

Inhibition of Acetaminophen Oxidation by Cimetidine and the Effects on Glutathione and Activated Sulphate Synthesis Rates

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Abstract: The aim of the present study was to examine the effects of the hepatotoxic drug, acetaminophen, on the synthesis rates of glutathione, activated sulphate (PAPS, adenosine 3'-phosphate 5'-phosphosulphate) and the acetaminophen metabolites, acetaminophen-glutathione and acetaminophen-sulphate after inhibition of cytochrome P-450 drug oxidation by cimetidine in isolated rat hepatocytes. The synthesis rates of glutathione and PAPS were determined simultaneously by an established method based on trapping of radioactivity (³⁵S) in the prelabelled glutathione and PAPS pools. Preincubation of the hepatocytes with 60 µg/ml cimetidine for 30 min. did not affect PAPS (1.71 versus 1.78 nmol/10⁶ cells) nor glutathione concentration (16.0 versus 16.4 nmol/10⁶ cells). The subsequent incubation with 5 mM acetaminophen resulted in decreased PAPS synthesis in the cimetidine treated cells [0.79 · 10³ versus 0.92 · 10³ nmol/(10⁶ cells · hr)] (P < 0.05). There was no difference in PAPS concentration or acetaminophen-sulphate synthesis [1.73 versus 1.79 nmol/10⁶ cells and 13.0 versus 12.9 nmol/(10⁶ cells · hr), respectively]. Decreased PAPS synthesis may be related to decreased ATP supply or may be the result of a feed-back regulation due to diversion of sulphur from glutathione synthesis to sulfoxidation. The glutathione synthesis was not significantly affected by cimetidine treatment [57 · 10³ versus 27 · 10³ nmol/(10⁶ cells · hr)]. As expected acetaminophen-glutathione synthesis decreased by 38% [1.66 versus 2.68 nmol/(10⁶ cells · hr)] (P < 0.01). Also the glutathione concentration was lower in cimetidine treated cells [15.2 versus 15.9 nmol/10⁶ cells] (P < 0.05). We have previously shown that glutathione synthesis was reduced if substrate availability decreased (acetaminophen concentration lowered). Thus, the unaltered glutathione synthesis observed in the present study in which N-acetyl-p-benzoquinoneimine formation was diminished suggests that cimetidine does not inhibit all acetaminophen metabolites which utilize reduced glutathione.

Cimetidine, a 4-5 substituted imidazole used as a histamine H₂-receptor antagonist, has been demonstrated to cause dose-related inhibition of cytochrome P-450-mediated oxidation *in vivo* and *in vitro* (Speeg *et al.* 1982; Knodell *et al.* 1982; Desmond *et al.* 1980). Hepatotoxicity due to acetaminophen overdose is related to formation of an acetaminophen P-450 oxidized reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which once formed, may alkylate hepatic macromolecules unless detoxified by conjugation with glutathione (Dahlin *et al.* 1984).

By means of a radioactive tracer method (Dalhoff & Poulsen 1992), simultaneously measuring PAPS and glutathione synthesis rates, we have previously shown that if glutathione synthesis is inhibited by buthionine sulphoximine preceding administration of a toxic acetaminophen concentration, sulphur is diverted from glutathione synthesis to sulfoxidation and PAPS synthesis maintaining the PAPS pool at a certain level (Dalhoff & Poulsen 1993).

The aim of the present study was to examine if sulphur could be diverted from glutathione synthesis to sulfoxidation if the demand for glutathione was diminished due to decreased production of NAPQI by inhibition of P-450 oxidation of acetaminophen with cimetidine. Secondly we wanted to examine the effect of cimetidine on sulphation, especially on PAPS synthesis, since very limited information

is available concerning the effect of histamine H₂-receptor antagonists on this essential cosubstrate in drug sulphation. Knowledge of the effect on sulphation is important, since cimetidine has been suggested as an adjunctive to N-acetylcysteine in the treatment of acetaminophen intoxication (Speeg 1987; Rolband & Marcuard 1991), and since sulphation and glucuronidation ultimately will be responsible for the majority of acetaminophen elimination.

Materials and Methods

Female Wistar rats (180-205 g, Møllegaards Avlslaboratorium, L.L. Skensved, Denmark) were fasted for 16 hr before the experiments, housed in an air-conditioned environment with a controlled 12 hr light-dark cycle. They had free access to water. One hr before hepatocyte isolation ³⁵S-L-cysteine (80-130 µCi, specific activity > 600 Ci/mmol, Amersham Denmark ApS) was given intraperitoneally.

