and haemodialyzed patients. Plasma total protein and albumin concentrations were lower in nondialyzed and haemodialyzed chronic renal failure patients compared to the levels in healthy subjects (Table 1). The lowest level of plasma proteins was observed in patients maintained on regular haemodialysis ($p<0.001$). However, a correlation between total plasma protein and albumin

Table 1. Plasma protein, carbonyl and SH group levels in chronic renal failure patients

	Controls	Chronic renal failure patients			
		Non-dialyzed patients			Haemodialyzed patients
Ccr (mL/min)	> 90	51-90	21-50	< 20	
Total proteins (g/L)	74±5.39[1] 100%	68± 3.91** 92%	64± 5.12*** 87%	66±7.21*** 89%	62±9.94*** 84%
Albumin (g/L)	44±5.25 100%	42±3.61* 94%	38±3.34** 86%	37±6.30** 84%	41±5.25* 92%
RCD (μmol/g proteins)	0.50±0.15 100%	1.16±0.19*** 231%	1.25±0.16*** 248%	1.25±0.22*** 248%	1.04±0.20*** 208%
P-SH (mmol/L)	0.57±0.09 100%	0.36±0.06*** 64%	0.43±0.08*** 75%	0.34±0.1*** 59%	0.29±0.08*** 51%
P-SH/P (mmol/g)	0.0077	0.0053	0.0067	0.0052	0.0042

[1] - values are presented as mean ± SD and in % of controls
* - $p < 0.05$ vs. controls
** - $p < 0.01$ vs. controls
*** - $p < 0.001$ vs. controls
Abbreviations: RCD, reactive carbonyl derivatives, P-SH, protein thiol groups; P-SH/P, ratio between protein thiol groups and plasma protein concentration.

level and the creatinine clearance (Ccr), as a parameter of renal insufficiency degree, was not observed ($r = 0.236$, $p > 0.05$).

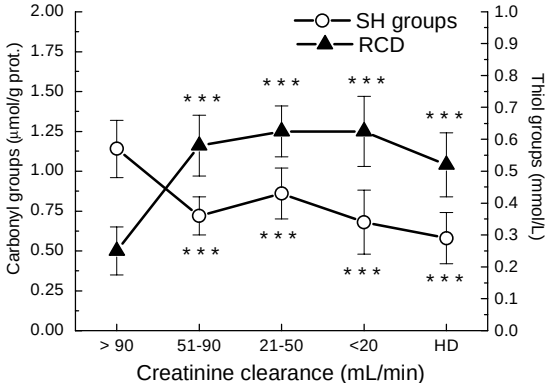

Fig. 1. Carbonyl and thiol groups levels in plasma of healthy controls and chronic renal failure patients. Abbreviations: HD – haemodialysis, RCD, reactive carbonyl derivatives, SH-thiol groups. Data are expressed as mean ±SD. Significance: * * * $p < 0.001$ vs. healthy subjects

Carbonyl groups content, measured as 2,4-dinitrophenylhydrazine-reactive carbonyl derivatives (RCD), increased in plasma of all chronic renal failure patients compared with healthy controls (Table 1 and Fig. 1). RCD level was 208% to 248% higher in plasma of chronic renal failure patients compared to healthy subjects (Table 1). However, plasma RCD level in haemodialyzed patients was significantly lower compared to the nondialyzed patients suffering from various degrees of renal insufficiency (Table 1 and Fig 1). Correlation analysis showed that plasma RCD level was not correlated with creatinine clearance ($r = -0.161$, $p > 0.05$, data not shown). However, statistically significant positive correlation ($r = 0.374$, $p < 0.05$) between the serum creatinine level and plasma RCD content in chronic renal failure patients was observed (Fig. 2).

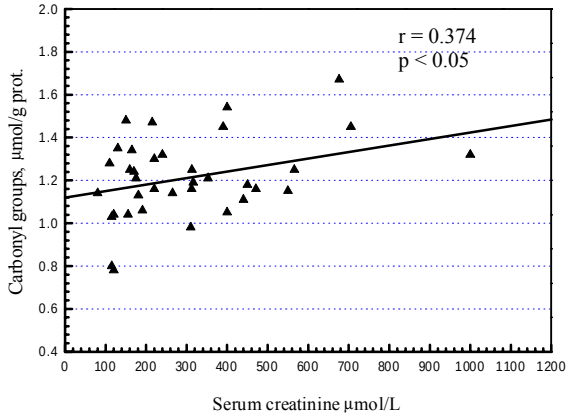

Fig. 2. Correlation between plasma carbonyl group content and serum creatinine in nondialyzed chronic renal failure patients

Plasma protein-thiol (P-SH) groups content and the ratio between protein thiol groups and plasma protein concentration (P-SH/P) were significantly lower in all patients with chronic renal failure compared with the control group (Table 1 and Fig. 1). The lowest level of plasma P-SH groups and P-SH/P ratio were observed in subjects with Ccr < 20 ml/min as well as in haemodialyzed patients compared with healthy controls (59% and 51%, respectively, Table 1). However, a correlation between the degree of renal insufficiency and plasma level of P-SH groups was not observed.

