TURNOVER OF CYTOCHROME *P*-450 AND CYTOCHROME b_5 HEMES IN GRISEOFULVIN-INDUCED MURINE PORPHYRIA

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1. Introduction

The fungistatic drug griseofulvin (GF) is a potent inducer of protoporphyria in the mouse [1]. Studies on the mechanism of GF-induced porphyria revealed an increased δ-aminolevulinic acid (ALA)-synthetase and a decreased hepatic ferrochelatase (heme-synthetase) activity, indicating that the inability to convert rapidly forming protoporphyrin into heme may account for the protoporphyrin accumulation [2]. It was shown in a previous paper [3] that GF-treatment leads to a significant fall in microsomal cytochrome *P*-450 and to a rise in microsomal cytochrome b_5 content whereas the total microsomal-heme remained unchanged. The present studies were designed to shed some light on the mechanism underlying the GF-induced microsomal cytochrome alterations. For this purpose, cytochrome *P*-450- and b_5-hemes were isolated after labeling with [^{14}C]ALA and the half-life times were determined.

2. Materials and methods

2.1. *Animals*

Male Swiss albino mice weighing 25–30 g (strain Him OF 1 SPF, Institute of Laboratory Animal Research of the University of Vienna, Himberg, Austria) were fed a standard diet (Altromin, Lippe, Germany) containing 2.5% GF (Glaxo, Greenford, England). The animals had free access to water and were housed under constant temperature conditions at 22°C. Control mice received the same diet without GF.

2.2. *Determination of cytochrome* P-*450 and* b_5 *alterations in GF-feeding duration*

Mice were fed the GF-diet for different time periods. Groups consisting of 5 animals each were sacrificed before and 2,4,7,9, and 11 days after commencement of GF-feeding together with a control group at each time point by cervical dislocation. The livers of each group were pooled and microsomal cytochromes *P*-450 and b_5 were determined (vide infra).

2.3. *Preparation of microsomes and cytochrome determinations*

Livers were removed, chilled on ice, weighed, perfused with ice-cold isotonic saline through the hepatic veins and homogenized in sucrose (250 mM)/Tris–HCl (50 mM) buffer, pH 7.6. Microsomes were prepared by the rapid Ca^{2+}-sedimentation technique [4]. Microsomal protein was determined by a modified biuret method [5]. Microsomal cytochromes b_5 and *P*-450 were measured by difference spectrophotometry in a PYE-UNICAM SP 1800 ultraviolet spectrophotometer and calculated according to Omura and Sato [6].

2.4. *Determination of half-life times of cytochrome* P-*450 and cytochrome* b_5 *hemes in GF-treated mice and controls*

[^{14}C]ALA-hydrochloride (Radiochemical Centre, Amersham, England) with a specific activity of 18 mCi/mmol was administered intraperitoneally to GF-treated (GF-treatment for 8–11 days) and control mice in doses of 3.5 μCi/100 g body weight and the animals were killed 5, 10, 20, 48, 72, 86 and 96 h after ALA-injection, all at the eleventh day after commence-

ment of GF-feeding. Heme half-life times in GF-treated animals were determined in a single experiment in which four groups of mice, each consisting of five animals, were sacrificed at each time point, and the livers of the animals belonging to one group were pooled. With control mice two experiments were performed, in each of which two groups of mice consisting of five animals were sacrificed at each time point after ALA-injection. Half-life times were determined from the decay of specific-heme radioactivity by regression analysis.