Hepatocyte isolation was performed after collagenase perfusion (Berry & Friend 1969; Seglen 1975) as previously described in detail (Dalhoff & Poulsen 1992). The hepatocytes were divided into two fractions; one was preincubated for 30 min. with 60 µg/ml cimetidine in Krebs-Ringer buffer, the other was preincubated for 30 min. with an identical amount of buffer. After preincubation the experiments were started by addition of 5 mM acetaminophen to each cell fraction and synthesis rates of PAPS, glutathione, acetaminophen-sulphate, and acetaminophen-glutathione were measured together with the concentrations of PAPS and glutathione during a 30 min. incubation period.

Table 1.

Median (interquartile distances) turnover rates, concentrations and synthesis rates of activated sulphate (PAPS), glutathione, acetaminophen-sulphate and acetaminophen-glutathione in isolated hepatocytes *preincubated* in the presence or absence (controls) of 60 µg/ml cimetidine and subsequently *incubated* with 5 mM acetaminophen.

	Turnover rate of activated sulphate hr ⁻¹ (· 10 ³)	Concentration of activated sulphate nmol/10 ⁶ cells	Synthesis rate of activated sulphate nmol/(10 ⁶ cells · hr) (· 10 ³)	Synthesis rate of acetaminophen-sulphate nmol/(10 ⁶ cells · hr)
Controls	0.67 (0.18–0.72)	1.79 (1.52–2.36)	0.92 (0.30–2.03)	12.9 (10.8–15.5)
Cimetidine	0.56* (0.11–0.66)	1.73 (1.48–2.38)	0.79* (0.19–1.52)	13.0 (11.8–14.3)
	Turnover rate of glutathione hr ⁻¹ (· 10 ³)	Concentration of glutathione nmol/10 ⁶ cells	Synthesis rate of glutathione nmol/(10 ⁶ cells · hr) (· 10 ³)	Synthesis rate of acetaminophen-glutathione nmol/(10 ⁶ cells · hr)
Controls	1.7 (0.8–3.4)	15.9 (13.8–20.4)	27 (9–54)	2.68 (2.37–3.11)
Cimetidine	3.6 (0.8–6.5)	15.2* (13.4–19.1)	57 (17–127)	1.66* (1.44–2.03)

* Significant different from control experiments (P < 0.05).

Analytical methods. All the analytical analyses were performed according to our previous study (Dalhoff & Poulsen 1992). PAPS was measured using a modification of a method of Hazelton *et al.* (1985). Total glutathione was measured by the glutathione reductase 5,5'-dithiobis (2-nitrobenzoic acid) assay of Tietze (1969). Acetaminophen metabolites were measured and fractionated on HPLC (Waters Associates) and radioactivity of the peaks containing acetaminophen-sulphate and acetaminophen-glutathione metabolites was determined by liquid scintillation spectrometry (Packard Instruments) with quench correction by the channels ratio technique.

Calculations. Turnover rates of PAPS and glutathione were determined from the decline in specific activities in acetaminophen-sulphate and acetaminophen-glutathione by calculating the turnover rate constants k_{PAPS} and $k_{\text{glutathione}}$. These rates were multiplied with the mean concentrations of PAPS and glutathione, respectively, during the incubation period to give the synthesis rates (Dalhoff & Poulsen 1992).

All values are expressed as medians with interquartile distances. The differences between turnover rates, concentrations and synthesis rates were tested by a Wilcoxon-test for paired observations. The level of significance was 0.05.

Results

At the end of the preincubation period the PAPS concentration was 1.78 (1.50–2.42) nmol/10⁶ cells and the glutathione concentration was 16.4 (13.1–18.4) nmol/10⁶ cells in the fraction of hepatocytes treated with cimetidine. The corresponding values in the fraction of cells which were not treated with cimetidine were 1.71 (1.51–2.37) nmol/10⁶ cells and 16.0 (14.1–20.4) nmol/10⁶ cells, respectively.

Table 1 shows the difference in turnover rates, concentrations and synthesis rates of PAPS, glutathione, acetaminophen-sulphate and acetaminophen-glutathione between hepatocytes preincubated with cimetidine and subsequently incubated with 5 mM acetaminophen, and cells which were preincubated with Krebs-Ringer buffer instead of cimetidine (controls) and subsequently with 5 mM acetaminophen.

PAPS synthesis rate decreased by 14% due to cimetidine, corresponding to decreased PAPS turnover rate. There was no difference in PAPS concentration between cells preincubated with cimetidine and cells which were preincubated by Krebs-Ringer buffer alone, nor was there any difference in

acetaminophen-sulphate synthesis rate between the cimetidine and the control cell fraction.

