Cephalosporin-induced Alteration in Hepatic Glutathione Redox State

A Potential Mechanism for Inhibition of Hepatic Reduction of Vitamin K₁,2,3-epoxide in the Rat

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Abstract

Hypoprothrombinemia is a serious adverse effect of antimicrobial therapy that occurs after administration of some second- and third-generation cephalosporins which contain the methyltetrazole-thiol (MTT) group. Previous studies have shown that in vitro MTT directly inhibits micromolar γ-carboxylation of a synthetic pentapeptide. Since MTT is a thio-carbamide, a type of compound that can increase oxidation of glutathione, the present studies were carried out to determine whether alterations in hepatic glutathione redox state might interfere with vitamin K metabolism.

Dose-related increases in biliary efflux and hepatic concentration of oxidized glutathione (GSSG) occurred after intravenous administration of MTT or MTT-containing antibiotics to rats. This finding suggested that these compounds could alter the hepatic glutathione redox state in vivo. Micromolar reduction of vitamin K epoxide occurred in the presence of 100 μM dithiothreitol (DTT), but was inhibited by preincubation with GSSG at concentrations as low as 10 μM. At higher concentrations of DTT (1.0 mM) inhibition by GSSG persisted, but higher concentrations were required, suggesting that the thiol/disulfide ratio, rather than the absolute concentration of GSSG was important. By contrast, GSSG did not effect micromolar γ-carboxylation of a pentapeptide, using either vitamin K₁ or its hydroquinone as a cofactor. These findings suggest a novel mechanism for the hypoprothrombinemia occurring after administration of MTT-containing antibiotics. (J. Clin. Invest. 1990, 86:1589–1594.) Key words: γ-carboxylation • drug metabolism • methyltetrazole thiol • prothrombin time • thio-carbamides

Introduction

Hypoprothrombinemia occurs in some patients treated with β-lactam antibiotics containing the methyltetrazole-thiol (MTT) group. This group is contained in cefamandole, moxalactam, cefoperazone, and other second- and third-generation cephalosporins (Fig. 1). In some instances, clinically significant bleeding episodes have occurred, particularly in patients who are fasting, malnourished, immediately postoperative, or have decreased renal function (1).

The mechanism for development of hypoprothrombinemia after administration of MTT-containing antibiotics is the subject of controversy. Some investigators have speculated that the effect is related to alterations in colonic flora which result in vitamin K deficiency (2). Another hypothesis is that these drugs inhibit the vitamin K-dependent step in clotting factor synthesis, the γ-carboxylation of glutamic acid (3, 4). Failure of carboxylation of the clotting factors could be due either to inhibition of vitamin K metabolism or by direct inhibition of the carboxylation reaction. Metabolism of vitamin K includes a cycle that involves reduction of vitamin K₁ (a quinone), to a hydroquinone, oxidation of the hydroquinone to an epoxide, and finally the reduction of the epoxide to vitamin K₁ (5).

Previous studies have shown that MTT inhibits in vitro microsomal γ-carboxylation of a synthetic peptide (Ph–Leu–Glu–Glu–Ile) (3, 4). This finding suggests that the MTT group, or an active metabolite of this compound might be responsible for the anticoagulant effects. The finding of increased vitamin K epoxide in plasma of patients treated with moxalactam and cefamandole is consistent with an inhibition of the reduction of vitamin K epoxide (6), an effect which cannot be due to vitamin K deficiency.

MTT is a thio-carbamide, a class of compounds which may be metabolized by a flavin-containing monooxygenase to more reactive intermediates, such as sulfenic acids (7). Krieter and his colleagues (8) have shown that infusion of thio-carbamides into the isolated perfused rat liver causes in nonenzymatic oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) with subsequent release of GSSG into bile (8). Such alterations in the ratio of GSH to GSSG may effect the activity of various enzymes (8–12). Under physiologic conditions, the ratio of GSH to GSSG is tightly controlled, but after pharmacologic intervention, large shifts can occur.

