Accumulation of cyanide and thiocyanate in haemodialysis patients

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Abstract

Background. Cyanide is a toxic agent, and its detoxification product, thiocyanate, may be a major pathogenetic substance in uraemia. Recent studies examining the myeloperoxidase(MPO)/thiocyanate system have suggested a link between thiocyanate and atherosclerosis. However, inaccuracies in conventional assays for cyanide and thiocyanate have limited the understanding of their metabolism in haemodialysis (HD) patients.

Methods. We used high-performance liquid chromatography to measure cyanide in erythrocytes and thiocyanate in plasma in 43 HD patients and in a group of 46 healthy controls that included 15 current smokers. To clarify the metabolic conversion of cyanide to thiocyanate in uraemic patients, we also measured cysteine and sulfate. We then used stepwise regression analysis to analyse factors that determine erythrocyte cyanide and plasma thiocyanate.

Results. Mean cyanide and thiocyanate were significantly greater in HD patients than in non-smoking controls. However, cyanide was far below lethal concentrations in dialysis patients. Thiocyanate was six to seven times greater in HD patients than in non-smoking controls, and decreases in thiocyanate following dialysis were only 19.3±3.5%. Multiple regression analysis showed a positive correlation between cyanide and thiocyanate in controls, but a negative correlation in HD patients. In patients, an inverse relationship between thiocyanate and BUN was also observed.

Conclusions. The elevation of thiocyanate in patients undergoing dialysis probably is secondary to both limited efficiency of HD and deranged metabolism of cyanide and thiocyanate. Because thiocyanate is a preferred substrate for MPO, it may play a role in uraemic complications including cardiovascular events.

Keywords: chronic renal failure; cyanide; cysteine; haemodialysis; thiocyanate

Introduction

The accumulation of cyanide (CN) results in a fatal breakdown of mitochondrial oxidative phosphorylation. In uraemic patients as well as the general population, CN exposure occurs from tobacco smoke, some drugs (sodium nitroprusside) and certain foods (cassava, chokecherry, pin cherry, wild black cherry, peach, apricot and bitter almond). CN is metabolized to thiocyanate (SCN) by the enzyme rhodanese. This reaction is essential to life through its detoxification of CN, and SCN synthesis can be accelerated under CN-loaded conditions [1]. However, the human capacity for detoxification of CN in uraemia has not been investigated fully.

It is well documented that SCN is the strongest atherogenic factor in smokers. In addition, serum SCN is positively correlated with the formation of advanced atherosclerotic plaques within coronary arteries [2]. Furthermore, a recent study showed that risk of cerebral infarction stroke was significantly increased in individuals having high serum SCN concentrations, with an odds ratio of 3.00 [3]. However, the mechanisms through which SCN accelerates atherosclerosis have not been well clarified. SCN in plasma is a substrate for leukocyte peroxidase [4]. Recent studies demonstrated that the peroxidase/SCN/H2O2 system might promote lipid peroxidation [5]. As a substrate for myeloperoxidase (MPO), SCN is favoured 400 times over chloride. Zhang et al. [4] demonstrated that lipid peroxidation in the presence of MPO was accelerated by SCN in a dose-dependent manner [4]. In addition, uraemic patients receiving haemodialysis (HD) therapy have consistently shown significant increases in plasma MPO levels [6], which may accelerate the oxidative pathway [7]. Therefore, it has been suggested that SCN is closely associated with uraemic complications such as atherosclerosis.

Despite the importance of both CN and SCN, there is little available data describing their metabolism in the uraemic state. This may have been due to unreliable analytic methods for determining CN and SCN in uraemia. For example, a non-specific turbidity method (Bowler’s method) had been used for the...
estimation of SCN. This assay may be inappropriate for the estimation of SCN in plasma from HD patients, which presumably contains a number of uremic substances. To measure both CN and SCN in HD patients in the present study, we adapted the sensitive and specific method of Chinaka et al. [8], using ion chromatography. To further examine CN metabolism in HD patients, we also determined plasma (p) concentrations of cysteine (p-Cys), a rate-limiting cofactor in the conversion of CN to SCN [9], and of sulfate (p-sulfate), a final degradation product of sulfur-containing amino acids (SAA). We used a multiple regression analysis to assess independent predictors of plasma levels of SCN.

