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EFFECT OF CIMETIDINE ON δ-AMINOLEVULINIC ACID SYNTHASE AND MICROSOMAL HEME OXYGENASE IN RAT LIVER

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Abstract—Cimetidine is a well known inhibitor of the heme-containing enzyme cytochrome P-450. We have found that it also inhibits δ-aminolevulinic acid synthase (ALA-S) and microsomal heme oxygenase, the rate-limiting enzymes for heme synthesis and heme degradation respectively. Cytochrome P-450 content was decreased but microsomal heme concentration remained unaltered for a period of 30 min after *in vivo* cimetidine administration to rats. *In vitro* incubation of cimetidine with each of the above enzymes revealed no direct effect of cimetidine on ALA-S but about 50% inhibition of heme oxygenase and 20% reduction in cytochrome P-450 content. This suggests that a metabolite of cimetidine inhibits ALA-S activity *in vivo* , while the drug itself or a metabolite inhibits heme oxygenase both *in vivo* and *in vitro*. A rise in ALA-S activity seen after its early inhibition and its return to approximate control values after 60 min suggest a reversible inhibition of ALA-S by a metabolite of cimetidine and may correspond to its clearance from the animal. An elevation in microsomal heme content paralleled the rise in ALA-S activity while microsomal heme oxygenase activity returned to only 65% of control value 60 min after cimetidine treatment. Cytochrome P-450 content did not change after its initial decrease, suggesting that irreversible alteration had occured.

Cimetidine has been reported to reduce the activity of cytochrome P-450 in both humans and animals [1–5]. Rendic *et al.* [6] demonstrated that cimetidine inactivates cytochrome P-450 by binding reversibly to its heme moiety. They also found cimetidine to be a competitive inhibitor of 7-ethoxycoumarin-*O*-dealkylation in rat liver microsomes [6]. Reilly *et al.* [7] have recently demonstrated the existence of a high-affinity binding site on microsomal cytochrome P-450 which explains the potent inhibitory effect of low doses of cimetidine on the monooxygenase-dependent clearance of a variety of drugs *in vivo*.

Cytochrome P-450, a heme-containing enzyme, is dependent on the availability of heme for its biologic activity. In hepatic cells, the production of heme is regulated by the exquisite balance between heme synthesis, controlled by δ-aminolevulinic acid synthase (ALA-S) [8], and heme degradation, controlled by microsomal heme oxygenase [9].

This study was performed to examine the effect of cimetidine on heme synthesis, heme degradation, and heme levels in rat liver.

MATERIALS AND METHODS

Cimetidine, USP grade, was obtained from Smith, Klein & French Laboratories, Philadelphia, PA; NADPH and hemin were obtained from the Sigma Chemical Co., St. Louis, MO. All other chemicals were of the highest grade commercially available.

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Animals. Male Sprague–Dawley rats (150–200 g) were used in this study. They were starved for 24 hr prior to receiving treatment regimens and then decapitated. Livers were removed and placed in ice-cold 0.15 M KCl, 0.1 M phosphate buffer (pH 7.4). Each liver was weighed and homogenized in 1.5 vol. of 0.15 M KCl, 0.1 M phosphate buffer (pH 7.4) with five strokes on a Dounce homogenizer. Rats were injected intraperitoneally with the indicated dose of cimetidine 30 min prior to sacrifice. Control rats received an equal volume of isotonic saline.

Preparation of microsomes. The homogenate was centrifuged at 9000 *g* for 20 min. The supernatant fraction was centrifuged at 105,000 *g* for 1 hr, and the resulting microsomal pellet was resuspended in 0.1 M phosphate buffer (pH 7.4). The protein concentration of both supernatant fraction and microsomal pellet was adjusted to 10 mg/ml.

ALA-synthase assay. ALA-synthase activity was assayed colorimetrically according to the method of Sassa *et al.* [10] employing a modified Ehrlich reagent [11]. The concentration of ALA-pyrrole formed was determined based on the difference in absorbance between 553 and 650 nm using and extinction coefficient of 58 $mM^{-1}cm^{-1}$ [11].