The present studies were carried out to evaluate whether MTT-containing antibiotics could inhibit vitamin K metabolism through effects on the hepatic thiol/disulfide ratio. Changes in the hepatic content of GSSG and vitamin K₁,2,3-epoxide were measured to provide evidence that MTT-containing antibiotics alter both the hepatic thiol redox state and vitamin K metabolism in vivo. The effects of GSSG on microsomal reduction of vitamin K₁,2,3-epoxide were studied to

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Abbreviations used in this paper: GSSG, oxidized glutathione; MTT, methyltetrazole-thiol.
determine whether increases in the ratio of GSSG/GSH could explain the impairment in reduction of vitamin K epoxide observed after administration of cephalosporins containing MTT.

**Methods**

**Microsomal γ-carboxylation of glutamic acid residues.** Rat liver microsomes were prepared for determination of the carboxylation of glutamic acid by the method of Houser et al. (14) with the previously described modifications (3). In brief, reactions were carried out in buffer containing 25 mM imidazole, 250 mM sucrose, and 20 mM KCl, pH 7.4, which contained the following substrates: l-phenylala

**Fig. 1. Structures of MTT and the antibiotics cefamandole and moxalactam which contain this side group.**

after administration of test compounds, and flow was determined gravimetrically. After centrifugation to remove precipitated protein, bile was assayed for GSSG and "total" GSH (reduced GSH + GSSG) as described (15). A specific assay for GSSG monitored the oxidation of NADPH at 340 mm in the presence of glutathione reductase. Total GSH was measured using the NADPH glutathione reductase coupled oxidation of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) monitored at 412 nm.

**Hepatic concentrations of glutathione after administration of cefamandole.** Male Sprague-Dawley rats weighing 200–250 g, allowed free access to food and water, were anesthetized with sodium pentobarbital 50 mg/kg of i.p. The abdomen was opened and the liver was rapidly freeze-clamped with aluminum tongs precooled in liquid nitrogen. Frozen tissue was pulverized under liquid nitrogen and extracted with 5–10 vol of perchloric acid, 2 mM EDTA for total glutathione (reduced + oxidized) or 1.0 M perchloric acid containing 2 mM EDTA and 50 mM N-ethylmaleimide for GSSG. For analysis of total glutathione, extracts were diluted into 100 mM sodium phosphate buffer, pH 7.5 and assayed as described above. For analysis of GSSG, the acid extract was carefully neutralized with 2.0 M KOH containing 0.3 M 3-[N-morpholinio]-propanesulfonic acid as described (15). Neutralized supernatants were extracted on solid-phase C18 columns (Bakerbond, J. T. Baker, Inc., Phillipsburg, NJ). Columns were washed with 1.0 ml of 100 mM sodium phosphate buffer, pH 6.0. Removal of N-ethylmaleimide was verified by checking the optical density at 315 nm. GSSG was assayed using the DTNB recycling assay as described above. Recovery was > 90% routinely.

**Effects of MTT-containing antibiotic on hepatic vitamin K1, 2,3-epoxide.** Male Sprague-Dawley rats weighing 200–250 g allowed free access to food and water, were given 3 g/kg of cefamandole by intraperitoneal injection each day for 5 d. 24 h after the last injection, animals were killed under pentobarbital anesthesia. Liver (100 mg) was homogenized in 2 ml of sucrose-imidazole buffer and extracted for 10 min with 2 vol of hexane/isopropanol (1:1, vol/vol) (16). The organic phase was dried under a stream of nitrogen and the residue dissolved in 100 µl of methanol. 20-µl aliquots were injected for measurement of vitamin K1 and vitamin K2, 2,3-epoxide.