Subjects and methods

Subjects and sample preparation

We included 43 patients with chronic renal failure that were undergoing maintenance HD (HD group), and 46 healthy controls with normal renal function (control group). This control group consisted of 31 non-smokers and 15 smokers. HD was performed three times weekly for 4 h with polysulfone or polyamide membranes. Ultra pure dialysate was generated using water that had undergone dual pass exchange chromatography (TSKgel IC-Anion-PW, Tosoh). Ultra pure dialysate solution were added to a 250-m/1 mixture of 2,3-naphthalenedialdehyde (NDA) and 50 mmol/l taurine for 10 min. We used the sensitive and specific method of Chinaka et al. [8], using ion chromatography. To further examine CN metabolism in HD patients, we also determined plasma (p) concentrations of cysteine (p-Cys), a rate-limiting cofactor in the conversion of CN to SCN [9], and of sulfate (p-sulfate), a final degradation product of sulfur-containing amino acids (SAA). We used a multiple regression analysis to assess independent predictors of plasma levels of SCN.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>Non-smoking</th>
<th>Smoking</th>
<th>HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>31</td>
<td>15</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>11/20</td>
<td>10/5</td>
<td>22/21</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.9±2.4</td>
<td>41.4±2.9</td>
<td>60.0±2.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Duration on HD (years)</td>
<td>N/A</td>
<td>N/A</td>
<td>6.7±0.9</td>
<td></td>
</tr>
<tr>
<td>BUN (mmol/l)</td>
<td>4.9±0.2</td>
<td>5.1±0.1</td>
<td>28.6±1.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>72.4±1.6</td>
<td>73.6±1.5</td>
<td>1043.1±44.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td>134.5±2.8</td>
<td>146.8±7.1</td>
<td>102.6±1.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>73.0±0.7</td>
<td>74.5±1.6</td>
<td>66.9±0.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>44.2±0.6</td>
<td>46.3±1.2</td>
<td>40.8±0.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>TACurea (mmol/l)</td>
<td>N/A</td>
<td>N/A</td>
<td>19.10±0.88</td>
<td></td>
</tr>
<tr>
<td>Kt/V</td>
<td>N/A</td>
<td>N/A</td>
<td>1.12±0.03</td>
<td></td>
</tr>
</tbody>
</table>

Mean±SE. TACurea, time-averaged concentration of urea; N/A, not available.
<sup>a</sup>Significantly different vs non-smoking subjects. <sup>b</sup>vs smoking subjects (<i>P</i>&lt;0.05).

Determination of erythrocyte CN (e-CN) and plasma SCN (p-SCN)

For determination of e-CN and p-SCN, we carried out ion chromatography using a modification of the method by Chinaka et al. [8]. In our preliminary experiment, plasma CN was below detection limits, which was expected as 98% of blood CN exists within erythrocytes [10]. We then prepared erythrocytes for determination of CN by osmotically lysing 0.5-ml aliquots of erythrocytes in 1 ml of distilled water. We added 350 μl of methanol to 150 μl of haemolysate. After mixing with a vortex agitator, the mixture was centrifuged at 1600 g for 10 min. For derivatization, 50 μl each of 2 μmol/l 2,3-naphthalenedialdehyde (NDA) and 50 mmol/l taurine solution were added to a 250-μl aliquot of supernatant or standard solution. Samples were injected into a chromatographic system consisting of an L-6000 high-performance liquid chromatographic (HPLC) pump (Hitachi, Tokyo, Japan), a DGU-3A on-line degasser (Uniflows, Tokyo, Japan), an L-4000 ultraviolet detector (Hitachi), an RF-10A fluorescence detector (Hitachi) and a D-2500 Chromato- Integrator (Hitachi), in which the detectors were connected in tandem. The CN derivative was detected by fluorescence (418 nm excitation, 460 nm emission). A TSKgel IC-Anion-SW anion-exchange column (50 × 4.6 mm; Tosoh, Tokyo, Japan) was used as a separation column, using 10 mmol/l phosphate buffer (pH 6.1) with methanol (1:1, v/v) as the eluent. The column was kept at room temperature, and the flow rate was 1.0 ml/min. The sample injection volume was 20 μl.