Cytochrome P-450 determination. Cytochrome P-450 was determined on microsomal suspensions. The heme protein content was determined by the method of Omura and Sato [12]. An extinction coefficient of 91 $mM^{-1}cm^{-1}$ between 450 and 490 nm was used to calculate the concentration of the heme protein.

Microsomal heme determination. The concentration of microsomal heme was determined by the

pyridine hemochromogen method of Paul *et al.* [13] using the reduced minus oxidized difference spectrum between 557 and 575 nm and an extinction coefficient of 32.4 $mM^{-1}cm^{-1}$.

Heme oxygenase assay. Preliminary assays were carried out according to the method of Maines and Kappas [14] which employed the following components of an NADPH-generating system: glucose-6-phosphate (0.85 mM); glucose-6-phosphate dehydrogenase (3 units); NADP (final concentration 0.8 mM) in 1% $NaHCO_3$ (added after preincubation). This method was replaced with authentic NADPH as described by Tenhunen *et al.* [15]. Bilirubin formation was calculated using an extinction coefficient of 40 $mM^{-1}cm^{-1}$ between 468 and 530 nm [14].

Protein assay. Protein content of the various cell fractions was determined by the method of Lowry *et al.* [16] using crystalline bovine serum albumin as standard.

RESULTS

Figure 1 shows the effect of cimetidine on δ-aminolevulinic acid synthase. Fifty percent (50%) inhibition of ALA-S activity was obtained when rats were administered 20 mg of cimetidine/100 g body wt 30 min prior to being killed. Larger doses of the drug produced little, if any, further inhibition of ALA-S activity. This dose was used in all subsequent studies.

Figure 2 shows the *in vivo* effect of cimetidine on the indicated parameters which are involved in the production and maintenance of cellular heme levels. ALA-S activity was reduced to 56% of control, cytochrome P-450 content was reduced to 60% of control, heme oxygenase was reduced to 50% of control, and microsomal heme levels were unaltered.

Cimetidine was incubated in a range of concentrations with the enzyme assays so that any direct effect of the drug could be detected (Table 1). It is of particular interest that cimetidine itself has no direct *in vitro* effect on ALA-S activity throughout the concentration range tested. A direct inhibitory effect on heme oxygenase was seen in the range of 10^{-2} to 10^{-4} M. Cytochrome P-450 content ranged from 82.5 to 94.5% of control when microsomes were incubated with 10^{-2} to 10^{-4} M cimetidine.

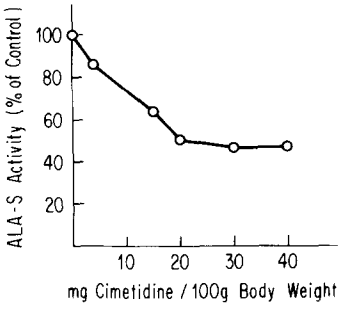

Fig. 1. *In vivo* effect of cimetidine on rat liver δ-aminolevulinic acid synthase. Each point represents the activity obtained from a pool of three livers. One hundred percent (100%) of control equals 0.11 ± 0.02 nmoles ALA/mg protein.

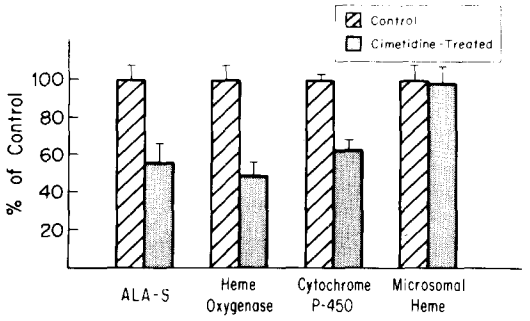

Fig. 2. Effect of cimetidine on the parameters involved in the production and maintenance of cellular heme levels. Rats were treated with 20 mg cimetidine/ 100 g body wt 30 min prior to being killed. Each determination was carried out as described in Materials and Methods. The error bars represent the standard error of the mean. Key: (▨) control animals; and (▩) cimetidine-treated. Control values: ALA-S, 0.11 ± 0.02 nmoles ALA/mg protein; heme oxygenase, 2.33 ± 0.11 nmoles bilirubin/mg protein/hr; cytochrome P-450, 0.93 ± 0.03 nmoles/mg microsomal protein; and microsomal heme, 1.07 ± 0.09 nmoles/mg microsomal protein.

