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INHIBITION OF CYTOCHROME P-450p (P450IIIA1) GENE EXPRESSION DURING LIVER REGENERATION FROM TWO-THIRDS HEPATECTOMY IN THE RAT

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Abstract—Regenerating liver from partial hepatectomy (HPX) is known to exhibit a strong and transient deficiency in both spectrally detectable microsomal cytochrome P-450 (P-450) and related monooxygenase activities. Male Wistar rats (250–300 g) were HPX or sham operated and liver was excised at different times after operation. The time course of accumulation of five different forms of P-450 (including P-450b/e, P-450c, P-450d, P-450p and P-450UT-A) was determined in the regenerating liver, by Western blots developed with specific antibodies. With the exception of P-450c, whose level was not affected, the accumulation of other forms strongly decreased during the first 24 hr after HPX. For P-450b/e and P-450d, 80% of initial level was restored at 96 hr, whereas for P-450p and P-450UT-A, two major forms in control rat liver, the accumulation was only 20–25% of the initial, 1 week after HPX. No significant decrease was observed in sham operated animals. Plasmid pDex 12 containing a cDNA insert coding for P-450p was used to further investigate the effects of HPX on P-450p mRNA level and gene transcription. Northern blot analysis of RNA from regenerating liver (cDNA insert of pDex 12 being used as a probe) demonstrated that P-450p mRNA level decreased strongly to a minimum 12 hr after operation. This was correlated with a strong and transient decrease in P-450p gene transcription determined from nuclear run on experiments, the time course of which, however, did not account for the early decrease in mRNA level. We conclude that P-450p deficiency in the regenerating liver results from a combination of transient inhibition of gene transcription and early increase of mRNA degradation. Time course and amplitude of the decrease in P-450 UT-A accumulation suggest an inhibition of gene transcription as observed with P-450p.

Cytochromes P-450‖ constitute a superfamily of genes comprising at least eight distinct gene families expressed in mammalian species, four of which are encoding monooxygenases involved in the detoxication function of the liver [1]. A number of cDNA and/or genes coding for these enzymes were recently cloned and sequenced and regulation of their expression investigated specially in response to different classes of inducers including phenobarbital: P450II subfamilies B and C; methylcholanthrene (aromatic polycyclic hydrocarbons): P450I family; troleandomycine (macrolide antibiotics), rifampicin and glucocorticoids: P450III family (9–11); ethanol: P450IIE subfamily; clofibrate: P450IV family [2]. These inducers were shown to increase expression of cytochrome P-450 genes by acting either at transcriptional or post-transcriptional levels.

Fewer studies were devoted to the opposite situation, that is, decrease in level of cytochrome P-450 under specific physiological or non-physiological conditions. Partial (two-thirds) hepatectomy (HPX) provides such a situation. A number of investigators have reported that the level of cytochrome P-450 as well as some related monooxygenase activities are strongly decreased a few hours and for several days after HPX [3–7]. Although the data appear to be in reasonable agreement from one group of investigators to another, the interpretations of this decrease are conflicting and not satisfactory. Thus for example Presta *et al.* [4] concluded that the decrease in monooxygenase activities from regenerating liver was the consequence of the mere surgical stress, whereas Yoshida *et al.* [5] proposed that these observations were to be related to the increase in heme oxygenase activity which occurs in the regenerating liver. However, the possibility that regeneration could transiently affect the expression of cytochrome P-450 genes was only recently considered [8]. We therefore decided to test this hypoth-

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‖ Abbreviations: P-450, cytochrome P-450 from liver microsomes; HPX, two-thirds hepatectomy. Correspondence between trivial and newly proposed official nomenclature [1] of cytochromes P-450 studied in this work is: P-450b/e, P450IIB1/B2; P-450c, P450IA1; P-450d, P450IA2; P-450p, P450IIIA1; P-450 UT-A, P450IIC (gene number is not known).

esis by investigating the effect of HPX on P-450 apoprotein and mRNA accumulation and on gene transcription. Our results indicate that the apoprotein level of various forms of P-450 is strongly decreased during the regeneration period. In the case of P-450p, for which a cDNA probe could be used, this was correlated with a decrease in specific mRNA accumulation which was shown to result from both increased degradation and inhibition of P-450p gene transcription.

