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Studies on the mechanism of induction of microsomal cytochrome P452 and peroxisomal bifunctional enzyme mRNAs by nafenopin in primary cultures of adult rat hepatocytes

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A causal inter-relationship has been proposed to explain the good correlation between the induction of the peroxisomal and the microsomal compartments which is characteristic of the hepatomegaly produced in rodents by treatment with peroxisome proliferators [1–5]. The peroxisomal bifunctional enzyme (PBE*), a protein which catalyses both the hydration and dehydrogenation steps in the peroxisomal fatty acid β-oxidation system, may be used as a marker of the peroxisome induction. The lauric acid ω and ω-1 hydroxylase activity or cytochrome P452 (P450 IV A) may be used to demonstrate the induction of the endoplasmic reticulum.

The effects typically induced by this class of compounds in short-term studies, including the species differences, could be reproduced in primary cultures of adult hepatocytes [6–12].

Consequently primary hepatocyte cultures may be used to investigate *in vitro*, and therefore directly, the interrelationship between the induction of a typical peroxisomal gene (PBE) and of a typical microsomal gene (cytochrome P452 or P450IVAI). The aim of the present study was to compare the time courses of the induction of PBE and cyt.P452 mRNAs, in confluent monolayers of adult rat hepatocytes maintained in a chemically-defined medium and exposed to a non-toxic concentration of nafenopin. Pretreatment of the cultures with selected inhibitors was performed to shed some light on the nature of the events involved in the accumulation of the mRNAs in the presence of nafenopin.

Materials and Methods

Materials. Rats, culture vessels, cell culture medium, hormones, other additions and nafenopin (Su 13647), were as previously described [7, 11, 12]. Cycloheximide was from Fluka (Buchs, Switzerland) and sterile Actinomycin D was from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

5′-[α-^{32}P]dATP (3000 Ci/mmol) was from Amersham International (Amersham, U.K.). The 2.2 kb full length cDNA probe to the rat PBE gene and the 2.1 kb full length cDNA to the rat cytochrome P452 (P-450IVAI) were kindly provided by Dr T. Osumi, Shinshu University School of Medicine, Matsumoto, Japan and Dr G. Gibson (University of Surrey, U.K.), respectively.

In vivo experiments were as described previously [11].

Cell culture experiments. Cell culture experiments were performed as described in Ref. 7, with the additions to the culture medium used in Ref. 12. Nafenopin was added 20–22 hr after the seeding. At various time points, the cells were rapidly chilled, washed, harvested in Guanidium isothiocyanate buffer and stored at −70° until they were processed for RNA purification [11]. Each treatment group contained 2.1×10^7 hepatocytes originally seeded in three petri dishes.

Northern blotting. Purified RNA fractions (5 μg) were submitted to electrophoresis and transferred by capillarity to nitrocellulose filters (Gene Screen Plus, New England Nuclear, Boston, MA, U.S.A.) as previously described [11]. Filters were then hybridized to ^{32}P randomly-labelled cDNA probes. After stringent washes and autoradiography to identify the radioactivity bands, equal areas of the filters corresponding to the required specific mRNA band were cut out and the radioactivity was measured by liquid scintillation counting.

Results and Discussion

Co-induction of microsomal cyt. P452 mRNA and peroxisomal PBE mRNA in the rat liver by nafenopin treatment and in rat hepatocyte cultures exposed to nafenopin. In agreement with our previously published report [11], the amount of PBE mRNA assessed in hepatocytes which were isolated at various time points after peroral treatment of rats with nafenopin, was found to increase after a slight decrease at the first hour. A maximum was reached within 8 to 24 hr. On the other hand, the amount of cyt.P452 mRNA increased gradually from the time of nafenopin treatment (Fig. 1A).

The amount of these mRNAs was measured in confluent hepatocyte monolayers. The basal amount of the microsomal cyt.P452 mRNA and the peroxisomal PBE mRNA was found to decrease with time in rat hepatocytes maintained in primary culture in a chemically-defined medium as expected from the usually-observed losses in the content of cytochrome P450 and in the activity of peroxisomal β-oxidation. However, the addition of a non-toxic dose of nafenopin (32 μM) to 22-hr-old cultures triggered a true induction of the transcription of both the microsomal and the peroxisomal gene, since the concentrations of mRNAs present in nafenopin-treated 46-hr-old cultures were higher than those present in freshly isolated hepatocytes (Fig. 1B). Moreover, the induction of cyt.P452 mRNA by nafenopin occurred repeatedly earlier than the induction of PBE mRNA which appeared to be delayed as compared to the *in vivo* situation. The level of both mRNAs was not further increased by continuation of the treatment for up to 3 days either *in vivo* or *in vitro*.

