Cyclophosphamide-impaired regulation of hepatic heme metabolism

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Summary. In male rats hepatic cytochromes b_5 and P-450 were reduced at different times after treatment with cyclophosphamide (CP) (200 mg/kg i.p. for 3 days). In contrast, microsomal heme did not change until 48 h after the last dose of CP, leading to accumulation of heme in a 'non-cytochromal' form. Parallel to the above changes the heme metabolism showed derangement: δ-aminolaevulinate synthase, the rate-limiting enzyme in heme synthesis, was depressed and heme oxygenase, the enzyme which catalyzes the oxidative degradation of heme, was increased.

Key words. Rat liver; liver, rat; heme metabolism; cytochrome; cyclophosphamide; δ-aminolaevulinate synthase; heme oxygenase.

Cyclophosphamide is a valuable drug used in the treatment of many tumors and as an immunosuppressive agent[1-3]; it is ineffective per se and requires metabolic activation by the hepatic microsomal mixed function oxidase system (MFO) to exert its cytotoxic activity[4]. Besides leukopenia and extensive urinary bladder damage[5,6] an important feature of CP liver toxicity is the loss of the hemoprotein cytochrome P-450 and the accompanying depression of the metabolism of various drugs including CP itself. This effect has been reported after treatment of rats with high ($\geq$ 100 mg/kg) or repeated doses of CP[7,11].

In rat liver, cytochrome P-450 incorporates about 60% of the newly synthesized heme[12] and several xenobiotics known to alter in vivo levels of this cytochrome have parallel effects on the activity of enzymes of the heme pathway[13-18]. As suggested by the variety of the substances reducing the concentration of cytochrome P-450, the mechanisms underlying the inactivation are necessarily different.

In vitro studies strongly suggest that one of the metabolites of CP, acrolein, may form adducts with sulphydryl group(s) in the apoprotein and lead to a destruction/denaturation of cytochrome P-450 similar to that produced by sulphydryl reagents, such as p-hydroxy-mercuribenzoate[19].

The present study was undertaken to clarify some temporal aspects of the response to CP treatment of microsomal cytochromes and heme levels and to investigate any changes in liver heme metabolism and particularly in the activity of δ-aminolaevulinate synthase (E.C.2.3.1.37) (ALA-S) and heme oxygenase (E.C.1.14.99.3) (HO).

Materials and methods. Charles River CD-COBS male rats (b.wt 175–200 g) were used. Animals were treated with cyclophosphamide dissolved in 0.9% NaCl, at a dose of 200 mg/kg i.p. daily for 3 days. Control rats were given the same volume of saline. A different times after the last dose rats were fasted overnight before being killed by decapitation. Livers were perfused in situ via the inferior vena cava with ice-cold 0.9% NaCl and homogenized (20% w/v) using a Teflon-glass Potter homogenizer in 0.25 M sucrose. Liver microsomes were prepared according to the method of Kato and Takayanaghi[20]. ALA-S activity was assayed on the crude homogenate with the radiochemical method described by De Matteis et al.[21]. HO activity was assayed using a post-mitochondrial supernatant as reported by Schacter et al.[22] with the modifications of De Matteis and Gibbs[23]. Tryptophan pyrrolase activity was determined on the crude homogenate as described by Feigelson and Greengard[24]. Microsomal cytochromes P-450 and b_5

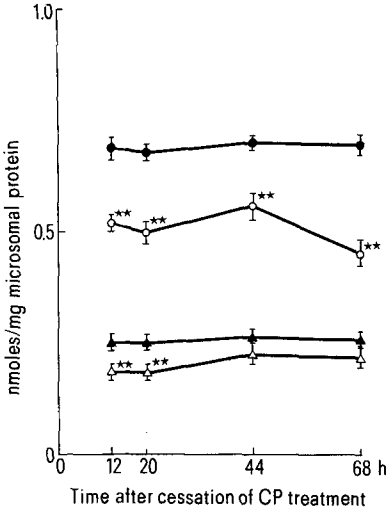

Figure 1. Concentration of the microsomal cytochromes P-450 and b_5 at various times after cessation of CP treatment. Cytochrome P-450: ●, control; ○, treated. Cytochrome b_5: ▲, control; △, treated. Values are expressed as mean ± SE of 10 determinations. Student's t-test: $^{**}p \leq 0.01$.