MATERIALS AND METHODS

Animals. Male Wistar rats (250–300 g) were maintained in individual cages with free access to food and water. Two-thirds hepactectomy was carried out according to the procedure of Higgins and Anderson [9], the animals being anesthetized under ether. Sham operated rats were simply laparotomized under similar conditions. In all cases the animals were fasted overnight either before operation or sacrifice. Each group referred to in the following as unoperated, hepatectomized or sham operated contained three animals.

Microsomes, cytochrome P-450 and monooxygenase activities. Liver microsomes were prepared as described previously [10]. Protein concentration was determined by the method of Lowry with appropriate modifications for buffer components [11]. Total cytochrome P-450 concentration was estimated spectrally according to Omura and Sato [12].

Benzphetamine, ethylmorphine and erythromycin demethylases were determined by the method of Nash [13], the final substrate concentration being 1 mM. Ethoxycoumarin and ethoxyresorufin deethylases were determined fluorometrically according to previously published procedures [14, 15], the final substrate concentration being 200 μM. Microsomes were diluted to 1 mg/ml (except for ethoxyresorufin deethylase: 0.5 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.4. After addition of the substrate, the reaction was initiated by addition of 0.5 mM NADPH at 37°. For demethylase activities, the assays were performed for 10 min whereas for the deethylase activities turnover was deduced from the initial linear part of the recordings of fluorescence change against time. In all cases the use of appropriate standards allowed the turnover to be determined in nmol product formed per min per mg of microsomal proteins. Absolute uncertainties were estimated so as to take into account both experimental precision of measurements and inter-individual variability.

Western blotting and anti P-450 antibodies. Western blotting analysis was carried out as previously described [16] except that 10–30 μg of microsomal proteins (depending on the antibodies used) were submitted to electrophoresis on a 10% polyacrylamide gel in the presence of SDS. After electrophoretic transfer onto nitrocellulose filters, the various P-450 forms were revealed with specific antibodies diluted to 0.1 mg/ml. Antibodies against P-450c, P-450d and P-450UT-A were kindly supplied by Dr P. Beaune (INSERM U-75 Paris, France). Cytochromes P-450b/e and P-450p were revealed with antibodies directed against the orthologous forms in the rabbit, P-450 2 and 3c, respectively [1, 17–19]. Control experiments including comparative analysis of microsomes from control and induced animals (phenobarbital for P-450b/e and troleandomycin for P-450p) and immunoinhibition assays benzphetamine and erythromycin being used as substrates, confirmed the adequate reactivity of these antibodies. In all cases HRP-labeled species specific antibodies allowed the staining of the blots, diaminobenzidine being used as the substrate. Relative concentration of the various forms of P-450 was estimated from a densitometric analysis of the blots with a Shimadzu dual-wavelength scanner.

RNA preparation. RNA was isolated from freshly excised rat liver by the procedure of Auffray and Rougeon [20]. RNA was precipitated with ethanol and redissolved in pure water before Northern blot analysis. RNA concentration was estimated spectrophotometrically at 260 nm.

cDNA probes and Northern blots. Plasmid pDex 12 [21] containing a lkb cDNA insert coding for cytochrome P-450p was kindly provided by Dr P. S. Guzelian (MCV, Richmond, VA). Plasmid pRGAPDH-13 [22] containing a 1.4kb cDNA insert coding for glyceraldehyde-3P-dehydrogenase was used in control experiments. Northern blotting was carried out as previously described [16, 17]. Briefly, 20 μg of total RNA from control, hepatectomized and sham operated animals were submitted to electrophoresis on a 1.2% agarose gel and transferred to a nylon (Hybond) filter. Hybridization and washing were carried out as described [16, 17]; pDex 12 was radiolabeled by nick translation (1–2 $\times$ 10^8 dpm/μg). In some control experiments, the filter was dehybridized and rehybridized with pRGAPDH-13 under the same conditions. Filters were autoradiographed for 16 hr at −80°.

Nuclei preparation and in vitro *transcription experiments*. Nuclei were prepared from freshly excised liver of control, hepatectomized or sham operated animals by the method of Schibler *et al.* [23]. *In vitro* transcription was carried out with approximately 10^7 nuclei as previously described [24]. The extracted nascent RNA chains were hybridized with 5 μg of either pDex12 or pRGAPDH-13 (for control experiments) which had been previously denatured and blotted onto nitrocellulose filters. The filters were autoradiographed for 16 hr at −80°.