Figure 3 shows the time course of the effect of *in vivo* administration of cimetidine on ALA-S and heme oxygenase activities, and cytochrome P-450 and microsomal heme content. The initial effect of cimetidine appears to be on heme oxygenase activity and cytochrome P-450 content, for both of these were reduced to 60% of their control values within 5 min. ALA-S activity appeared to decline more slowly, reaching a minimum value at 30 min and then rising to 120% of control by 1 hr. Microsomal heme level rose to approximately 140% of control during the latter time period, while heme oxygenase activity slightly increased to about 65% of control levels. Multiple repetitions of this experiment indicated that approximately 50% inhibition of ALA-S activity indeed occurred at 30 min.

DISCUSSION

The present study was undertaken to determine whether cimetidine treatment affected the level of

Table 1. Effect of cimetidine on the *in vitro* determination of ALA-S, heme oxygenase and cytochrome P-450*

	Percent control activity remaining		
	ALA-S	Heme oxygenase	Cytochrome P-450
No addition (control)	100	100	100
Cimetidine			
10^{-2} M	96.7	48.0	82.5
10^{-3} M	103.2	54.0	80.0
10^{-4} M	95.5	78.0	94.5
10^{-5} M	96.7	114.0	95.0
10^{-6} M	93.8	108.0	95.0
10^{-7} M	103.2	104.0	102.8

* Each enzyme was prepared, incubated with the indicated concentration of cimetidine for 30 min at 37° and then assayed according to Materials and Methods.

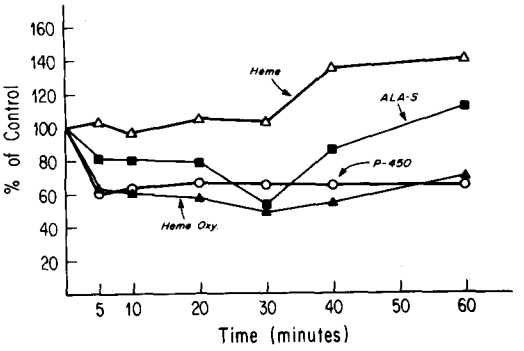

Fig. 3. Time course of the effect of cimetidine on the components of the heme regulatory pathway. Rats were treated with cimetidine, and the enzymes were assayed as in Fig. 2. Key: (△) microsomal heme; (■) ALA-S; (○) cytochrome P-450; and (▲) heme oxygenase. Each point represents the value obtained for pooled livers from three rats.

microsomal heme and the activities of ALA-S and heme oxygenase at a time when cytochrome P-450 activity is inhibited.

As shown in Fig. 1, 50% inhibition of ALA-S activity occured after a dose of 20 mg of cimetidine/100 g body wt was administered 30 min prior to sacrifice. Table 1 shows that there is no direct *in vitro* effect of cimetidine on the activity of this enzyme through the entire concentration range tested. The *in vivo* inhibition of this enzyme suggests that one or more of the biotransformation products of the drug might be responsible for this inhibition, namely, the sulfoxide or the hydroxymethyl derivative [17, 18].

The effect of cimetidine on cytochrome P-450 activity has been examined by a number of investigators [5–7, 9], and a K_i of 1–10 mM for 50% inhibition of aminopyrine *N*-demethylase activity reported. Pelkonen and Puurunen [5] reported that, when rats were treated with cimetidine (75 mg/kg) three times daily for 7 days and killed 24 hr after the last injection, there was no significant difference in the cytochrome P-450 content between the treated and control rats as measured by the method of Omura and Sato [12]. The above dose of 75 mg/kg three times daily, or 225 mg/kg in a single dose, is very close to our dose of 200 mg/kg. However, in our experiments, the animals were killed 30 min after the administration of the drug, while the above authors killed their animals 24 hr after the last cimetidine administration, which corresponds to 14.4 times the reported plasma half-life of 100 min for cimetidine [19, 20]. In our *in vitro* experiments, there is some inhibition of cytochrome P-450 content by cimetidine (Table 1). However, there appears to be a rapid *in vivo* decrease in cytochrome P-450 content which occurs as early as 5 min (Fig. 3) and does not change during the 60-min time course. This suggests that non-competitive inhibition or an irreversible change has occurred.