**Synthesis, purification, and analysis of vitamin K1, 2,3-epoxide and vitamin K1 by high pressure liquid chromatography (HPLC).** Vitamin K1, 2,3-epoxide was synthesized chemically from vitamin K1, by the method of Tisher et al. (16). The epoxide was further purified by HPLC separation on a reversed phase C18 preparative column (30 cm x 4.8 mm i.d.) (Waters Associates, Milford, MA) using methanol as a mobile phase. For analytical purposes, an Excellopak C-18 column (15 mm x 4.6 mm i.d.) (R. G. Gourley Co., Laurel, MD) was used to separate vitamin K1 and vitamin K1 epoxide. Retention times of vitamin K1 and vitamin K1 epoxide were 4.8 and 3.9 min, respectively. Peaks were detected at 254 and at 220 nm using a Spectromonitor III (Lab Data Control, Riviera Beach, FL), or a diode array detector (LKB, Uppsala, Sweden). Quantitation was carried out by integration of peak areas using a recording integrator (Hewlett-Packard Co., Palo Alto, CA).

**Preparation of KH2 by reduction of vitamin K1.** Vitamin K1 was reduced chemically by sodium dithionite (hydrosulfite) as described (17). Reduction was confirmed by UV spectral analysis just before use.

**Microsomal reduction of vitamin K1, 2,3-epoxide.** Rat hepatic microsomes were prepared using a modification of the method described (18) in which the microsomal pellet was suspended in 25 mM imidazole, pH 7.2, containing 0.5% [3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and diluted to a concentration of 10 mg of microsomal protein/ml. Vitamin K epoxide reductase activity was assayed by a modification of the method of Wallin and Martin (19). The solubilized microsomal fraction was incubated with varying concentrations of vitamin K1, 2,3-epoxide in the presence of 0.1–5.0 mM DTT at 22°C for 30 min. The reaction was terminated by the addition of 2 vol hexane/isopropanol (1:1, vol/vol). Vitamin K metabolites were extracted by vortexing for 30 s followed by centrifugation at 2,000 g for 10 min. The organic phase was transferred to glass tubes and dried under a stream of N2 (N-Evap, Organomation Asso-
concentrations, ion, exhibited y-carboxylation of vitamin K, since neither compound is endogenously present in the microsomal preparation. Recovery was > 90% in all experiments.

Statistics. Analysis of variance for repeated measures was used with Duncan's test for multiple comparisons to compare changes in biliary efflux of GSSG after antibiotics in each experiment. Student's t-test was used to compare group means.

Results

Inhibition of microsomal y-carboxylation of glutamic acid. Since previous work had shown that MTT is able to inhibit microsomal carboxylation of glutamic acid (3, 4), concentration inhibition curves for this effect were established for the intact MTT-containing antibiotics, cefamandole and moxalactam, and compared to the previous results obtained with MTT. Neither moxalactam nor cefamandole inhibited y-carboxylation > 20% when added in concentrations up to 2.5 mM (Fig. 2). Addition of MTT to microsomes inhibited y-carboxylation of glutamic acid in a dose-dependent fashion as shown in Fig. 2 with an IC50 concentration between 0.5 and 0.6 mM.

The effect of GSSG upon carboxylation was examined. As shown in Table I, GSSG had only minimal effect upon carboxylation. When the hydroquinone (KHSO3) was used to initiate the reaction in the absence of NADH, no inhibition was observed, indicating no direct effect of GSSG upon the carboxylation reaction itself.

Effects of MTT and antibiotics on biliary efflux and hepatic concentrations of GSH and GSSG. After i.v. administration of MTT there was a rapid and striking, dose-related increase in both biliary GSSG concentration and efflux (Fig. 3 A) (P < 0.05 for 2.5 and 5.0 mmol/kg vs. baseline). Baseline GSSG output was 0.36±0.07 nmol/min per g liver. After 60 min from the time of injection, the increase in GSSG efflux returned toward baseline.

After administration of cefamandole, but not moxalactam or MTT, there was increased biliary efflux of GSH (P < 0.01 vs. baseline), which was not accounted for by the increase in efflux of GSSG alone (Fig. 4). This finding was unexpected since administration of other thiosemicarbazides such as methimazole does not alter biliary GSH output (10, M.C. Mitchell, unpublished observations).