Materials. Substrates were purchased from the following sources: benzphetamine (Upjohn, Paris, France), erythromycin (Roussel, Paris, France), ethylmorphine and ethoxycoumarin (Sigma, St Louis, MO), ethoxyresorufin (Pierce Eurochimie, Ond-Beijerland, Holland). Species specific HRP-labeled antibodies were from Sigma (St Louis, MO). [^{32}P]dCTP and [^{32}P]UTP were from Amersham International (Amersham, U.K.). Nitrocellulose filters used in Western blots and for *in vitro* transcription experiments were from Bio-Rad (Richmond, CA) and Schleicher and Schuell (Dassel, F.R.G.), respectively. Northern blots were carried out on Hybond filters from Amersham (Amersham, U.K.). Autoradiography was carried out on X-omat AR Kodak films.

RESULTS

Cytochrome P-450 and related monooxygenase activities during liver regeneration

The data reported in Table 1 show that in the liver microsomes from hepatectomized animals, spectrally detectable cytochrome P-450 decreased by 50% during the first 24 hr and reached a minimum between 48 and 96 hr following the operation. The cytochrome P-450 level then started to increase after 1 week and reached (or approached) its initial value by 2 weeks. In the meantime sham operated animals did not exhibit such a large decrease, if any.

In agreement with these observations various monooxygenase activities appeared to be similarly depressed during liver regeneration. The various substrates selected are known to be specific to various P-450 forms: ethylmorphine, P-450 UT-A; ethoxycoumarin and ethoxyresorufin P-450c; erythromycin, P-450p; and benzphetamine, P-450b/e [25]. As observed, for the P-450 level, these activities decreased sharply during the first 24 hr to reach a minimum between 48 and 96 hr and then increased back to their original level 2 weeks after operation, with the exception of ethoxycoumarin deethylase which was only 57% of control at this time. Here again no significant decrease was observed with the liver microsomes from sham operated rats. These results are in good agreement with previous reports [3–7] and illustrate quite conclusively the dramatic transient decrease in detoxication capacity of the regenerating liver.

Immunoquantitation of five different forms of microsomal P-450 during liver regeneration

Since a number of different forms of microsomal P-450 coexist in the liver it was of interest to determine whether their concentration was similarly or differently affected during liver regeneration.

For this purpose liver microsomes from control, sham operated and hepatectomized animals were comparatively analyzed by Western blotting using various antibodies directed against five different forms of P-450, including P-450b/e, P-450c, P-450d, P-450p and P-450UT-A. The results are reported in Fig. 1. Three different patterns were exhibited: (i) P-450p and P-450UT-A apoproteins accumulation decreased sharply after HPX and this effect lasted for at least 1 week; in both cases the original level was not fully restored after 2 weeks; (ii) P-450b/e and P-450d apoprotein level decreased sharply during the first 24 hr but increased again quickly so that at least 80% of the original level was restored at 96 hr; and (iii) P-450c was not affected significantly during regeneration. With the exception of anti P-450c and P-450d antibodies which cross-reacted with both forms, only one band comigrating with authentic standards was revealed on each Western blot with the other antibodies. The reason why data from sham operated animals differ from those relative to unoperated animals in panels (B) and (C) is not clear. This most likely results from inter-individual differences.

In considering these data it must be realized that P-450p and P-450UT-A are two major forms of P-450 in the control rat liver, whereas the sum of P-

Table 1. Effect of sham operation and two-thirds hepatectomy on spectrally detectable cytochrome P-450 and monooxygenase activities in rat liver microsomes

Activities	UT	Sham operation		Hepatectomy				
		48 hr	96 hr	24 hr	48 hr	72 hr	96 hr	1 week	2 weeks
P-450	0.92 ± 0.10	1.05 ± 0.10	0.75 ± 0.10	0.46 ± 0.07	0.30 ± 0.5	0.38 ± 0.07	0.31 ± 0.08	0.51 ± 0.10	0.82 ± 0.10
ETDM	7.0 ± 0.6	5.8 ± 0.5	5.8 ± 0.6	4.0 ± 0.1	2.8 ± 0.4	2.1 ± 0.5	3.1 ± 0.5	3.4 ± 0.7	7.2 ± 0.1
ERDM	0.9 ± 0.1	0.79 ± 0.05	1.0 ± 0.1	0.51 ± 0.06	0.55 ± 0.07	0.34 ± 0.05	0.38 ± 0.02	0.41 ± 0.07	0.88 ± 0.07
BDM	4.3 ± 0.5	3.4 ± 0.5	4.4 ± 0.5	1.4 ± 0.4	1.5 ± 0.3	1.2 ± 0.3	2.0 ± 0.5	3.3 ± 0.5	6.1 ± 0.5
ECDE	0.33 ± 0.05	0.34 ± 0.04	0.35 ± 0.04	0.16 ± 0.05	—	0.16 ± 0.04	0.17 ± 0.03	0.15 ± 0.05	0.20 ± 0.04
ERDE	0.5 ± 0.1	0.68 ± 0.10	0.6 ± 0.1	0.19 ± 0.07	0.26 ± 0.05	0.39 ± 0.10	0.36 ± 0.08	0.39 ± 0.07	0.43 ± 0.05