Microsomes were prepared as described above and washed twice in 0.15 M KCl. They were then resuspended in 0.1 M potassium phosphate buffer, pH 8.6. Microsomal protein and cytochromes were measured as described above. To separate microsomal cytochromes, the microsomal suspension was incubated aerobically with subtilase (Type IX, Sigma Chem. Co., St Louis, Mo.) in a concentration of 25 μg/mg microsomal protein at room temperature for 30 min. Thereafter, the suspension was centrifuged at 150 000 × *g* in a Christ-Heraeus Vacufuge ultracentrifuge using a Ti 60 rotor. Cytochrome content was determined in supernatant and pellet. The supernatant contained mostly cytochrome b_5, the pellet consisted almost entirely of CO-binding particles in which cytochrome *P*-450 was converted to the *P*-420 form. Cross-contamination was below 10% in every instance. The hemes were extracted from the supernatant and the pellet as described previously [7]. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter with Insta-Gel (Packard Instrument Co.) as scintillation medium. Quenching was corrected by the internal standard method. Heme was determined by the pyridine hemochromogen technique [8] and calculated according to Omura and Sato [6].

3. Results

GF-Treatment resulted in a significant increase of cytochrome b_5 within 3 days of GF-feeding to about 150% and in a decrease of microsomal cytochrome *P*-450 to 60–70% of the control level. From the third day on, the cyto-chrome content of the microsomes remained constant for at least 11 days of GF-feeding, b_5 in an elevated and *P*-450 in a depressed state (fig.1). Half-life times of *P*-450 and b_5 hemes were then determined under these steady-state conditions. In control microsomes, decline of specific radioactivity of *P*-450 heme was biphasic in agreement with the results of Levin et al. [9] and Meyer and Marver [10] with a fast-phase between 0–24 h and a slow-phase thereafter. The fast-phase showed a half-life time of 7.1 h (mean value of two experiments) and the slow-phase of 23.9 h (mean value). In GF-treated mice the respective half-life times were doubled to 15.4 h and 50.2 h. A similar situation was found with respect to the b_5 hemes. In controls, the decay of specific radioactivity was linear after the twelfth hour following ALA-injection with a half-life time of 23.1 h (mean value), but there was a trend towards a faster decline within the first 12 h. In GF-treated mice, the decay of specific radioactivity was linear throughout the observation period with a half-life time of 45.6 h. The results are summarized in table 1.

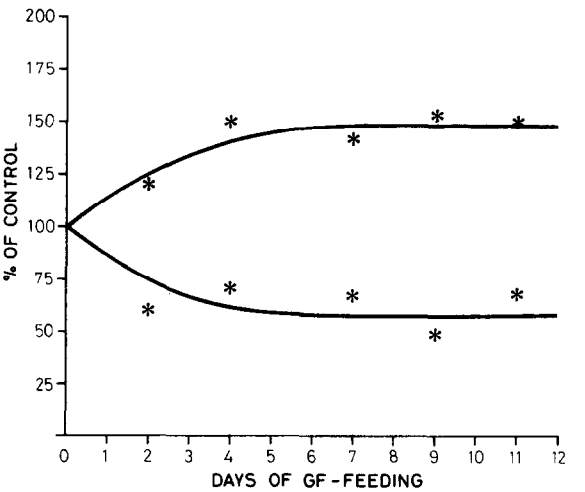

Fig.1. Alterations in the levels of microsomal cytochrome b_5 (upper line) and cytochrome *P*-450 (lower line) in relation to the duration of griseofulvin feeding.

4. Discussion

The GF-induced microsomal cytochrome b_5 elevation and the concomitant decline of cytochrome *P*-450 [3] was an unexpected finding since the GF-induced protoporphyria is thought to result from increased ALA synthetase activity and depressed con-

Table 1
Decay of specific radioactivity (regression lines) and half-life times of cytochrome *P*-450 and cytochrome b_5 hemes in griseofulvin-treated and control mice

		Regression lines and half-life times (t ½)[a]		
		Cytochrome *P*-450 heme		Cytochrome b_5 heme
		Fast-phase	Slow-phase	
Griseofulvin[b]		$y = -0.019x + 3.598$ t ½ = 15.4	$y = -0.006x + 2.939$ t ½ = 50.2	$y = -0.007x + 3.123$ t ½ = 45.6
Control[c]	Exp.1	$y = -0.047x + 3.445$ t ½ = 6.4	$y = -0.011x + 2.449$ t ½ = 26.6	$y = -0.011x + 2.818$ t ½ = 26.2
	Exp.2	$y = -0.039x + 2.720$ t ½ = 7.8	$y = -0.014x + 2.127$ t ½ = 21.2	$y = -0.015x + 2.343$ t ½ = 20.0