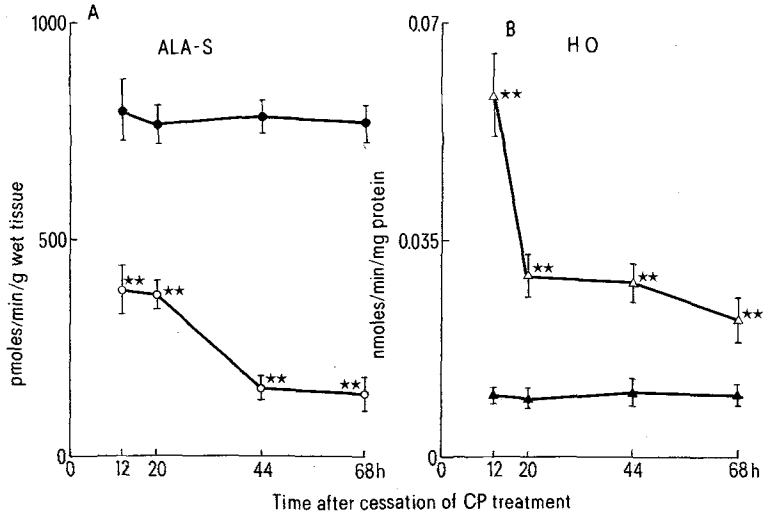

Figure 2. Activity of ALA-S (A) and HO (B) at various times after cessation of CP treatment. *A* ALA-S: ●, control; ○, treated. *B* HO: ▲, control; △, treated. Values are expressed as mean ± SE of 8 determinations. Student's t-test: $^{**}p \leq 0.01$.

Effect of CP on the concentrations of various heme components in rat liver microsomal fraction

Treatment	Time of killing (h after treatment)	Total heme (B)	Sum of cytochrome p-450 + cyt b_5 (A)	% A/B	Non-cytochromal heme (B–A)
None	(zero time)	1.10 ± 0.053	0.95 ± 0.052	86.3	0.15 ± 0.04
CP	12	0.98 ± 0.061	0.69 ± 0.039**	70.4	0.28 ± 0.03*
CP	20	0.98 ± 0.035	0.70 ± 0.038**	71.4	0.29 ± 0.02**
CP	44	1.08 ± 0.040	0.78 ± 0.037*	72.2	0.30 ± 0.04*
CP	68	0.90 ± 0.042**	0.66 ± 0.046**	73.3	0.24 ± 0.03

Values are expressed in nmoles/mg microsomal protein (means ± SE of 10 determinations). Student's t-test: * $p \leq 0.05$; ** $p \leq 0.01$ in comparison to control value.

were measured according to Omura and Sato[25] and heme content by the pyridine hemochromogen method as described by Falk[26]. Proteins were determined by the method of Lowry et al.[27] using bovine serum albumin as standard.

Results. Figure 1 shows the effect of CP treatment on the concentrations of microsomal cytochromes P-450 and b_5 in rat liver. Cytochrome P-450 was already significantly reduced 12 h after the last dose of CP (75% of control value) and this decrease reached a maximum at 68 h after the last dose (65% of control value). Cytochrome b_5 levels were less affected and were significantly lower than in control animals only 12 and 20 h after the last injection of CP (72% of control value).

Despite these changes total heme microsomal concentration (table), determined from pyridine hemochrome, did not show major changes during the experiment except at the late time of 68 h after the last dose of CP (81% of control value). Thus most of the heme which cannot be accounted for as cytochrome P-450 and b_5 is still in the microsomes with a doubling of the amount of heme present in a 'non cytochromal' form (table). This increase was significant up till 24 h after the last injection of CP.

The effect of the same treatment schedule with CP on ALA-S (A) and HO (B) activities was also studied (fig. 2). The 2 enzymes behaved in opposite ways. After 12 h HO had a peak of activity (6 times the control) while ALA-S activity was already reduced to 45% of the control value; then the former established values from 3–2 times the control value and the latter continued to decrease, reaching as little as 16% of the control value at 68 h.

Discussion. The present study shows that CP, a drug already known to depress cytochrome P-450[8,9], alters cellular heme metabolism in rat liver at the same time. The characteristic focus of this effect appears to be an induction of microsomal HO and concomitant inhibition of heme synthesis through an effect on ALA-S, the rate limiting enzyme in this process.

All the effects, including P-450 loss, were long-lasting after treatment and appear to be important since they could influence the clinical efficacy of CP itself or of drugs given in combination whose therapeutic effects depend on the integrity of the cytochrome P-450 system.

Cyclophosphamide requires activation in vivo by the MFO system of cytochrome P-450 in order to exert the alkylating activity[3,28] which is believed to be the basis of its antitumor effect.

In a non-enzymic reaction, acrolein can be generated[29], which seems to be responsible for some of CP's adverse effects, such as extensive urinary bladder damage[6]. In vitro results point to the possibility that acrolein is the cyclophosphamide metabolite responsible for the loss of cytochrome P-450 through alkylation of the sulphydryl groups in the active site of cytochrome P-450, and not through interaction with the porphyrin nucleus or iron of the heme moiety[19]. This proposed mechanism is in agreement with findings that in vivo P-450 degradation induced by cyclophosphamide can be reduced by simultaneous administration of n-acetyl-cysteine[7,8].

