The effect of preincubation with cimetidine on the *N*-hydroxylation of dapsone by human liver microsomes

M. D. TINGLE, M. D. COLEMAN & B. K. PARK

Department of Pharmacology and Therapeutics, New Medical Building, Ashton Street, P.O. Box 147, Liverpool L69 3BX

We have examined the ability of cimetidine to inhibit the oxidative metabolism and hence haemotoxicity of dapsone *in vitro*, using a two compartment system in which two Teflon® chambers are separated by a semi-permeable membrane. Compartment A contained a drug metabolizing system (microsomes prepared from human or rat liver $\pm$ NADPH), whilst compartment B contained human red cells. Preincubation (30 min) of human liver microsomes with cimetidine (0–1000 μ M) and NADPH prior to the addition of dapsone (100 μ M) and NADPH (1 mM) resulted in a concentration-dependent decrease in the concentrations of dapsone hydroxylamine (from 179 ± 47 to 40 ± 6 ng) in compartment B. This reduction of hydroxylamine metabolite was reflected in the concentration-dependent reduction in methaemoglobin measured (from 7.1 ± 0.7 to $3.5 \pm 1.5\%$) in parallel experiments. Preincubation of microsomes with cimetidine in the absence of NADPH had no effect. The effect of cimetidine pretreatment on dapsone-dependent methaemoglobin was confirmed using microsomes prepared from a further three sources of human liver, as well as from rat liver.

Keywords dapsone metabolism cimetidine inhibition methaemoglobinaemia

Introduction

Dapsone causes dose-dependent methaemoglobinaemia and a reduction in erythrocyte life span in all individuals (Zuidema *et al.*, 1986). These haematological effects are a result of the *N*-hydroxylation of dapsone by cytochrome P-450 enzymes in the liver to yield dapsone hydroxylamine, which can oxidize haemoglobin (Cucinell *et al.*, 1972; Israili *et al.*, 1973).

However, clinical administration of cimetidine has been shown to inhibit the oxidative metabolism and thus reduce the haemotoxicity of dapsone in man, the rat and the rat isolated perfused liver (Coleman *et al.*, 1990a, b, c), although cimetidine is a poor inhibitor of methaemoglobin formation *in vitro* (Tingle *et al.*, 1990). Cimetidine has been reported widely to interfere with cytochrome P-450-mediated metabolism *in vivo* (Galbraith & Michnovicz, 1989; Serlin *et al.*, 1980; Somogyi & Muirhead, 1987) but it is only a weak inhibitor *in vitro* (Ioannoni *et al.*, 1986; Jensen & Gugler, 1985). The purpose of this study was to examine the basis of the inhibition of *N*-hydroxylation and methaemoglobin formation by cimetidine *in vitro* using a two compartment system as described previously (Tingle *et al.*, 1990).

Methods

Materials

Dapsone (4,4'-diaminodiphenyl sulphone) and potassium ferricyanide were purchased from Sigma Chemical Co. Ltd (Poole, U.K.). Cimetidine was obtained from Aldrich Chemical Co. Ltd (Poole, U.K.). Reduced nicotinic adenine dinucleotide phosphate (NADPH; tetrasodium salt), potassium cyanide and all other reagents were purchased from BDH Chemicals Ltd (Poole, U.K.).

Methodology

Washed microsomes were prepared from four histologically normal human livers obtained from renal transplant donors and from the pooled livers of four rats as described previously (Purba *et al.*, 1987). Microsomes (8 mg protein) were incubated with cimetidine (0, 30, 100, 300 or 1000 μ M) and NADPH (1 mM) in 0.1M phosphate buffer, pH 7.4 (final volume 1600 μ l) for 0, 15 or 30 min at 37° C with constant shaking. After the preincubation period an aliquot (400 μ l) of the microsomal suspension

was added to one compartment (compartment A) of a two compartment system in which two Teflon® chambers are separated by a semi-permeable membrane, as described previously (Riley *et al.*, 1990; Tingle *et al.*, 1990). Dapsone (100 μM) was added to compartment A in dimethyl sulphoxide (5 μl) followed by the addition of NADPH (1 mM). Compartment B contained 0.1 M phosphate buffer, pH 7.4. The final volume of each half cell was 500 μl . All concentrations refer to the concentration in compartment A. After 60 min at 37° C, the contents of compartment B were expelled into microcentrifuge tubes containing 50 mM ascorbate (50 μl) in order to stabilize dapsone hydroxylamine, and aliquots (75 μl) assayed for dapsone and its major metabolite dapsone hydroxylamine by reversed-phase h.p.l.c. using the method of Uetrecht *et al.* (1988).

In a separate series of experiments, microsomes, pre-incubated with cimetidine and NADPH as described above, were added to compartment A with dapsone (100 μM) and a further aliquot of NADPH (1 mM). Compartment B contained washed human red blood cells (RBC) suspended to approximately 50% haematocrit. The apparatus was incubated for 60 min at 37° C with constant rotation (8 rev min⁻¹), after which time the RBC were expelled and assayed for methaemoglobin content by the method of Harrison & Jollow (1986).

The inhibition of methaemoglobin formation by cimetidine was investigated further by preincubation (0 or 30 min) of microsomes prepared from three different human livers (LII, LIII and LIV) and pooled rat livers ($n = 4$) with cimetidine (0 or 300 μM) in the presence of NADPH (1 mM), as described above. Aliquots (400 μl) of the microsomal suspension were incubated in compartment A along with dapsone (100 μM) and NADPH (1 mM), whilst compartment B contained washed RBC. Incubations were for 60 min, after which time the methaemoglobin content of the RBC was determined.