One possible reason for the appearance of this non-competitive inhibition has been suggested as the unusually high affinity of an inhibitor for the binding site of an enzyme [21]. Recently, Reilly *et al.* [7] have demonstrated the existence of two distinct and independent classes of binding sites on rat liver microsomes for cimetidine, of dissociation constants 8.3 and 105 μM. The presence of this high-affinity binding site is a suggestive explanation for the potent inhibitory effect of cimetidine in decreasing the monooxygenase-dependent clearance of a variety of drugs *in vivo*. However, an irreversible alteration in the steady levels of cytochrome P-450 by cimetidine or its metabolite is a more likely explanation for the present findings.

Cimetidine binds to cytochrome P-450 to produce a characteristic type II spectral change which is considered to result from the binding of a ligand N atom to the sixth coordination position of the heme iron of the cytochrome [22]. The demonstration of the presence of a high-affinity binding site on cytochrome P-450 by Reilly *et al.* [7] and the concentration of cimetidine used in the experiments (Table 1 and Fig. 2) also suggest that the drug and/or its metabolite might bind tightly so that carbon monoxide fails to displace the ligand which is bound to cytochrome P-450, as has been shown for the binding of benzphetamine [23]. However, complex formation requires metabolism of the initial compound to products with high affinity for reduced cytochrome P-450 [24], and in most cases the high-affinity ligand produced by metabolism has not been clearly identified. High-affinity binding of cimetidine or its metabolite could prevent formation of the oxygen complex of the cytochrome and act as an inhibitor of P-450-dependent reactions [1–5]. It also appears that the binding of cimetidine to P-450 is less reversible than originally proposed [6].

The rise in ALA-S activity seen after 30 min approximately parallels a similar rise in microsomal heme levels (Fig. 3). Heme oxygenase activity rises only slightly by 1 hr, from 50 to 65% of control values. The "regulatory heme pool" has been thought to be responsible for the regulation of ALA-S and heme oxygenase [8, 9, 25], as well as for the supply of heme to the apo form of various heme proteins, including cytochrome P-450. Under conditions where excess heme is present in the cell, microsomal heme oxygenase activity is increased by the direct inductive effect of heme on this enzyme [9]. At the same time, heme synthesis is reduced by inhibition by heme of the synthesis of ALA-S. Yamamoto *et al.* [26] have suggested that heme may inhibit the intracellular translocation of ALA-S in addition to inhibiting its synthesis.

As shown in Fig. 3, relatively little change in microsomal heme levels occurs during the 30 min after cimetidine administration. At the same time, ALA-S and heme oxygenase activities are reduced to about 50% of control. During the period from 30 to 60 min after cimetidine administration, microsomal heme levels rise and begin to plateau. A similar rise in ALA-S activity also occurs, while heme oxygenase activity remains at essentially 60% of its control value. Hence, as ALA-S activity returns to normal levels, heme production in excess of heme utilization occurs and microsomal heme levels rise until the intracellular heme concentration reaches a level high enough to inhibit synthesis of ALA-S, giving rise to a new steady-state heme level. This level would be maintained until heme oxygenase

activity returns to its normal level. The events occurring between 30 and 60 min probably coincide with the onset of clearance of cimetidine and/or its metabolites from the animal. Figure 3 suggests that the drug has a higher affinity for heme oxygenase than it does for ALA-S. Figure 3 also shows that there is no elevation in P-450 levels even though there is an enhanced availability of intracellular heme. This finding is in agreement with the concept that heme does not regulate the synthesis of cytochrome P-450 hemoproteins [27–30] and implies that an alternate "heme sink" is available such as tryptophan pyrrolase as has been suggested by Badaway [31].