We used plasma samples to measure blood SCN, as most blood SCN is bound to albumin [11]. For SCN determination, 100 μl of plasma was added to 400 μl of methanol. The mixture was centrifuged at 1600 g for 10 min. We used the same HPLC procedure as above except that the eluent was 10 μmol/l phosphate buffer (pH 6.1) with methanol at 3:2 (v/v), and SCN was quantified using an ultraviolet detector (210 nm). The intra-assay coefficient of variance (CV) was 3.0% and the inter-assay CV was 3.8%.

Determination of sulfate and thiosulfate in plasma

We analysed p-sulfate and thiosulfate concentrations by ion exchange chromatography (TSKgel IC-Anion-PW, Tosoh) with a conductivity meter (CM-8020, Tosoh) and a UV detector (L-4000) according to our previous report [12]. Plasma thiosulfate concentrations in control subjects and HD patients were below detection limits.

HPLC for determination of Cys in plasma

We performed the HPLC separation of Cys after derivatization with the fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulphonate (SBD-F), by using a modified method of Toyo’oka et al. [12].
Reagents

NDA was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). SBD-F and L-cysteine (Sigma, St Louis, MO) and methanol (Wako, Tokyo, Japan) were HPLC grade. Potassium CN, potassium SCN and taurine were analytic reagent grade (Wako). All other reagents used were of analytic reagent grade.

Statistics

Statistical analyses were performed with a DOS/V computer using StatView for Windows (version 5.0, SAS institute, Cary, NC). Results are reported as the mean±SEM. P values <0.05 were considered to indicate significance. Normally distributed variables were analysed by analysis of variance followed by Bonferroni’s post-hoc test. Simple linear regression analysis was performed to assess the relationships between p-SCN and several variables including age, e-CN, p-Cys, p-sulfate, BUN, current smoking in the control group and duration of HD in the HD group. Stepwise multiple regression analysis was used for determining independent predictors of p-SCN levels. Factors that were not significant in the univariate analysis were excluded from the latter analysis.

Results

Patient characteristics

In the HD group, BUN and serum creatinine were significantly higher and haemoglobin, serum total protein and albumin were lower than in the control group (Table 1). These parameters did not differ between non-smoking and smoking controls.

Fig. 1. e-CN and plasma SCN, Cys and sulfate were measured in 31 non-smoking healthy subjects, 15 healthy smoking subjects and in 43 chronic renal failure patients treated with maintenance HD. a P < 0.05 vs non-smoker; b P < 0.05 vs smoker. c P < 0.05 vs pre-HD.

e-CN, p-SCN, p-Cys and p-sulfate concentrations

Mean concentrations of e-CN and p-SCN were significantly greater in smoking subjects than in non-smoking subjects (Figure 1). e-CN levels were higher in pre-HD samples than in samples from non-smoking subjects, and did not change after HD (Figure 1). There was no difference in e-CN levels between smoking subjects and dialysed patients. p-SCN levels were significantly greater in pre-HD samples than in samples from both non-smoking and smoking subjects. Although the post-HD p-SCN concentrations were significantly lower than pre-HD concentrations, they were still higher (four times over) than in non-smoking subjects.