Administration of the intact antibiotics cefamandole and moxalactam also increased the biliary efflux of GSSG (P < 0.05 vs. baseline) to a similar extent as MTT on a molar basis (Fig. 3 B). In addition, both cefamandole and moxalactam significantly increased bile flow: from 1.36±0.22 to 3.74±0.45 μl/min per g liver and from 1.57±0.16 to 3.40±0.33 μl/min per g, respectively (P < 0.05). Hepatic levels of GSSG were significantly higher 30 min after intravenous injection of 2.5 mmol/kg of cefamandole, confirming the increased biliary efflux of GSSG was accompanied by higher tissue levels. The ratio of GSH to GSSG was significantly lower at the same time, indicating a change in the intracellular thiol/disulfide ratio (Table II).

Effects of cefamandole on hepatic vitamin K1,2,3-epoxide. Administration of MTT-containing antibiotics to rats has been shown to prolong plasma prothrombin times (20). Cefamandole was given daily to rats for 3 d to determine whether this treatment was also associated with changes in the relative amounts of vitamin K1 and vitamin K1,2,3-epoxide. After administration of cefamandole, the amount of vitamin K epoxide expressed as a percentage of vitamin K metabolites was significantly higher, 7.87±1.03 compared to 4.10±0.50 in control animals (P < 0.05). These observations confirm that both prolongation of the prothrombin time (20) and inhibition of the reduction of vitamin K epoxide occurs in rats as well as in humans receiving MTT-containing cephalosporins (6).

Effects of GSSG on microsomal reduction of vitamin K1,2,3-epoxide. Reduction of vitamin K1,2,3-epoxide to vitamin K1, in solubilized microsomes required the presence of a thiol as well as K epoxide. The physiological thiol required for this reaction remains unknown. Data in Table III give comparisons of activity for various concentrations of thiols. On a molar basis, the dithiol, DTT, was significantly better than

![Figure 2. Effects of intact cephalosporins and MTT on microsomal y-carboxylation of a synthetic pentapeptide. Microsomal y-carboxylation was performed as described in Methods. Addition of MTT inhibited carboxylation of glutamic acid residues in a dose-related fashion, while neither moxalactam nor cefamandole inhibited > 20% in concentrations up to 2.5 mM.](image)

Table I. Effects of GSSG on Microsomal y-Carboxylation of a Synthetic Pentapeptide

<table>
<thead>
<tr>
<th>GSSG</th>
<th>Vitamin K form</th>
<th>NADH</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>1.0</td>
<td>Quinone</td>
<td>2.0</td>
<td>5±6</td>
</tr>
<tr>
<td>2.5</td>
<td>Quinone</td>
<td>2.0</td>
<td>3±6</td>
</tr>
<tr>
<td>5.0</td>
<td>Quinone</td>
<td>2.0</td>
<td>2±7</td>
</tr>
<tr>
<td>5.0</td>
<td>Hydroquinone</td>
<td>0</td>
<td>0±6</td>
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Rat hepatic microsomal y-carboxylation of a synthetic peptide (Phe-Leu-Glu-Glu-ile) was determined by the incorporation of 14CO2 into the peptide as described in Methods. Varying concentrations of GSSG were preincubated with microsomes for 2 min before addition of vitamin K to initiate the reaction. As shown, GSSG had no statistically significant effect on y-carboxylation. Data shown are the mean±SE results of three experiments.

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GSH in supporting this reaction. Similar findings have been reported for the human liver enzyme (18). Some activity could be detected at concentrations of DTT as low as 0.05 mM.

Preincubation of solubilized microsomes for 2 minutes with GSSG blocked the reduction of vitamin K epoxide at 0.1 mM DTT in a concentration-dependent manner (Fig. 5). Under these conditions inhibition was detected at concentrations as low as 0.01 mM GSSG. At a higher concentration of DTT (1.0 mM), a higher concentration of GSSG was required to achieve the same degree of inhibition.