In the cultured hepatocytes, the increase in the mRNA levels was followed by an increase in the amount of the corresponding gene products as visualized by Western blotting, although this increase was only observed 1–2 days later (not shown).

Such a true induction of the mRNAs could be observed only when the hepatocytes were allowed to recover from the isolation stress for 22 hr before the addition of nafenopin.

Effect of RNA synthesis inhibition on the mRNA induction by nafenopin. The addition of actinomycin D (1 μg/mL) to the culture medium completely inhibited RNA synthesis assessed by the incorporation of [^{14}C]uridine into RNA. In this situation the level of PBE mRNA was unchanged (91, 101 and 90% of control, respectively, at 3, 9 and 25 hr after the addition of the inhibitor), whereas the level of cyt.P452 mRNA almost doubled (being 174, 181 and 192% of control at the same time points). Although this effect is at present unexplained, it does suggest that the uninduced expression of the microsomal cyt.P452 and the peroxisomal

* Abbreviations: cyclo, cycloheximide; cyt.P452, cytochrome P450 isoenzyme which catalyses the ω-hydroxylation of lauric acid; naf, nafenopin; PBE, enoyl-CoA hydratase-3-hydroxyacylCoA dehydrogenase, peroxisomal bifunctional enzyme.

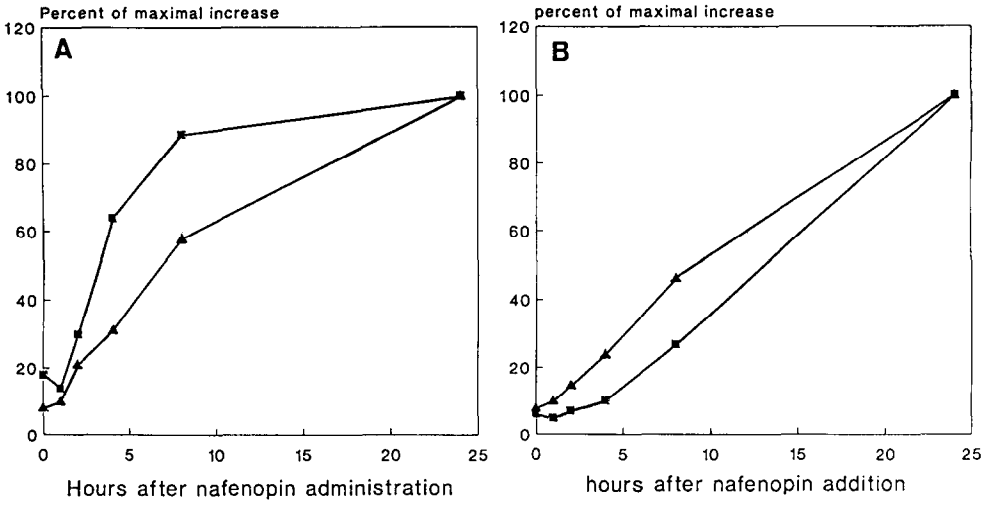

Fig. 1. Time course of the induction of PBE and cyt.P452 mRNAs: (A) In rat hepatocytes following a single peroral treatment with nafenopin. Following Northern blotting of the purified RNA fractions, the filters were separately hybridized to the randomly labelled cDNA probes (sp. act. $> 2.5 \times 10^6$ cpm/ng cDNA or 15×10^6/mL). Values in controls ranged between 300 and 900 cpm for PBE bands (—■—) and between 1260 and 2600 cpm for cyt.P452 band (—▲—). (B) In cultured hepatocytes exposed to 32 μM nafenopin. Control values correspond to the amount of mRNA present at the initiation of the treatment, i.e. in 22-hr-old cultures. They were 63 ± 10% and 24 ± 17% of the amount present in freshly isolated hepatocytes for cytP452 mRNA and PBE mRNA, respectively.