Liver microsomes prepared from unoperated animals (UT), sham operated animals and two-thirds hepatectomized animals at different times after operation were diluted to 1 mg/ml in 0.1 M potassium phosphate, pH 7.4, and assayed for cytochrome P-450 (nmol/mg) and monooxygenase activities (nmol/mg/min) as described in Materials and Methods. ETDM, ethylmorphine demethylase; ERDM, erythromycin demethylase; BDM, benzphetamine demethylase; ECDE, ethoxycoumarin deethylase; ERDE, ethoxyresorufin deethylase. Data reported are average values from three animals in each group.

450b/e, P-450c and P-450d only accounts for less than 5% [25]. It is therefore obvious that the strong decrease observed on the accumulation of P-450p and P-450 UT-A contributes predominantly to the spectrally detectable P-450 deficiency reported in Table 1.

These results correlated reasonably well with those reported for erythomycin, ethylmorphine and benzphetamine demethylases. The finding that both ethoxycoumarin and ethoxyresorufin deethylases strongly decrease after HPX, whereas P-450c was not affected, is not inconsistent. Although these substrates are rather specific to P-450c it has to be emphasized, as mentioned above, that this form is a very minor one in control rat liver so that both activities are more likely to reflect the level of other P-450 forms.

At least two explanations might be proposed to account for the results presented in Fig. 1: either transient stimulation of P-450 apoprotein degradation or inhibition of expression of P-450 genes during the regeneration process. Further experiments were therefore designed to decide tentatively in favor of one of these two alternatives.

Cytochrome P-450p mRNA level and gene transcription during liver regeneration

Plasmid pDex12 (kindly provided by Dr P. S. Guzelian, MCV Richmond, VA), containing a 1kb cDNA insert coding for the C-terminal half of the protein [21] was used to investigate the influence of HPX and liver regeneration on cytochrome P-450p mRNA level and gene transcription. For this purpose RNA was isolated from the liver of control, sham operated and hepatectomized rats and analyzed by Northern blot. The transcriptional state of the P-450p gene was determined in parallel on the same liver samples using a run on transcription assay. The results are shown in Figs 2 and 3.