In separate experiments, 0.1 mM GSSG reduced the activity of epoxide reductase to 57% of control in the presence of 0.1 mM DTT. Further addition of DTT to achieve a final concentration of 1.1 mM DTT restored activity to 95% of control, whereas addition of buffer did not alter the inhibition observed at 30 min.

To determine whether GSSG causes nonenzymatic oxidation of DTT, thereby depleting a necessary cofactor for vitamin K epoxide reductase; oxidation of DTT was monitored spectrophotometrically as described by Ciecielski (21). In the presence of 0.2 mM GSSG, 50% of 0.1 mM DTT was oxidized over 30 min. At this concentration of GSSG, the activity of epoxide reductase was completely inhibited (Fig. 5). If the observed inhibition of the reductase was due entirely to a decrease in the concentration of reduced DTT, one would expect to find no activity in the presence of 0.05 mM DTT. However, activity of the reductase was 63% of that observed in the presence of 0.1 mM DTT. These observations suggest that, although GSSG may cause nonenzymatic oxidation of DTT, its effects upon the activity of epoxide reductase are not due entirely to this mechanism.

Discussion

The results of this study provide new insight into how METT, a thio carbamide side group of several β-lactam antibiotics, may cause hypoprothrombinemia. Administration of METT-containing antibiotics can produce hypoprothrombinemia (20) and alter vitamin K metabolism in the rat (22). Vitamin K

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total GSH</th>
<th>GSSG</th>
<th>2 GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/g liver</td>
<td>nmol/g liver</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>6.73±0.65</td>
<td>52±8.1</td>
<td>318±62</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>6.11±0.44</td>
<td>71±5.3*</td>
<td>182±21*</td>
</tr>
</tbody>
</table>

Livers of rats were freeze-clamped 30 min after treatment with intravenous cefamandole (2.5 mmol/kg) or saline. Total glutathione (reduced and oxidized) and GSSG were measured as described in the methods. As shown, hepatic GSSG was increased significantly and the ratio of GSH/GSSG decreased significantly by cefamandole. Data shown are the mean±SE of nine animals in each group. * P < 0.05.

Figure 4. Effects of intravenous administration of cefamandole (2.5 mmol/kg) on biliary efflux of GSH (•) and GSSG (○). Cefamandole resulted in a marked increase in biliary efflux of both "total" glutathione (GSH + GSSG) and GSSG. Baseline GSH efflux was 1.68±0.23 nmol/min per g liver. Baseline GSSG efflux was 0.32±0.06 nmol/min per g liver. The larger increase in "total" glutathione (reduced + oxidized) compared to GSSG alone shows that cefamandole increased efflux of reduced GSH as well as GSSG. Neither moxalactam nor METT increased efflux of reduced GSH (data not shown). Results shown are the mean±SE expressed as a percentage of baseline for eight animals.

Table II. Effects of Cefamandole on Hepatic Concentrations of Glutathione
metabolism may be indirectly altered by MTT through formation of GSSG in addition to the previously reported direct effects of MTT upon γ-carboxylation of clotting factors (3–5, 22). The increased amount of vitamin K_1,2,3-epoxide as a percentage of vitamin K metabolites observed after cefamandole is consistent with the increase in plasma vitamin K_1,2,3-epoxide seen in humans receiving these same drugs (6). Both observations strongly suggest that the reduction of vitamin K epoxide to vitamin K_1 is inhibited by these cephalosporins and could contribute to development of hypoprothrombinemia. The similarity of findings in the rat and humans indicates that the rat is an appropriate animal model for exploring further the mechanism underlying these effects.

The increased hepatic concentration and biliary efflux of GSSG after administration of MTT and cefamandole demonstrated in these studies is consistent with previously reported findings with other thio carbamides (8). Therefore, we investigated the possibility that GSSG, an endogenous disulfide, might interfere with reduction of vitamin K epoxide or directly inhibit carboxylation of glutamic acid or both. Although we did not find evidence for direct inhibition of carboxylation by GSSG, our results show that GSSG can inhibit reduction of vitamin K_1,2,3-epoxide in a concentration-dependent manner. The fact that there is less inhibition seen when GSSG is incubated in the presence of higher concentrations of DTT suggests that the thiol/disulfide ratio rather than the absolute concentration of GSSG may be an important determinant of the activity of the vitamin K epoxide reductase.