[a] Half life times (t ½) in h
[b] Regression lines based on 4 values at each time point (5-96 hours) each obtained from 5 pooled mouse livers
[c] Regression lines in each experiment based on 2 values at each time point (5-96 hours) each obtained from 5 pooled mouse livers

version of protoporphyrin into heme [2]. But under these conditions both cytochromes should be equally affected since their heme portions come from a common pool. One explanation of our results put forward in the past [3] was that the specificity of the GF effect lies in an alteration of apocytochrome *P*-450 synthesis rather than of heme synthesis. However, the findings of De Matteis and his group [2] do not fit this hypothesis. The results of the turnover studies presented in this paper suggest another mechanism. The half-life times of *P*-450 and b_5 hemes are prolonged which, at the first glance, seems to be a paradoxial finding. However, one has to consider that half-life times are determined at steady-state conditions which are, in our situation, characterized by a constant elevation of cytochrome b_5 and a constant depression of cytochrome *P*-450. Under these circumstances, rates of heme synthesis match those of heme degradation. From the results presented in this paper and those reported in the literature [2] the mechanism underlying the cytochrome b_5 and *P*-450 changes in GF-treated mice can be deduced as follows:

(i) GF-Treatment exerts an as yet unexplained stabilizing effect on microsomal cytochrome b_5 heme which results in a prolongation of the half-life time and a cytochrome b_5 elevation.

(ii) The constant level of elevated cytochrome b_5 from day 3 of GF-feeding on is only possible if b_5 synthesis is suppressed to match decreased b_5-heme degradation, possibly by inhibition of heme-synthetase. This assumption agrees with the findings of De Matteis et al. [2] that heme-synthetase activity is low in GF-treated mice.

(iii) Since cytochrome *P*-450 and cytochrome b_5 hemes come from a common pool, the inhibition of heme synthesis following b_5 heme stabilization leads to a decrease of cytochrome *P*-450. Protoporphyrin accumulation results from step 2 and step 3.

(iv) Under steady-state conditions, a decrease in the degradation-rate of cytochrome *P*-450 heme is inevitable in order to keep cytochrome *P*-450 at the constant low level.

In conclusion, the GF-induced protoporphyria seems to be triggered by a peculiar and as yet unexplained interaction between GF or GF-dependent mechanisms and cytochrome b_5 thus initiating a chain reaction which results in protoporphyria.

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References

[1] De Matteis, F. and Rimington, O. (1963) Br. J. Dermatol. 75, 91–104.
[2] De Matteis, F. and Gibbs, A. H. (1975) Biochem. J. 146, 285–289.
[3] Denk, H., Eckerstorfer, R., Talcott, R. E. and Schenkman, J. B. (1977) Biochem. Pharmacol. in press.
[4] Cinti, D. L., Moldeus, P. and Schenkman, J. B. (1972) Biochem. Pharmacol. 21, 3249–3256.
[5] Layne, E. (1957) in: Methods in Enzymology Vol. III, pp. 447–454, Academic Press, New York.
[6] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385.
[7] Denk, H., Greim, H., Hutterer, F., Schaffner, F. and Popper, H. (1973) Exp. Molec. Pathol. 19, 241–247.
[8] Paul, K. G., Theorell, H. and Akeson, A. (1953) Acta Chem. Scand. 7, 1284–1287.
[9] Levin, W., Jacobson, M. and Kuntzman, R. (1972) Arch. Biochem. Biophys. 148, 262–269.
[10] Meyer, U. A. and Marver, H. S. (1971) Science 171, 64–65.