PBE genes could be regulated by different transcriptional control mechanisms.

In the absence of transcription, no nafenopin-induced accumulation of the mRNAs studied was observed (not shown), suggesting that the increase in the mRNA levels in response to nafenopin was due to an induction of the transcription of the genes.

Effect of protein synthesis inhibition on the mRNA induction by nafenopin. The addition of cycloheximide (5 μg/mL) to the culture medium inhibited the incorporation of [^{3}H]leucine into proteins by 80–90% within less than 30 min (data not shown). In control cultures, the level of either mRNAs was not significantly altered by the inhibition of protein synthesis (legend of Table 1). The addition of nafenopin in this situation led to a transient increase in the amount of cyt.P452 mRNA which was significantly higher in the first 2 hr than the increase measured in the presence of nafenopin alone. Such an effect was not observed with PBE mRNA. The latter finding is inconclusive, since by the first time point where induction would have been apparent (at least 4 hr) the cycloheximide-induced general toxicity had already altered the cyt.P452 induction. A non-specific activation of RNA polymerase by cycloheximide has been reported previously [13, 14] and cannot be excluded here. But it is unlikely that such an "unspecific" effect would only alter the cyt.P452 gene and only in nafenopin-treated hepatocytes.

In previous reports [15, 16], the existence of a labile repressor has been suggested to explain the transient overexpression or superinduction of the cyt.P450IA mRNA during partial inhibition of protein synthesis. More directly, the discrepancy observed between the gene transcription

Table 1. mRNA levels in cultures of adult rat hepatocytes exposed to nafenopin alone or in the presence of a protein synthesis inhibitor (per cent of the mRNA amount found at the beginning of the treatment)

Time after nafenopin addition (hr)	cyt.P452 mRNA (% of control)		PBE mRNA (% of control)	
	naf	naf + cyclo*	naf	naf + cyclo*
1	125 ± 52	290 ± 47	108 ± 21	155 ± 14
2	191 ± 39	290 ± 98	120 ± 23	140 ± 12
4	320 ± 132	265	144 ± 39	165 ± 37
8	707 ± 376	255	565 ± 310	136
24	1560 ± 921	142 ± 42	2240 ± 1268	104 ± 20

* Cycloheximide was added 1 hr before the addition of nafenopin. The amounts of cyt.P452 mRNA found in the presence of cycloheximide were 139, 110, 88 and 148% of their respective control at 1, 4, 8 and 24 hr after nafenopin addition, respectively.

The amount of PBE mRNA found in the presence of cycloheximide were 105, 100, 84 and 79% of their respective control at 1, 4, 8 and 24 hr after nafenopin addition, respectively.

If standard deviation is given, at least three experimental values have been averaged.

rates and the amount of mRNAs present in cultured hepatocytes following exposure to 3-methylcholanthrene or phenobarbital also suggested that the cyt.P450 mRNA concentrations were regulated by both transcriptional and post-transcriptional controls [17, 18].

In this study, the transient overexpression was restricted to the cyt.P452 gene in the presence of nafenopin and absent in cycloheximide-treated "control" cultures. The existence of two cyt.P452 genes is conceivable: a constitutive gene which would not be affected by protein synthesis inhibition and an inducible gene, expressed in the presence of nafenopin and overexpressed when the amount of a hypothetical "repressor" is decreased as a result of protein synthesis inhibition.

Studies of the regulatory regions of the cyt.P452 and PBE genes could help to determine the number of genes per genome and to understand their respective involvement in mRNA induction by peroxisome proliferators.

In summary, the amount of the two mRNAs although lower in cultured hepatocytes than in freshly isolated cells was found to be rapidly inducible upon the addition of 32 μM nafenopin. The induction of cyt.P452 mRNA always preceded the induction of PBE mRNA, but for both, the maximal induction (10–20-fold over control) was obtained within 24 hr and was achieved by transcriptional activation. At early time points (1 and 2 hr after the addition of nafenopin), in the absence of on-going protein synthesis, the amount of cyt.P452 mRNA (and not of PBE mRNA) was transiently higher in the presence of cycloheximide and nafenopin than in the presence of nafenopin alone.

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