Smoking did not affect p-Cys and p-sulfate levels in control subjects. Mean concentrations of p-Cys and p-sulfate were significantly greater in pre-HD samples than in control subject samples (Figure 1). The pre-HD concentrations of both p-Cys and p-sulfate were greater than in controls. The post-HD concentrations of p-Cys and p-sulfate were significantly lower than pre-HD concentrations, declining to levels observed in the control group samples.

Determination of significant predictors for SCN

Univariate correlations and multiple regression analyses were performed to establish the principal determinants of SCN. Age, duration of HD (HD group only), BUN, three variables related to conversion from CN to SCN (CN, Cys and sulfate) and current smoking status (control group only) were analysed by simple regression analysis (Table 2, upper portion). In the control group, CN (Figure 2A), Cys and smoking
were significantly correlated with SCN. In the HD group, CN (β = 0.34), Cys (β = 0.42) and smoking (β = 0.38) were independently associated with SCN in the control group, while CN (β = -0.31) and BUN (β = -0.33) were significant predictors of SCN levels in the HD group (Table 2, lower portion).

**Discussion**

In the present study, we used the rapid and sensitive method of Chinaka et al. [8], that simultaneously determines, after a simple pre-treatment, both CN and SCN levels in blood. For the determination of CN, the microdiffusion-König spectrophotometric method had been used previously in forensic toxicology and wastewater regulation. However, SCN causes serious positive
interference in this assay. SCN is inevitably high when blood CN levels are increased. However, even forensic scientists have often mistakenly estimated CN because of interference from SCN [13]. Conventional methods for CN determination require an acidification procedure to extract volatile HCN from blood, which results in considerable artifactual production of CN from SCN. This positive interference by SCN, which is abundant in the plasma of smokers and uraemic patients, greatly affects accuracy of CN blood determinations. In addition, the conventional turbidity method for SCN determination is thought to have poor specificity. In the present study, we used ion chromatography to make non-interference determinations of e-CN and p-SCN. To our knowledge, this is the first report showing simultaneous determinations of CN and SCN in HD patients using this specific method.

CN loading is caused by the intake of tobacco smoke and various foods and drugs. In the present study, the control group included 15 current smokers. Low levels of CN from smoking are primarily produced by the incomplete combustion of tobacco smoke. The mean e-CN in the smokers (3.71 ± 1.22 nmol/g Hb) was significantly higher than in the non-smokers (2.62 ± 0.13). In the HD group, the mean e-CN was 4.04 ± 0.20, which was comparable with the levels in smokers but significantly increased compared with the control group. The significantly higher e-CN levels in smokers compared with non-smokers (P < 0.001) is consistent with previous reports using HPLC with fluorescence detection [14], spectrophotometry [15] and a fluorimetric method [16].

e-CN levels were not significantly altered by HD (Figure 1). In the blood, 98% of CN is contained in erythrocytes and only 2% is in plasma [10]. Accordingly, in our preliminary experiments, plasma CN was below detection limits. Because of this localization of CN, HD would have little effect on total concentration in blood.

CN poisoning can be lethal. To estimate the potential toxicity of CN in blood, e-CN is multiplied by the haemoglobin value to determine whole-blood CN [14]. Calculated whole-blood CN concentrations were 335 ± 8 nmol/l in non-smoking controls, 548 ± 123 nmol/l in smoking controls and 416 ± 22 nmol/l in pre-HD samples, which were all far below lethal amounts (over 115 nmol/l) [13]. The total body burden of CN is kept within the safe range even in HD patients, since CN is normally metabolized to SCN, a less toxic compound.