Except for the concentration values the variation of the glutathione data was considerably higher than the variation of the PAPS data. However, cimetidine did not seem to affect glutathione synthesis rate corresponding to unaltered glutathione turnover rate. However, both the glutathione concentration and the acetaminophen-glutathione synthesis rate decreased in the cimetidine cell fraction by 4% and 38%, respectively.

Fig. 1 shows the relative changes in PAPS, glutathione, acetaminophen-sulphate and acetaminophen-glutathione synthesis rates together with the concentrations of PAPS and glutathione during the preincubation and the incubation periods of each individual experiment.

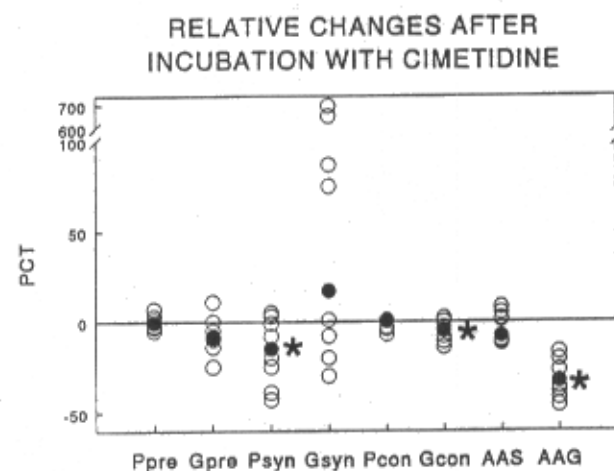


Fig. 1. Relative changes in concentrations of PAPS (Ppre) and glutathione (Gpre) in eight single paired experiments with isolated hepatocytes after *preincubation* with cimetidine, and relative changes in synthesis rates of PAPS (Psyn) and glutathione (Gsyn), in concentrations of PAPS (Pcon) and glutathione (Gcon) and in synthesis rates of the acetaminophen metabolites, acetaminophen-sulphate (AAS) and acetaminophen-glutathione (AAG) after subsequent *incubation* with acetaminophen. Filled out circles represent median values. Asterixes indicate significant changes (P < 0.05).

Discussion

The present study demonstrated decreased PAPS synthesis when a toxic acetaminophen concentration was given to hepatocytes preincubated with cimetidine. Cimetidine itself did not cause any significant change in hepatocellular PAPS concentration nor did it influence acetaminophen-sulphate synthesis. Furthermore we demonstrated that hepatocellular glutathione synthesis was unaffected by cimetidine, but that the glutathione concentration in the cimetidine preincubated cells were slightly lower than in cells only treated with a toxic acetaminophen concentration. Finally we were able to show significantly decreased acetaminophen-glutathione synthesis.

De novo PAPS synthesis rate was reduced by 15% in the cimetidine treated hepatocytes. In freshly isolated hepatocytes cimetidine has been shown to be relatively non-toxic based on measurements of cellular enzyme leakage, oxygen consumption and inhibition of protein synthesis (Oldham *et al.* 1985). However, in more recent studies cimetidine analogues have been shown to be cytotoxic, the toxicity evident in a variety of different cimetidine analogues (Rush *et al.* 1988) and related to their lipid solubility. Later it has been demonstrated that the cimetidine analogue oxmetidine, which produces a marked and rapid inhibition of cellular respiration and depletion of ATP, may inhibit mitochondrial electron transport by "short circuiting" the flow of electrons between NADH dehydrogenase and ubiquinone (Hoke *et al.* 1990). PAPS synthesis is highly dependent on mitochondrial ATP production (Roy 1971) which may have been affected, at least to some extent, by cimetidine in the present study. Although PAPS synthesis decreased, the cellular PAPS concentration was maintained on a level sufficient to keep the acetaminophen sulphation capacity constant. This is in accordance with studies of hepatocytes maintained in cultures containing acetaminophen (0.5 and 5 mM) and cimetidine (1 mM) or acetaminophen without cimetidine (Emery *et al.* 1985). After 24 hr acetaminophen sulphation was identical in the two cell cultures.

Alternatively the reduction in PAPS synthesis after cimetidine administration might be the result of a feed-back regulation due to a diversion of sulphur from cysteine to sulfoxidation, resulting in decreased *de novo* synthesis of PAPS in order to maintain the PAPS concentration constant. More sulphur from cysteine is available when the demand in the glutathione synthesizing gamma-glutamyl cycle for cysteine is reduced due to a reduced concentration of NAPQI to be detoxified.

Finally cimetidine might be able to inhibit other sulphation pathways, hereby making more PAPS available for acetaminophen sulphation, resulting in decreased *de novo* PAPS synthesis.

