Plasma cysteine and sulphate levels in patients with cirrhosis of the liver

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1. Fasting levels of plasma cysteine, plasma sulphate and the plasma cysteine/sulphate ratio were measured in patients with primary biliary cirrhosis and compared with those in patients with other liver disease, general intensive therapy unit patients and healthy subjects.

2. Plasma cysteine was significantly elevated in patients with primary biliary cirrhosis (median 0.364 nmol/mg of protein, \( P < 0.0001 \)) and patients with other liver disease (median 0.445 nmol/mg of protein, \( P < 0.0001 \)), compared with healthy control subjects (median 0.125 nmol/mg of protein) and increased progressively with the severity of liver disease. Plasma cysteine was also elevated in intensive therapy unit patients (median 1.564 nmol/mg of protein) compared with healthy control subjects \( (P < 0.0001) \) and patients with other liver disease \( (P < 0.0001) \).

3. Plasma sulphate was reduced significantly only in patients with primary biliary cirrhosis (median 0.822 nmol/mg of protein) compared with healthy control subjects (median 1.37 nmol/mg of protein, \( P < 0.05 \)). There was no significant difference in plasma sulphate between disease groups.

4. The plasma cysteine/sulphate ratio was significantly elevated in patients with primary biliary cirrhosis (median 0.448, \( P < 0.0001 \)) and patients with other liver diseases (median 0.394, \( P < 0.0001 \)) compared with healthy control subjects (median 0.095). The ratio was also elevated in intensive therapy unit patients (median 1.650, \( P < 0.0001 \)) compared with healthy control subjects and liver disease groups \( (P < 0.0001) \).

5. In conclusion, plasma cysteine rises in primary biliary cirrhosis and other forms of liver disease. This effect is not specific to liver disease, since cysteine is elevated in an heterogeneous group receiving intensive care. Impairment of trans-methylation and trans-sulphuration pathways does not explain the finding of increased plasma cysteine. Since cysteine is elevated in non-hepatic disease, it may reflect the effect of muscle breakdown and the catabolic state. Impaired activity of cysteine dioxygenase and impaired mitochondrial function may be contributory, but this requires further study. These metabolic changes may reflect progressively diminished detoxification capacity within the liver and other tissues.

INTRODUCTION

Liver disease is associated with diverse metabolic derangements, involving both synthetic pathways and xenobiotic detoxification. The function of the trans-methylation pathway is impaired, with resulting hypermethioninaemia [1]. Generation of sulphate via the trans-sulphuration pathway is also suboptimal [2]. The capacity to sulphate endo- and exogenous compounds is dependent upon a continued supply of 3'-phosphoadenosine 5'-phosphosulphate (PAPS) [3], which therefore partially regulates sulphation activity [4]. If the demand for sulphate exceeds the supply of PAPS, alternative conjugation pathways will be utilized, so the proportion of glucuronide conjugates increases. This can be particularly disadvantageous in the case of some hepatotoxic secondary monohydroxy bile acids and oestrogens, since glucuronide conjugates may be far more hepatotoxic than their respective sulphate conjugates [5–8]. The rate-limiting step in the formation of sulphate is S-oxidation of cysteine to cysteine sulphinic acid, a reaction which is predominantly dependent upon activity of the enzyme cysteine dioxygenase [9,10].

Primary biliary cirrhosis (PBC) is a disease of unknown aetiology, which has been associated with an increased incidence of impaired S-oxidation, attributed putatively to impaired activity of cysteine dioxygenase [11]. This could play an important role in disease pathogenesis, since S-oxidation and, in particular, activity of cysteine dioxygenase, is responsible for the intracellular supply of inorganic sulphate, the precursor of PAPS [12]. Impaired S-oxidation, and reduced activity of cysteine dioxygenase may be reflected by elevation of plasma cysteine and reduction of plasma sulphate [13].

The aim of this study was to examine whether patients with PBC have an increase in the plasma cysteine/sulphate ratio, as further evidence of

Key words: cirrhosis, cysteine, trans-methylation, trans-sulphuration, sulphate.

Abbreviations: ITU, intensive therapy unit; OLD, other liver disease; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; PBC, primary biliary cirrhosis.

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impaired sulphur metabolism. Comparison was made with patients suffering other forms of liver disease and patients with severe non-hepatic disease requiring treatment on an intensive therapy unit (ITU).

MATERIALS AND METHODS

Subjects

Group 1. This comprised eight male and 32 female healthy subjects (median age 30 years), who all worked at the Queen Elizabeth Medical Centre. These controls did not take medication and they did not abuse alcohol. One-third were cigarette smokers, which was not significantly different from the disease groups.