We also found that the mean concentration of p-SCN in smokers was significantly higher than in non-smoking control subjects (8.94 ± 1.04 μmol/l vs 4.83 ± 0.47; P < 0.0001, Figure 1). This result is consistent with previous findings that p-SCN in smokers was two times higher than in non-smokers [2]. An important observation in the present study was that p-SCN in the HD group was six to seven times higher than in non-smoking controls. Although p-SCN was significantly decreased following the HD session, it remained more than four times higher than in non-smokers. A possible cause of the elevated p-SCN in the HD patients could be a low efficiency removal by HD. Although the molecular weight of SCN (≈58) is sufficiently small to permit dialysis, the reduction rate of SCN in the present study was 19.3 ± 3.5%. This insufficient removal may have resulted from binding of the majority of blood SCN to albumin [11]. However, further investigations will be necessary to clarify the degree of protein binding, as this varied in previous reports [11,17].

SCN is converted by leukocyte peroxidase into hypohiocyanite (OSCN−) [5], and radical species are therefore generated [4,5]. The regulation of MPO activity relies upon other co-substrates. For instance, the specificity constants for chloride, bromide and SCN are 1:60:730, respectively, indicating that SCN is by far the most favoured substrate for MPO [18]. Recently, Zhang et al. [4], found that SCN and Cl− are distinct substrates for MPO, which results in a dose-dependent initiation of lipid peroxidation. In HD patients, there have been several reports of neutrophil activation accompanied by elevated MPO levels [19], as well as increases in neutrophil production of reactive oxygen species [7], which may promote subsequent oxidation to increase the risk for atherosclerosis. These observations suggest that SCN may be a critical factor causing atherogenesis in HD patients as well as in smokers.

Stepwise regression analyses were performed to establish significant predictors of p-SCN. These analyses revealed that e-CN significantly predicted p-SCN in the control group (Table 2). The positive correlation between p-SCN and e-CN suggested that SCN might be synthesized in parallel with the synthesis of CN (Figure 2A). This notion is consistent with previous findings in other investigations [20]. In contrast, e-CN significantly predicted p-SCN in the HD group (Table 2), but in this case, p-SCN was inversely correlated with e-CN (Figure 2B). It is unclear which mechanism may describe the inverse correlation between CN and SCN in the HD group. We suspect that a deranged excretion of SCN may explain these findings. In HD patients, p-SCN can be removed only by dialysis, while in the control group it is excreted continuously in the urine. The sum total of CN and its metabolite SCN may be relatively constant in HD patients, which could explain the negative correlation between e-CN and p-SCN.

The conversion of CN to SCN is catalysed by the enzyme rhodanese. Serum and erythrocyte activities of rhodanese are very low in healthy subjects and the enzyme is easily inactivated [21]. Consequently, the activity of this enzyme is commonly estimated by measuring amounts of the substances involved in the reaction. The plasma concentration of thiosulfate, which is required as a sulfur donor, was below the detection limit even by our ion chromatography method and this is because thiosulfate has an intracellular localization [22]. Thiosulfate availability can also be determined from the levels of Cys and SAA [23]. We additionally determined p-sulfate levels
as an index of SAA intake, as most SAA is ultimately degraded to sulfate [24]. In the control group, p-Cys was a significant predictor of p-SCN. SCN synthesis was likely to depend on the rhodanese pathway. Nevertheless, there was no correlation between p-Cys and p-SCN in HD patients. Although we are uncertain why controls differed from HD patients, plasma levels of Cys and sulfate were remarkably elevated in the HD group, and the abundant supply of thiosulfate may affect this relationship.

We also observed an inverse relationship between p-SCN and BUN in the HD group. We suspected that BUN or other uremic toxins might have been associated with the inhibition of SCN synthesis. However, a possible link between CN or SCN metabolism and protein intake could not be ruled out.

In summary, we observed significant elevations in both e-CN and p-SCN in HD patients. The p-SCN levels were six to seven times higher than in non-smoking controls. However, concentrations of e-CN were far below the lethal level in HD patients. The elevations in p-SCN in the HD group reflected not only low efficiency of removal by HD, but also a deranged metabolism of CN and SCN. As SCN is a favoured substrate for MPO, further investigation is needed to examine the relationships between p-SCN and uremic complications, and to determine their relation to cardiovascular events.

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Conflict of interest statement. None declared.

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