Discussion

Evidence of an imbalance between pro-oxidants and antioxidants, as well as that the chronic renal failure appears to be a state of increased oxidative stress (9 - 15), has been abundantly documented in ESRD patients. Massive oxidative stress might increase both lipid peroxidation and protein oxidation level. Alterations in lipid metabolism are well recognized as major risk factors for long-term complications in chronic renal injury (23, 24). However, under the conditions of severe oxidative stress, radical generation at inappropriate sites also leads to protein modification since they are essential targets for free radical attack, both intracellularly and extracellularly (4, 5). Proteins may be damaged directly, by specific interactions of oxidants or free radicals with particularly susceptible amino acids. Proteins are also modified indirectly, with reactive carbonyl compounds formed by the autooxidation of carbohydrates and lipids, with the eventual formation of the advanced glycation/lipoxidation end products (4–6, 20). The end products of advanced glycation (pentosidine and carboxymethyllysine) and lipid peroxidation (malondialdehyde-lysine) are elevated in plasma and matrix proteins of uraemic patients several times above normal subjects (20). Thus, precursor carbonyl compounds derived from carbohydrates and lipids are elevated in circulation of uraemic persons. The consequences of such damage may be impaired enzymatic activity and modified membrane and cellular function depending on the nature of the vulnerable protein component and the attacking radical species.

In this study, we found significant changes in the DNPH-reactive carbonyl derivatives (RCD) in plasma proteins of chronic renal failure patients. DNPH activity of proteins is postulated to indicate the presence of RCD by free radical-initiated reactions of side chains of amino acids residues (4, 22). The pattern and intensity of RCD formation in plasma of chronic renal failure patients were similar to that of plasma malondialdehyde (the end product of lipid peroxidation) formation in the same groups of CRF patients which have been observed in our earlier study (14). The increasing gap between the activities of superoxide dismutase (SOD) and peroxidase scavenger enzymes observed in plasma of patients with various degree of chronic renal failure (14,15), supports the idea that in chronic renal failure accumu-

lation of free radical species could result in unscheduled oxidation of proteins and an increase in plasma RCD. The other possible mechanism underlying the increases in RCD content in plasma of CRF patients might be a lower rate of degradation of proteins (4,5). However, plasma protein concentrations were lower in all groups of CRF, indicating that the accumulation of RCD in chronic renal injury did not depend on the amount of plasma proteins. These results support hypothesis of Inagi et al. (21), that uraemia appears to be in a state of carbonyl overload with potentially damaging proteins.

The results presented in this study also demonstrated that the concentration of protein sulphydryl groups (P-SH) in plasma were markedly reduced in individuals with CRF compared with healthy subjects. However, the degree of renal impairment does not appear to influence plasma P-SH in our data. Decreased plasma P-SH levels may be a consequence of enhanced free radicals in chronic renal failure. In normal healthy controls, albumin is an important chain breaking extracellular antioxidant which contains an exposed cysteine-SH group (25). Since albumin is present in plasma at concentrations approaching 0.5 mM (concentration of albumin-SH groups is about 500 μM), it provides the bulk of "total plasma thiols". Although the thiol groups are oxidized during oxidative stress, the high plasma level and high turnover rate make albumin a "sacrificial" antioxidant. This, however, is probably not the case for uraemic individuals with low albumin concentration and high production of free radicals. Thus, under these conditions, free radicals mediated oxidation and poor degradation of albumin may lead to accumulation of oxidatively modified albumin with lowered capacity to bind uraemic toxins and other protein bound substances. Our data on reduced level of protein thiol groups in plasma of CRF patients suggest that this mechanism may be involved in the worsening of oxidative stress at different stages of the evolution of chronic renal diseases. Oxidative modifications of proteins in uraemia might be relevant to several long-term complications associated with chronic renal failure and dialysis, such as atherosclerosis, aging, amyloidosis (21, 24).

The present results appear to prove the first demonstration that increased oxidative damage of plasma proteins (measured as reactive carbonyl derivatives content) correlates with the degree of renal insufficiency. Besides, the results presented also show that one of the features of uraemia is the presence of signs of oxidative stress before haemodialysis.

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OKSIDATIVNE MODIFIKACIJE PROTEINA PLAZME U RAZLIČITIM STADIJUMIMA HRONIČNOG OŠTEĆENJA BUBREGA

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Kratak sadržaj: *U radu je ispitivano oksidativno oštećenje proteina krvne plazme u nedijaliziranih bolesnika sa različitim stepenom hroničnog oštećenja bubrega i bolesnika koji se leče ponavljanim hemodijalizama. Prikazani rezultati pokazuju da je u plazmi svih bolesnika sa hroničnom insuficijencijom bubrega, u odnosu na zdrave osobe, značajno povećana koncentracija 2,4 dinitrofenilhidrazon-reaktivnih karbonilnih derivata (RCD), koji su jedan od markera oksidativne modifikacije proteina. Postoji statistički značajna korelacija izmedju koncentracije kreatinina u serumu i sadržaja RCD u plazmi hroničnih bubrežnih bolesnika. Sadržaj proteinskih tiol grupa (P-SH), koje su značajni "žrtveni" antioksidanti u prekidanju lanca reakcija izazvanih slobodnim radikalima, značajno je smanjena u plazmi ispitivanih bolesnika. Medjutim, ne postoji povezanost izmedju stepena oštećenja bubrega i smanjenja nivoa P-SH grupa. Prikazani rezultati govore u prilog tvrdnje da se u hroničnom oštećenju bubrega akumuliraju slobodni radikali koji mogu oksidativno modifikovati i oštetiti molekule proteina. Oksidativno oštećenje proteina krvne plazme zabeleženo je već u ranim stadijumima hroničnog oštećenja bubrega.*

Ključne reči: *Hronično oštećenje bubrega, karbonilne grupe, tiol grupe, oksidativni stres, slobodni radikali*