The concentrations of GSSG shown to inhibit reduction of vitamin K epoxide are pharmacologically relevant, occurring as low as 10–25 μM. With increasing thiol concentration, inhibition by GSSG was prevented, perhaps through reduction of mixed disulfides of glutathione and protein. Since the physiologically active thiol in this reaction is unknown, it is difficult to extrapolate this finding to in vivo conditions. Regulation of enzyme activity by the thiol/disulfide ratio has been proposed for several enzymes. Although the regulation of enzymes by physiologic alterations in thiol/disulfide status is a subject of controversy (12), the magnitude of changes in hepatic content of GSSG such as occurs after pharmacologic challenges may be sufficient to alter enzyme activity through such a mechanism. Recent work has also suggested that cellular resistance to cancer chemotherapeutic drugs may be related to alterations in the cellular glutathione reductase (23).

Glutathione can prevent, but not reverse, inhibition of γ-carboxylation in vitro by MTT (using vitamin K_1 as a cofactor) even when MTT is added in 40-fold excess (4). These findings also suggest that the thiol status may be important in maintaining activity of an enzyme(s) involved in γ-carboxylation or the vitamin K cycle. Alternatively, GSH may react directly with a metabolite of MTT to prevent inhibition under in vitro conditions. Rapid oxidation of GSH to GSSG is likely to lower hepatic levels of GSH allowing MTT-related inhibition of γ-carboxylation in vivo. This alteration in the thiol/disulfide ratio could indirectly enhance sensitivity of an enzyme to MTT in vivo. The observation that MTT containing antibiotics as well as MTT cause a disulfiram effect (24) suggests this may occur, since disulfiram reacts with critical sulfhydryl groups on aldehyde dehydrogenase, resulting in loss of activity which can be prevented by thios (25). Disulfiram has also been shown to inhibit both γ-carboxylation of glutamic acid residues (4) and reduction of vitamin K_1 to the hydroquinone form (22). Furthermore, there is evidence that the disulfiram-like inhibition of aldehyde dehydrogenase by MTT is caused by a more reactive metabolite of MTT (26, 27).

It is of interest that lack of oral intake of food is considered a clinical risk factor for cephalosporin-associated hypoprothrombinemia (1). Although depletion of vitamin K stores has been postulated to explain this enhanced susceptibility, it seems equally attractive to speculate that decreased glutathione levels owing to fasting (28) are important in pathogenesis. At lower hepatic concentrations of GSH, oxidation of GSH to GSSG would result in a GSSG/GSH ratio which would be considerably higher than under normal conditions. In some patients, cephalosporin-associated hypoprothrombinemia is
This reversible within 24 h after administration of vitamin K₁. This response is consistent with inhibition of the reduction of vitamin K epoxide, since large doses of vitamin K₁ would overcome the requirement for the intact recycling of vitamin K. However, in other patients, a much longer time is required for reversal of this effect. In the latter situation, impaired reduction of vitamin K epoxide alone would be an inadequate explanation for these findings. Direct inhibition of γ-carboxylation of glutamic acid residues due to a metabolite of MTZ would offer a more satisfactory explanation.

In summary, the results presented here show that (a) administration of MTZ-containing antibiotics alters both the reduction of vitamin K₁, 2,3-epoxide and the thiol redox ratio in rats in vivo, and (b) GSSG can inhibit the microsomal reduction of vitamin K₁, 2,3-epoxide in vitro. These observations suggest that the hypoprothrombinemia occurring after administration of MTZ-containing antibiotics may be due, in part, to an indirect effect on vitamin K metabolism resulting from oxidation of GSH to GSSG during hepatic metabolism of MTZ.

Acknowledgments

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