Group 2. This comprised five males and 29 females (median age 52 years) with PBC. Their disease severity was graded according to Pugh's modification [14] of the Child's score [15] accordingly: grade A, 10; grade B, 11; grade C, 13.

Group 3. This comprised 13 males and 13 females (median age 46 years) with other liver diseases (OLD). These were graded by Child's Pugh score accordingly: grade A, 2; grade B, 10; grade C, 14. The diagnoses were: primary sclerosing cholangitis, 13; autoimmune chronic active hepatitis, four; cryptogenic cirrhosis, four; alcoholic cirrhosis, three; \( \alpha_1 \)-antitrypsin deficiency, one; haemochromatosis, one.

Group 4. This comprised 19 males and 14 females (median age 62 years) being treated on the ITU. Each had received no nutrition for at least 48 h, i.e. zero enteral or parenteral nutrition (excluding those calories infused for hydration or sedation). Liver function tests in these patients, including serum bilirubin, alkaline phosphatase and aspartate transaminase, were normal. Diagnoses included: laparotomy and abdominal surgery, 16; respiratory failure, eight; insertion of prosthetic heart valve, three; coronary artery by-pass surgery, three; repair of abdominal aortic aneurysm, two; Guillain Barré syndrome, one.

It was not possible to accurately match groups for age and sex, since the demographics of patient populations with different liver diseases (that is PBC compared with OLD) and on the general ITU vary greatly.

Written informed consent was obtained from all patients, and approval for the study was given by the local ethics committee.

All subjects were tested between 07.30 and 09.00 hours after an overnight fast. Venepuncture was performed and 7 ml of blood was drawn into a lithium heparin tube and put immediately on ice. Plasma was separated and frozen within 15 min in all cases, until the time of analysis.

Analyses

Cysteine. Cysteine was determined by the ninhydrin method of Gaitonde [16]. Plasma was deproteinized by 5% perchloric acid (1:1, v/v). This was centrifuged for 20 min at 2000g. Supernatant (1 ml) was added to a boiling tube containing 1 ml of acid ninhydrin reagent plus 1 ml of glacial acetic acid. Ninhydrin reagent was prepared using 250 mg of ninhydrin in 6 ml of glacial acetic acid and 4 ml of concentrated hydrochloric acid. Tubes were vortexed, placed into a boiling water bath for 10 min, before rapid cooling. The volume of each tube was made up to 10 ml with 99% ethanol. A reagent blank was prepared as above using distilled water. The pink colour was read in a u.v.-visible spectrophotometer at 560 nm against the blank. A calibration curve was constructed on each occasion that the assay was performed and unknown plasma concentrations were read from this.

Sulphate. The colorimetric analysis of Jackson and McCandless [17] was used. This is dependent on resultant turbidity from formation of a barium sulphate precipitate. A solution of BaCl\(_2\) in agarose was prepared. Agarose was dissolved in water by gently heating, to a concentration of 0.01% (w/v). To this was added 0.5 g of BaCl\(_2\)/100 ml of solution and allowed to stand overnight. Plasma was deproteinized by adding 0.7 ml of sample to the same volume of 8% trichloroacetic acid. The sample was spun for 10 min at 2000 g, supernatant was removed and spun again for 10 min. To 0.55 ml of the second supernatant, 0.55 ml of 8% trichloroacetic acid was added, followed by 0.3 ml of the agarose/BaCl\(_2\) solution. The purpose of agarose is to keep barium sulphate in suspension. A reagent blank was prepared using distilled water. Samples were vortexed and allowed to stand for 40 min, after which the absorbance at 595 nm was read. A calibration curve was constructed with potassium sulphate each time the assay was performed.

Protein. Protein determinations were performed using the Bio-Rad protein assay, [18], modified by Bradford [19]. A standard curve was constructed using standard BSA each time the assay was performed. Bio-Rad reagent (5 ml) was added to 10 ml of plasma and the test tubes were vortexed. After 30 min the blue colouring was fully developed and the absorbance of each sample was read at 595 nm in the u.v. spectrophotometer, against the blank.

In each case, samples were taken in triplicate. All analyses were preceded by formation of a standard curve. Samples were analysed blind and in duplicate. The intra-assay variation was <5% for analysis of cysteine, sulphate and protein.