It is clear from Fig. 2 that the accumulation of P-

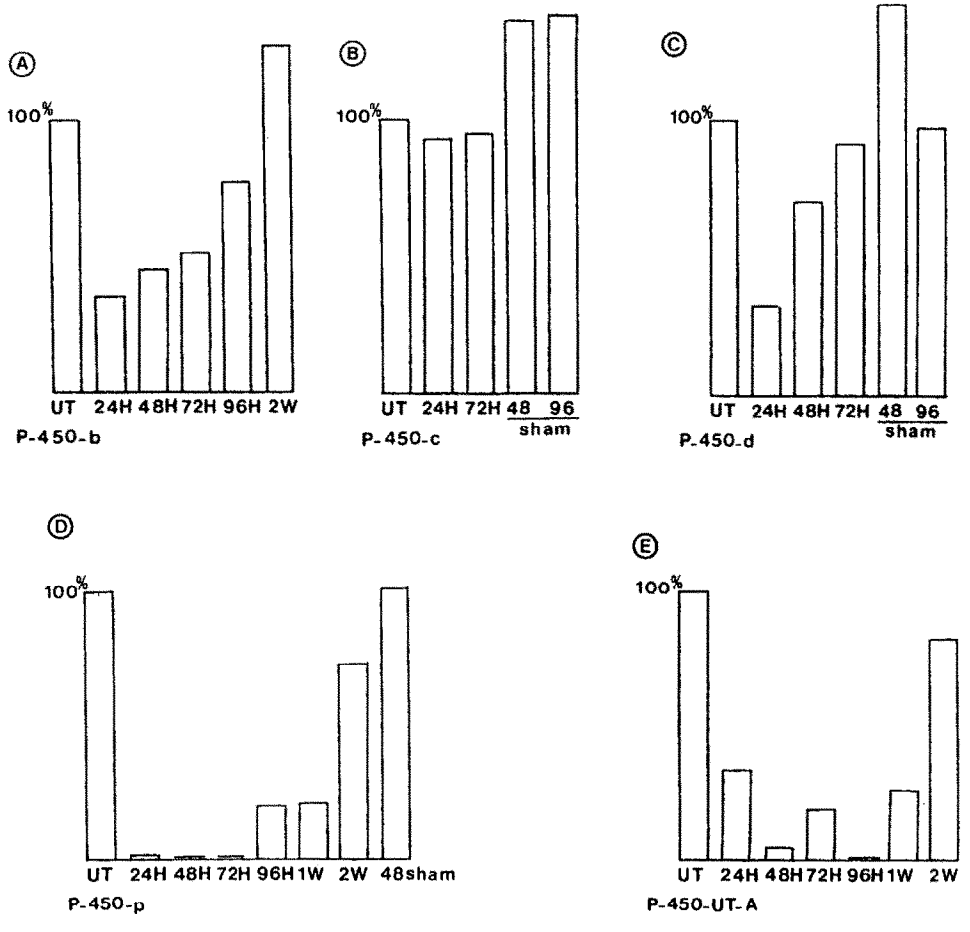

Fig. 1. Immunoquantitation of five different forms of cytochrome P-450 in rat liver microsomes after HPX. Liver microsomes prepared from unoperated (UT), two-thirds hepatectomized or sham operated animals at different times after operation were analyzed by Western blotting as described in Materials and Methods, for their content in five different forms of cytochrome P-450 by using specific antibodies. After revelation of the antibody–antigene complex by staining with HRP directed oxidation of diaminobenzidine, relative concentration of the various forms of P-450 was estimated from a densitometric analysis of the blots with a Shimadzu dual wavelength scanner. For the sake of clarity, only times at which significant effect was routinely observed (three series of animals) are presented here.

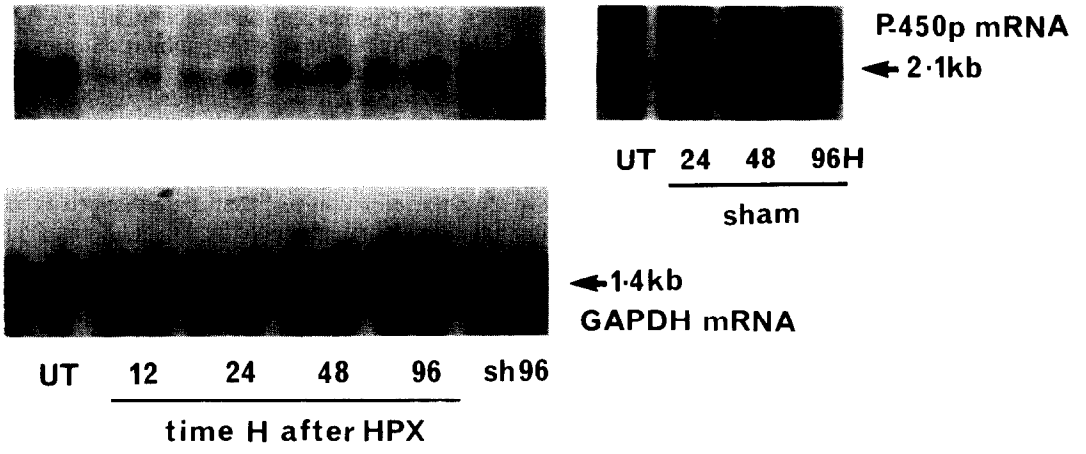

Fig. 2. Effect of HPX on the level of cytochrome P-450p mRNA in regenerating rat liver. RNA extracted from unoperated (UT), two-thirds hepatectomized and sham operated animals at different times after operation was analyzed in Northern blot probed with [^{32}P]pDex 12 (P-450p cDNA). After washing, the filter was autoradiographied for 16 hr at −80°. The filter was then dehybridized, rehybridized with [^{32}P]pRGAPDH and similarly analyzed.

450p mRNA (2.1 kb) was strongly decreased as early as 12 hr after HPX and remained low during liver regeneration, whereas in sham operated animals no decrease was apparent.