In an attempt to determine the stability and diffusibility of the inhibitory species formed from cimetidine, micro-

somes (2 mg protein) prepared from human liver (LIV) were added to compartment A and dialysed against rat liver microsomes (2 mg protein) which had been incubated previously (30 min, 37° C) with cimetidine (300 μM) and NADPH (1 mM, omitted from controls). After 30 min at 37° C, the contents of compartment B were removed and replaced with washed human RBC. Dapsone (100 μM) and NADPH (1 mM) were added to compartment A and the apparatus incubated for a further 60 min at 37° C, with constant rotation (8 rev min⁻¹). The final volume of each half cell was 500 μl .

Results and discussion

In this study we have examined the ability of cimetidine to inhibit the *in vitro* metabolism of dapsone using a two compartment system which has been used previously to investigate the role of metabolism in dapsone-induced methaemoglobinaemia (Tingle *et al.*, 1990). Preincubation of microsomes prepared from a human liver with cimetidine (0–1000 μM) and NADPH (1 mM) prior to the addition of dapsone (100 μM) and a further aliquot of NADPH (1 mM) led to a time- and concentration-dependent decrease in the amount of hydroxylamine and methaemoglobin measured in compartment B (Figure 1a and 1b respectively). Although the degree of inhibition increased with a longer preincubation time, control (preincubation with NADPH in the absence of cimetidine) concentrations of hydroxylamine fell from 470 ± 130 , to 316 ± 30 ng and 179 ± 47 ng after 15 and 30 min preincubation, respectively.

The reduction in hydroxylamine concentrations produced by cimetidine preincubation (Figure 1a) corresponded to a reduction in methaemoglobin formation observed in parallel experiments (Figure 1b). Control methaemoglobin (plus NADPH in the absence of cimetidine) values fell from $8.2 \pm 1.4\%$ to $7.3 \pm 0.9\%$ and $7.1 \pm 0.7\%$ after 15 and 30 min preincubation,

Figure 1 The effect of cimetidine preincubation for 0 (■), 15 (▲) or 30 min (●) on the formation of dapsone hydroxylamine (a) and methaemoglobin (b) by human liver (LI) microsomes. Mean $\pm$ s.e. mean ($n = 4$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values compared with respect to control (+NADPH in the absence of cimetidine) by ANOVA.

Table 1 The effect of preincubation (0 or 30 min) of human (LI, LII, LIII and LIV) or rat liver microsomes with cimetidine (300 μ M) and NADPH (1 mM) on dapsone-dependent methaemoglobin formation. Values are mean $\pm$ s.e. mean ($n = 4$).

Liver	Donor (age, years)	% control methaemoglobin	
		0 preincubation	30 min preincubation
LI	Male (45)	71.1 $\pm$ 1.8	38.4 $\pm$ 11.2**
LII	Male (18)	85.4 $\pm$ 13.5	53.4 $\pm$ 6.1**
LIII	Male (38)	73.7 $\pm$ 7.7	69.1 $\pm$ 11.1
LIV	Male (8)	90.9 $\pm$ 8.7	71.3 $\pm$ 9.9
Rat	Male (250–300 g)	84.3 $\pm$ 3.1*	55.9 $\pm$ 5.5**

* $P < 0.05$, ** $P < 0.01$

respectively. Although the determination of dapsone hydroxylamine concentrations appears to be more sensitive than methaemoglobin formation in order to assess the inhibitory effect of cimetidine *in vitro*, it is not possible to detect the hydroxylamine metabolite directly *in vivo* (Zuidema *et al.*, 1986). It was necessary to perform incubations for the determination of hydroxylamine and methaemoglobin concentrations separately as a previous study with radiolabelled compound showed that the hydroxylamine was taken up into the red cell and was non-extractable (Tingle *et al.*, 1990). Preincubation of microsomes with cimetidine but in the absence of NADPH did not lower methaemoglobin formation (100.1 $\pm$ 4.8%).

The effect of cimetidine preincubation on dapsone-dependent methaemoglobin formation was confirmed using microsomes prepared from a further three sources of human livers (LII, LIII and LIV) as well as from rat livers (Table 1). Although there was a trend for cimetidine to inhibit methaemoglobin formation, there was some inter-liver variation *in vitro*, in agreement with the inter-individual variation observed in man *in vivo* (Coleman *et al.*, 1990a). The need for preincubation of microsomes with cimetidine plus NADPH has been noted previously by Wild & Back (1989), who demonstrated that the inhibition of oestrogen 2-hydroxylase

activity in human liver microsomes by cimetidine was enhanced by preincubation.

In order to establish whether cimetidine is metabolized to a stable, diffusible inhibitory species, human liver microsomes were dialysed against cimetidine/NADPH-pretreated rat microsomes prior to incubation with red cells. However, this resulted in no significant change in methaemoglobin formation (118 $\pm$ 36% of control), which might suggest that the inhibition caused by cimetidine is a consequence of either irreversible binding or complex formation with the enzyme. Although inhibition by cimetidine *in vivo* persists after the drug can no longer be detected in plasma, the effect has been shown to be reversible (Teunissen *et al.*, 1985). Hence inhibition may be explained by the formation of a complex between cimetidine and cytochrome P-450 which requires the presence of NADPH (Jensen & Gugler, 1985), rather than covalent binding.

In summary, the two compartment system may be of value to assess not only clinically relevant metabolic interactions but also the nature and stability of the putative inhibitory species.

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