Accordingly our data show that a redundancy of heme is present in a 'non cytochromal' form in the liver of CP treated rats. Furthermore, 20 h after treatment we observed higher activity of the holoenzyme of tryptophan pyrrolase (1.89 ± 0.07 against 0.90 ± 0.10 μmoles kynurenine formed/h per g wet wt of liver, $p \leq 0.01$ by Student's t-test). Activation of this form of the enzyme has been described in several experimental conditions where increased amounts of heme are available to the apoenzyme[18,30–32].

Concomitantly we observed an inverse relationship in changes in ALA-S and HO enzymatic activities. This could be explained assuming that CP-induced P-450 degradation products containing the heme moiety raise the level of the heme of the 'regulatory pool'. It has in fact been postulated by various authors[14,33–35] that intracellular ALA-S and HO activities are modulated by a pool of free heme through mechanisms of respectively negative and positive feed-back.

More recently the use of adult rat hepatocytes in primary culture has provided direct evidence of a rapidly formed cytosolic heme fraction which appears to be identical to the previously postulated 'regulatory' or 'unassigned' heme pool of the liver[36]. However, at longer intervals after treatment, HO activity tends to return to normal according to a reduction in the level of 'non-cytochromal' heme, while ALA-S depression becomes striking. This suggests there is also a direct inhibitory effect on ALA-S and, as a consequence, on heme synthesis and P-450 formation. In this respect at least 2 possibilities must be considered. ALA-S could itself be a partial target for acrolein since it has been shown to contain an -SH group at its active centre and to be inhibited by the sulphydryl inhibitors N-ethylmaleimide and p-chloromercuribenzoate[37,38]. Furthermore the reaction of acrolein with available sulphydryl groups of enzymes has been described[39–41].

The other possibility is that CP metabolites interact with the genetic mechanism controlling ALA-S synthesis, bearing in mind that interaction of CP metabolites with nucleic acids is well established[42] and alkylation and inhibition of synthesis of RNA and DNA in vivo seem to occur at the peak after 24–48 h[43,44]. In this respect the short half-life of ALA-S (about 35 min)[45] would be in agreement with the finding that enzymatic activity decreased by about 65% in the interval between 12 and 68 h after the last CP dose.

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Difference of the inhibitory action of verapamil on the positive inotropic effect of Ca^{2+} between spontaneously hypertensive and normotensive rat myocardium

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Summary. Verapamil, a calcium entry blocker, had a greater inhibitory effect on the positive inotropic effect of excess Ca^{2+} in SHR than in NWR, suggesting that the cardiac responsiveness to verapamil was enhanced in SHR.
Key words. Rat myocardium; myocardium, rat; hypertensive rats, spontaneously; atrium; papillary muscle; verapamil; inotropic effect, calcium.

The differences between spontaneously hypertensive rats (SHR) and normotensive Wister rats (NWR) in the handling of calcium for contraction in vascular smooth muscle have previously been demonstrated. Calcium channel inhibitors, such as verapamil and nifedipine, had a greater inhibitory effect on the aortic contraction of SHR than on that of NWR[2]. Pederson et al[3] also found that in the SHR, excess calcium induced contraction of the aorta, which was more sensitive to nifedipine than the response in the NWR, suggesting a larger dependency of extracellular Ca^{2+} in the SHR vascular contraction. In fact, in patients with essential hypertension intra-arterial perfusion of verapamil and nifedipine caused a marked vasodilation, suggesting a functional abnormality in essential hypertension with increased dependency of arteriolar tone on calcium influx[4-6]. These findings demonstrate that the vascular smooth muscle in hypertension had an abnormality in calcium flux. Although such an abnormality in the vascular system is an important factor in the development of hypertension, changes in cardiac responsiveness are also involved in the disease. However, myocardial responsiveness of SHR to calcium entry blockers has not been reported yet. In the present study, the influence of verapamil on the positive inotropic effect of excess Ca in the isolated atrial and papillary muscles from SHR were compared to that in the preparations from NWR.

Materials and methods. Adult spontaneously hypertensive male Wister rats of the Kyoto strain (SHR) developed by Okamoto and Aoki[7], and normotensive Wistar rats (NWR) 3–5 months old were used. The blood pressure for SHR and NWR measured by the tail-plethysmographic method was 173 ± 1 mm Hg ($n = 10$) and 127 ± 3 mm Hg ($n = 10$), respectively. The animals were killed by a blow on the head. The hearts were quickly excised and dissected in Krebs-Ringer solution. The papillary muscles from the right ventricle and the left atrium were isolated. Each preparation was mounted in a tissue bath containing 20 ml of Krebs-Ringer solution of the following