Statistical analysis

The Wilcoxon rank sum test [20] was applied for the comparison of quantitative biochemical data of plasma samples for cysteine, sulphate and cysteine/sulphate ratio. The Kruskal–Wallis test was also applied to determine the statistical significance of variations of these parameters, within the liver disease groups, according to Child's Pugh score.
**RESULTS**

Plasma cysteine was expressed in nmol/mg of protein, plasma sulphate was expressed in nmol/mg of protein and the plasma cysteine/sulphate ratio was calculated. Results are represented graphically in Fig. 1. Plasma cysteine was lower in healthy subjects than in the disease groups. The differences were highly significant (healthy control subjects versus patients with PBC, \( P < 0.0001 \); versus patients with OLD, \( P < 0.0001 \); versus ITU patients, \( P < 0.0001 \)). Plasma cysteine was lower in PBC patients than OLD patients, but the difference was not statistically significant (\( P = 0.15 \)). Plasma cysteine in ITU patients was greatly elevated compared with all other groups (compared with healthy control subjects, \( P < 0.0001 \); with PBC patients, \( P < 0.0001 \) and OLD patients, \( P < 0.0001 \)).

Plasma sulphate was higher in healthy control subjects (1.37 nmol/mg of protein) than any disease group. The difference was significant only when compared with PBC patients (\( P < 0.05 \)) (compared with OLD patients, \( P = 0.10 \); compared with ITU patients, \( P = 0.14 \)). Plasma sulphate was lower in patients with PBC than patients with OLD, but not significantly (\( P = 0.57 \)). There was a non-significant reduction in patients with PBC compared with ITU patients (\( P = 0.44 \)).

The plasma cysteine/sulphate ratio in healthy control subjects (0.095) was significantly lower than that in all other groups (\( P < 0.0001 \) for all groups). The ratio in patients with PBC was not significantly different from that in patients with OLD (\( P = 0.98 \)). The ratio in ITU patients (1.650) was higher than that in all other groups (\( P < 0.0001 \)).

Because groups were poorly sex-matched, a separate analysis solely of females was performed. Females with PBC \( (n=29) \) and healthy control females \( (n=32) \) were compared using the Wilcoxon rank sum test [median plasma cysteine of PBC, 0.36, healthy control, 0.12 nmol/mg of protein (\( P < 0.0001 \)); median plasma sulphate PBC, 0.815, healthy control, 1.52 nmol/mg of protein (\( P < 0.05 \)); median plasma cysteine/sulphate ratio, PBC, 0.473, healthy control, 0.088 (\( P < 0.0001 \)).

### Analysis of plasma cysteine and sulphate levels by Child’s Pugh grade

Results are represented graphically in Fig. 2.

Plasma cysteine increased progressively with the grade of liver disease (grade A disease, 0.290 nmol/mg of protein; grade B disease, 0.350 nmol/mg of protein; grade C disease, 0.470 nmol/mg of protein). The difference was statistically significant between grades A and C (\( P < 0.005 \)) and between grades B and C (\( P < 0.05 \)), but not between grades A and B (\( P = 0.17 \)).

Plasma sulphate variations did not relate to the grade of liver disease. Plasma sulphate in grade A disease (0.68 nmol/mg of protein) was lowest, and in
Fig. 2. Scattergram of data points arising from analysis of plasma cysteine, plasma sulphate and plasma cysteine/sulphate ratio in the liver disease groups, according to Child's Pugh grade. Medians are represented by horizontal lines and 95% confidence intervals by bars transversing the median line. Median values and 95% confidence intervals, respectively, for the groups are as follows: plasma cysteine, Child's A, 0.29 and 0.25-0.37, Child's B, 0.35 and 0.28-0.47, Child's C, 0.47 and 0.40-0.56 nmol/mg of protein; plasma sulphate, Child's A, 0.68 and 0.19-1.15, Child's B, 1.19 and 0.59-2.10, Child's C, 0.82 and 0.45-1.41 nmol/mg of protein; plasma cysteine/sulphate ratio, Child's A, 0.59 and 0.237-1.088, Child's B, 0.36 and 0.159-0.445, Child's C, 0.57 and 0.356-1.000.

grade B (1.19 nmol/mg of protein) was highest. Differences were not significant [grade A compared with grade B, P = 0.07; grade A compared with grade C (0.82 nmol/mg of protein), P = 0.33; grade B compared with grade C, P = 0.28].

The plasma cysteine/sulphate ratio did not show consistent change with grade of liver disease. Grade A (ratio 0.591) was highest and grade B (ratio 0.358) the lowest. Only the difference between grades B and C (ratio 0.571) was significant (P < 0.05). The other differences were not significant (grade A versus grade B, P = 0.28; grade A versus grade C, P = 0.68).

