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Increased plasma protein homocysteinylation in hemodialysis patients

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Hyperhomocysteinemia, an independent cardiovascular risk factor, is present in the majority of hemodialysis patients. Among the postulated mechanisms of toxicity, protein homocysteinylation is potentially able to cause significant alterations in protein function. Protein homocysteinylation occurs through various mechanisms, among which is the post-translational acylation of free amino groups (protein-N-homocysteinylation, mediated by homocysteine (Hcy) thiolactone). Another type of protein homocysteinylation occurs through the formation of a covalent −S−S− bond, found primarily with cysteine residues (protein-S-homocysteinylation). Scant data are available in the literature regarding the extent to which alterations in protein homocysteinylation are present in uremic patients on hemodialysis, and the effects of folate treatment are not known. Protein homocysteinylation was measured in a group of hemodialysis patients ($n = 28$) compared to controls ($n = 14$), with a new method combining protein reduction, gel filtration and Hcy derivatization. Chemical hydrolysis was performed, followed by high-pressure liquid chromatography separation. The effects of folate treatment on protein homocysteinylation, as well as *in vitro* binding characteristics were evaluated. Plasma Hcy, protein-N-homocysteinylation and protein-S-homocysteinylation were significantly higher in patients vs controls. Plasma Hcy and protein-S-homocysteinylation were significantly correlated. After 2 months of oral folate treatment, protein-N-homocysteinylation was normalized, and protein-S-homocysteinylation was significantly reduced. Studies on albumin-binding capacity after *in vitro* homocysteinylation show that homocysteinylated albumin is significantly altered at the diazepam-binding site. In conclusion, increased protein homocysteinylation is present in hemodialysis patients, with possible consequences in terms of protein function. This alteration can be partially reversed after folate treatment.

Kidney International (2006) **69,** 869–876. doi:10.1038/sj.ki.5000070; published online 4 January 2006

KEYWORDS: homocysteine; protein homocysteinylation; folates; albumin; hemodialysis; uremia

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Received 10 December 2004; revised 20 June 2005; accepted 14 July 2005; published online 4 January 2006

In hemodialysis patients, high plasma homocysteine (Hcy) levels are commonly present.[1,2] Hyperhomocysteinemia has been linked to increased cardiovascular risk in the general population, where mild increases in Hcy levels apply.[3] In uremic patients on hemodialysis, high Hcy levels, usually in the moderate–intermediate range, have been linked to higher cardiovascular risk and mortality.[4]

The possible mechanisms through which increased Hcy levels exert a toxic effect in uremia are as follows: oxidation, nitrosylation and DNA and protein hypomethylation.[5,6] Another potential mechanism of Hcy toxicity is protein homocysteinylation. Only scant data are available in the literature on the extent of protein homocysteinylation in uremic patients.[7]

In general, protein homocysteinylation, that is, the binding of Hcy to proteins, can occur in the following ways:

(i) the acylation of free amino groups, termed protein-N-homocysteinylation, in particular, the binding of Hcy to the ε amino group of lysine residues and the terminal amino group of proteins (mediated by Hcy thiolactone, an Hcy derivative,[8]); and

(ii) oxidation of thiol groups, mediated by Hcy in its free form, termed protein-S-homocysteinylation (in particular directed towards cysteine (Cys) residues,[9]).

In this work, we refer to these two mechanisms as 'protein homocysteinylation' (see Figure 1 for a depiction of the moieties involved). The introduction of S-nitroso-Hcy (derived through the nitrosylation of Hcy by NO) in the covalent backbone of proteins[10] is a translational process and is considered part of the nitrosylation mechanism.

Formation of Hcy thiolactone, involved in protein-N-homocysteinylation, is a consequence of a *proof-reading* reaction that prevents the post-translational incorporation of Hcy into proteins,[11] or it can appear as a result of a reaction that binds Hcy to initiator methionyl tRNA. This complex is then methylated to methionine by a methylating factor. When hypomethylation is present, the Hcy–tRNA complex is hydrolyzed to form Hcy thiolactone.[12]

When plasma proteins are incubated in the presence of Hcy thiolactone, homocysteinylation occurs spontaneously, that is, through a non-enzymatic reaction, and rapidly, with the complete disappearance of Hcy thiolactone from the medium after 3 h.[13]

The consequences of increased protein homocysteinylation are represented by protein damage, monitored by altered electrophoretic mobility, and loss of enzymatic activity (with protein denaturation), found in several model systems, such as plasma proteins, methionyl tRNA synthetase, tripsyn, lysin oxidase, etc.[13] Technical difficulties may be encountered when determining Hcy thiolactone in the blood, probably because of the high molecular reactivity. Only recently, a high-performance liquid chromatography (HPLC) method has been published for an Hcy thiolactone assay in the plasma.[14] However, when there is an increase in the blood Hcy, an increase in homocysteinylated proteins can be observed.[15] Lately, it has been demonstrated that N-homocysteinylation affects the susceptibility of albumin to oxidation and proteolysis,[16] thus probably rendering this protein more prone to be cleared from circulation.