Since cimetidine administration may lead to a permanent inactivation of cytochrome P-450, *de novo* synthesis of the apoprotein may be required to restore its levels to normal. This then adds an additional precaution for the use of this drug in patients whose levels of cytochrome P-450 are already lowered, as in the elderly [32].

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REFERENCES

1. P. V. Desmond, R. V. Patwardhan, S. Schenker and K. V. Speeg, *Ann. intern. Med.* **93**, 266 (1980).
2. R. G. Knodell, J. L. Holzman, K. L. Crankshaw, N. M. Steele and L. N. Stanley, *Gastroenterology* **82**, 84 (1982).
3. U. Klotz and I. Reimann, *New Engl. J. Med.* **302**, 1012 (1980).
4. K. V. Speeg, R. V. Patwardhan, G. R. Avant, M. C. Mitchell and S. Schenker, *Gastroenterology* **82**, 89 (1982).
5. O. Pelkonen and J. Puurunen, *Biochem. Pharmac.* **29**, 3075 (1980).
6. S. Rendic, V. Sunjic, R. Roso, F. Kajfez and H. H. Raf, *Xenobiotica* **9**, 444 (1979).
7. P. E. B. Reilly, L. E. Carrington and D. J. Winzor, *Biochem. Pharmac.* **32**, 831 (1983).
8. S. Granick and S. Sassa, *Metab. Regulat.* **5**, 77 (1971).
9. R. Tenhunen, H. S. Marver and R. Schmid, *J. Lab. clin. Med.* **75**, 410 (1970).
10. S. Sassa, A. Kappas, S. Bernstein and A. Alvares, *J. biol. Chem.* **254**, 729 (1979).
11. D. Mauzerall and S. Granick, *J. biol. Chem.* **219**, 435 (1956).
12. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
13. K. G. Paul, H. Theorell and A. Akeson, *Acta chem. scand.* **7**, 1284 (1953).
14. M. D. Maines and A. Kappas, *J. biol. Chem.* **250**, 4171 (1975).
15. R. Tenhunen, H. S. Marver and R. Schmid, *J. biol. Chem.* **244**, 6388 (1969).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. R. S. Jacobs and H. Cantania, *Drug Intell. clin. Pharmac.* **11**, 723 (1977).
18. D. C. Taylor, P. R. Cresswell and D. C. Bartlett, *Drug Metab. Dispos.* **6**, 21 (1978).
19. R. Griffiths, R. M. Lee and D. C. Taylor, in *Cimetidine* (Eds. W. L. Burland and M. A. Simkins), p. 38. Excerpta Medica, Amsterdam (1977).
20. K. W. Ma, D. C. Brown, D. S. Masler and S. E. Silvis, *Gastroenterology* **74**, 473 (1978).
21. H. A. Sasame and J. R. Gillete, *Biochem. Pharmac.* **19**, 1025 (1970).
22. B. W. Griffin, S. M. Smith and J. A. Peterson, *Archs Biochem. Biophys.* **160**, 323 (1974).
23. J. Werringloer and R. W. Estabrook, *Archs Biochem. Biophys.* **167**, 270 (1975).
24. D. Mansuy, R. Beaune, J. C. Chottard, J. F. Bartolz and P. Gans, *Biochem. Pharmac.* **25**, 609 (1976).
25. S. Shibahara, T. Yoshida and G. Kikuchi, *Archs Biochem. Biophys.* **188**, 243 (1978).
26. M. Yamamoto, N. Hayashi and G. Kikuchi, *Archs Biochem. Biophys.* **209**, 451 (1981).
27. U. Giger and U. Meyer, *Biochem. J.* **198**, 321 (1981).
28. G. S. Song, H. L. Harold, A. S. Rosenthal, N. A. Gelb and A. Kappas, *J. exp. Med.* **134**, 1349 (1971).
29. F. DeMatteis and A. Gibbs, *Biochem. J.* **126**, 1149 (1972).
30. R. Druyan and A. Kelly, *Biochem. J.* **129**, 1095 (1972).
31. A. A. B. Badaway, *Biochem. J.* **202**, 807 (1982).
32. D. L. Schamcker and R. K. Wang, *Expl. Geront.* **15**, 321 (1980).