Application of Kruskall–Wallis analysis, with two degrees of freedom, showed a significant difference in plasma cysteine values between the Child's Pugh groups (P = 0.002), but no significant difference between plasma sulphate (P = 0.09) or the plasma cysteine/sulphate ratio (P = 0.18).

DISCUSSION

This study analyses plasma cysteine and sulphate in liver disease and an heterogeneous group of ITU patients. Plasma cysteine and sulphate varied widely in disease. PBC was associated with an increase in plasma cysteine, concurrent with a low plasma sulphate. However, the effect was not disease-specific, as plasma cysteine rose in all forms of liver disease studied, the increment correlating with disease severity, as measured by Child's Pugh grade. An additional group of patients, enduring a prolonged state of fasting on ITU, were studied. Plasma cysteine was more elevated in this group than in patients with liver disease.

Elevation of cysteine in plasma appears to be a non-specific reflection of 'disease', although the feature common to all groups was malnutrition. ITU patients were fasting, while patients with liver disease usually have muscle wasting and negative nitrogen balance [21,22], which will release amino acids, including cysteine, into the plasma from tissue breakdown. Although impairment of the trans-sulphuration pathway could lead to reduced concentrations of cysteine [22,23], in practice the impairment of oxidative degradation of cysteine derived from tissues appears to outweigh this effect.

Cysteine occurs in plasma as the 'free' amino acid, containing a thiol group, as the disulphide dimer, cystine, and also bound to protein, linked through a thiol grouping [24]. When plasma samples are first taken, most of the cysteine (≈95%) exists in the free form, although cystine and cysteine–protein dimers form if the sample is left to stand. Cysteine itself is not readily measured by the standard amino acid analyser method, which converts it to cystine and cysteic acid before determination. The inaccuracies of the amino acid analyser method have been discussed by Malloy et al. [25]. The colorimetric method used in our assays gives far higher readings for cysteine in plasma as it is designed to be specific
for the compound [25,26]. Rapid sample handling and freezing also increases the free plasma cysteine values. These factors may account for the 6-fold differences in plasma cysteine values obtained utilizing an automated analyser in consecutive studies [23,27]. It is noteworthy that Chawla et al. [28], in a study using Gaitonde’s method [16] of cysteine analysis, also found a significant increase in cirrhotic patients compared with healthy control subjects, although use of a derivatization technique incorporating S-carboxymethyl-L-cysteine had previously given decreased values [29]. The majority of other workers [2] have concentrated on determinations of cystine.

In our study, plasma sulphate did not correlate clearly with disease states, apart from the difference between patients with PBC and healthy subjects. Sulphate is utilized for formation of steroid, bile acid and connective tissue conjugates, so that values obtained must be determined by the relative activity of endogenous metabolic pathways. Further studies are required, looking exclusively at patients with very early disease.

Generally, liver disease has been associated with disturbances of sulphur compound metabolism, including trans-methylation [1] and trans-sulphuration [2], with reduced activity of S-adenosylmethionine synthetase, hypermethioninaemia and impaired capacity to metabolize a methionine load [2]. There are a number of pathways by which plasma cysteine can be oxidized to generate inorganic sulphate. The cytosolic enzyme cysteine dioxygenase is believed to be quantitatively the most important in man [9,11,12], although a mitochondrial pathway is also known to exist [30]. This mitochondrial pathway is enhanced by glutathione (glutamyl-cysteinyl-glycine), the synthesis of which is driven by cysteine concentrations. It seems possible, therefore, that a ‘feedback’ mechanism exists, in which high plasma cysteine indirectly activates a mitochondrial system of oxidative enzymes. Inhibition of either or both pathways in critical disease would lead to raised cysteine levels; the controlling factor may be the capacity of glutathione synthetase, as decreased glutathione is known to occur in cirrhosis [2], despite high cysteine levels. Whatever the precise aetiology of these defects, they will tend to impair detoxication pathways via glutathione conjugation or sulphate formation, and will therefore increase susceptibility to both endogenous and exogenous toxins.

In summary, major alterations occur in plasma levels of cysteine and sulphate in disease states. The plasma cysteine/sulphate ratio is greatly elevated both in liver disease and an heterogeneous ITU population. Because these effects are not specific to PBC, they are more likely to represent interference in the trans-sulphuration pathway and alterations in the balance of protein turnover, rather than a primary influence of the proposed genetic polymorphism in PBC. These metabolic changes may progressively increase the capacity of the liver and other tissues to detoxify xenobiotics and endogenously derived toxins as disease advances.

REFERENCES