Therefore, protein homocysteinylation could be one of the mediators of Hcy toxicity, contributing to structural and functional alterations at the molecular and cellular levels.

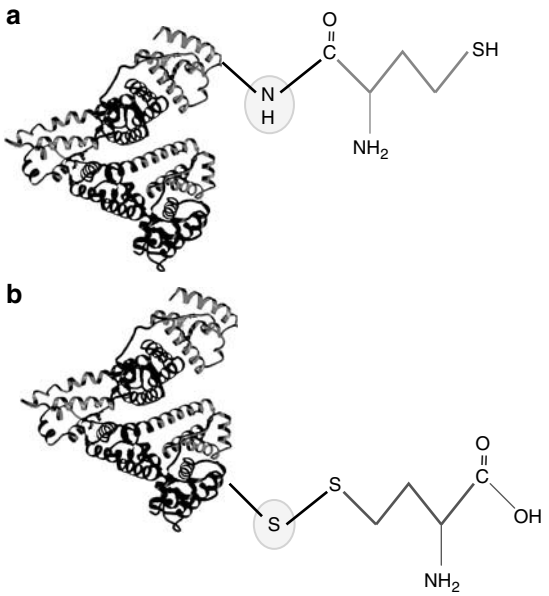

Figure 1 | Protein adducts of Hcy with plasma proteins. Protein-N-homocysteinylated derivatives, with either free N-terminus or the ε-amino group of lysine residues (particularly Lys^{525} in serum albumin). Protein S-homocysteinylated derivatives (particularly with Cys^{34} in serum albumin). Protein-N-homocysteinylated–S-derivatives are also possibly formed.[16] The shaded area indicates the group, which is homocysteinylated in proteins.

The aim of our study was to measure homocysteinylated proteins in uremic patients on hemodialysis, and to verify whether folate treatment would affect protein homocysteinylation. In addition, the consequences of protein homocysteinylation were investigated in relation to the *in vitro* binding properties of modified albumin.

RESULTS

Hcy levels, folate and vitamin B_6 and B_{12} in patients and controls

Results relative to plasma total, protein-bound and non-protein-bound Hcy (tHcy) are shown in Table 1. As expected, tHcy levels were significantly higher in uremics compared to controls ($P<0.001$).

The tHcy concentration range in the 28 patients in Table 1 was 14.50–221.34 μM. In uremic patients, folate levels were 13.3 (s.d. 5.4) nmol/l, while the vitamin B_6 level was 17.23 (s.d. 4.7) nmol/l and the vitamin B_{12} level was 475.2 (s.d. 65.5) pmol/l.

Protein-N-Hcy and protein-S-Hcy levels in patients and controls

Results relative to plasma protein-N-Hcy and protein-S-Hcy are shown in Table 1.

Protein-N-Hcy, expressed both as μM and as pmol/mg protein, was significantly different from controls in uremics. The concentration of Hcy bound to proteins through the disulfide bridge was also significantly different from control values, expressed both as μM and pmol/mg protein.

As for the correlations between tHcy levels and protein homocysteinylation, the results can be found in Table 2. The Pearson correlation coefficients and linear regression are relative to tHcy and protein-N-Hcy, and tHcy and protein-S-Hcy, both in controls and uremics. A significant value can be found considering the correlation between tHcy and protein-S-Hcy, both in controls and uremics, basically underscoring the fact that tHcy is the sum of protein-S-Hcy, Hcy-Hcy, Hcy-Cys and free Hcy in the blood. Therefore, in this case, total Hcy reflects the protein-S-Hcy content.

Folate treatment

Results relative to folate treatment are shown in Table 3.

The plasma tHcy concentration range was 16.47–178.88 μM. Plasma tHcy levels were significantly reduced after treatment, although tHcy levels were still above normal levels. Before therapy, in uremics, protein-N-Hcy and protein-S-Hcy were significantly higher with respect to control. After

Table 1 | Mean concentration levels (s.e.) of tHcy, protein-N-Hcy and protein-S-Hcy in controls and uremic patients on hemodialysis, the latter expressed as μM concentration and as pmol/mg protein

	tHcy (μM)	Protein-N-Hcy (μM)	Protein-N-Hcy (pmol/mg)	Protein-S-Hcy (μM)	Protein-S-Hcy (pmol/mg)
Controls (n=14)	11.35 (1.03)	0.35 (0.13)	4.65 (2.74)	9.46 (1.20)	139.52 (15.43)
Uremics (n=28)	57.84 (9.92)*	0.68 (0.10)*	12.40 (2.25)*	52.02 (9.16)*	825.53 (121.08)*
	*$P<0.001$ vs control	*$P<0.05$	*$P<0.05$	*$P<0.05$	*$P<0.001$

Protein-N-Hcy, Hcy bound to proteins through the amide bond; protein-S-Hcy, Hcy bound to proteins through the disulfide bridge.