In the present study cimetidine decreased acetaminophen-glutathione synthesis by 38%. This is in accordance with several other studies in which cimetidine inhibited the formation of the highly reactive electrophilic species NAPQI which normally is conjugated with glutathione forming acet-

aminophen-glutathione. Cimetidine has been demonstrated to cause dose-related inhibition of cytochrome P-450-mediated microsomal drug metabolism. In our study the P-450 inhibition did not influence glutathione synthesis which continued to be very high compared to acetaminophen-glutathione synthesis, indicating that in hepatocytes with normal glutathione content only a minuscule part of *de novo* glutathione synthesis is used for acetaminophen detoxification. Freshly isolated hepatocytes are under enormous oxidative stress (Sies *et al.* 1983; Jones *et al.* 1978), leading to increased glutathione peroxidase activity, increased glutathione consumption and increased glutathione synthesis.

The glutathione status in the liver is maintained mainly in the reduced state (reduced:oxidized glutathione 250), which is achieved by the export of a small but relative constant fraction of GSSG from the cell, and by the efficient glutathione peroxidase and reductase system coupled to NADP⁺/NADPH redox pair. At the expense of hepatocellular NADPH, oxidized glutathione is effectively reduced back to glutathione maintaining the thiol balance. H₂-Receptor antagonists seem to have an effect on mitochondrial homeostasis leading to decreased NADPH supply and consequently to changes in reduced:oxidized glutathione status resulting in decreased hepatocellular glutathione concentration available for acetaminophen detoxification, explaining the small difference in glutathione concentration between cimetidine preincubated cells and hepatocytes which were not preincubated with cimetidine. Oxidized glutathione content presumably increased but has not been measured in the present study.

We have previously shown that increased acetaminophen concentration resulted in increased *de novo* synthesis of glutathione presumably due to increased availability of NAPQI. It would therefore seem possible that diminished NAPQI formation would result in decreased *de novo* synthesis of glutathione. However, in the present study, in which decreased NAPQI formation is documented, we found *unaltered* glutathione synthesis indicating that glutathione is still needed for detoxification of an acetaminophen metabolite. It is possible that cimetidine prevented cytochrome P-450-mediated formation of some hepatotoxic metabolites but did not inhibit formation of all acetaminophen metabolites that bind to glutathione. This would support the finding by Peterson *et al.* (1983) who showed that cimetidine protected against acetaminophen toxicity by preventing hepatocellular necrosis, but that the covalent binding of acetaminophen to hepatocellular proteins and the glutathione depletion caused by acetaminophen were not prevented by cimetidine. Other studies reported conflicting results regarding the inhibitory effect of cimetidine on covalent binding of acetaminophen to liver proteins and glutathione depletion (Mitchell *et al.* 1984; Speeg *et al.* 1985). However, *de novo* synthesis of glutathione was not measured and different results may be related to differences in methodology, drug doses, pretreatment with microsomal drug oxidation inducers and animal species.

Cimetidine has been suggested as an adjunctive to N-

acetylcysteine in the antidotal regimen in the treatment of acetaminophen intoxication. It is therefore important to establish whether cimetidine causes any unwanted reactions to the non-toxic metabolic pathways of acetaminophen elimination, since sulphation together with glucuronidation ultimately will be responsible for most acetaminophen elimination. Animal and human studies have demonstrated that formation of acetaminophen-sulphate was not affected by cimetidine. However, the metabolism of PAPS which is the essential cosubstrate in sulphation of not only drugs but also of many endogenous substances has not been studied in detail. If the decreased PAPS synthesis seen in the present study is related to cimetidine treatment, and it progresses further resulting in reduced PAPS concentration, it could compromise the metabolism of many essential endogenous substances, depending on the supply of PAPS. Transfer of sulphate groups in the Golgi apparatus to macromolecules such as chondroitin sulphate, dermatan sulphate and keratan sulphate which maintain the extracellular matrix is highly dependent on a sufficient supply of PAPS (Schwarz *et al.* 1984; Hay 1981). It seems that the hepatocytes has a great ability to maintain the PAPS pool constant, but we recommend that great caution be observed when treatment with several drugs which undergo sulphation is considered.

In conclusion we found that cimetidine decreased PAPS synthesis in hepatocytes incubated with a toxic acetaminophen concentration without affecting neither PAPS concentration nor acetaminophen-sulphate synthesis. Glutathione metabolism, which was measured simultaneously with PAPS synthesis, showed slightly decreased glutathione concentration after cimetidine incubation but unaltered glutathione synthesis. As expected acetaminophen-glutathione synthesis decreased after cimetidine treatment.

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