The experiments reported in Fig. 3 show that cytochrome P-450p gene transcription was strongly inhibited during liver regeneration, reached a minimum near 96 hr and returned to its apparent initial level after 2 weeks. Although these results are in agreement with those concerning mRNA level at 96 hr and later on during regeneration (experiments not reported here), they do not explain the early decrease in mRNA level. Indeed, while no significant variation in transcription was observed during the first 24 hr after HPX, mRNA had reached a minimum in the same period of time. These observations suggest that the decrease in mRNA accumulation cannot simply result from inhibition of gene transcription; further contribution should operate, shortly after HPX.

As a control, this series of experiments was repeated with plasmid pRGAPDH-13 cDNA insert encoding glyceraldehyde-3P-dehydrogenase (GAPDH) [22]. In contrast to what we observed with cytochrome P-450p, neither GADPH mRNA level (Fig. 2) nor gene transcription (Fig. 3) was affected after HPX and during regeneration.

DISCUSSION

The results presented in this paper demonstrate that the dramatic decrease in detoxication capacity of the regenerating liver following HPX is attributable to reduced amounts of apoprotein of various forms of cytochrome P-450. Specially relevant was the observation that both P-450p and P-450 UT-A, two major forms in the control rat liver, exhibited the largest repression.

The use of a cDNA probe coding for P-450p allowed us to relate the decrease in P-450p apoprotein accumulation to a concomitant decrease in specific mRNA. Further experiments to characterize the effect of HPX on P-450p gene transcription revealed a strong inhibition of this activity during regeneration, the time course of which however, did not allow explanation of the early decrease in mRNA level. Taken together these observations suggest that shortly after HPX, P-450p mRNA degradation is highly accelerated so that even though the gene is still being transcribed at a normal rate (see Fig. 3 at 24 hr) the mRNA level is very low. We therefore conclude that during regeneration after HPX the expression of the cytochrome P-450p gene is (negatively) controlled at two different levels: increased degradation of mRNA and inhibited transcription of

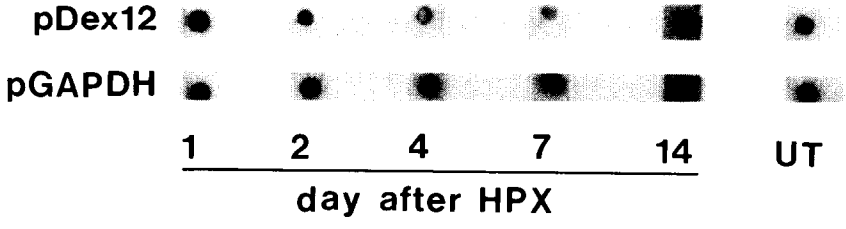

Fig. 3. Effect of HPX on cytochrome P-450p gene transcription in nuclei from regenerating rat liver. Nuclei were extracted from freshly excised liver of unoperated (UT), two-thirds hepatectomized animals at different times after operation. Equivalent amounts of newly synthesized radioactive transcripts were hybridized to nitrocellulose-bound cDNA from pDex 12 (P-450p) and pRGAPDH. After washing, filters were analyzed by autoradiography.

the gene. Increased apoprotein degradation after HPX cannot presently be ruled out and could also contribute to the observed decrease in cytochrome P-450 although this further contribution does not appear to be necessary to explain the results observed here. In the absence of a specific cDNA probe, similar investigation could not be carried out with P-450 UT-A. However, the results of Fig. 1E showing a pattern and amplitude of apoprotein deficiency quite similar to that exhibited by form P-450p suggest that transient inhibition of gene expression could occur with P-450 UT-A as well.

Interestingly our results showed that other quantitatively minor forms of cytochrome P-450 in control rat liver including P-450b/e, P-450c and P-450d (whose total concentration does not account for more than a few percent of P-450) were not affected to a similar extent both quantitatively and qualitatively at the apoprotein level (Fig. 1). In this respect our results on P-450c and P-450d are in qualitative agreement with a previous study [8] where it was observed that P-450c expression was increased after HPX while P-450d exhibited opposite behavior. These observations suggest that the expression of different P-450 genes is differently controlled in the regenerating liver.

On the basis of our and other's [5] results on sham operated animals the surgical stress theory proposed by Presta *et al.* [4] to explain the decrease in monooxygenase activities in the regenerating liver is no longer tenable. Clearly, this effect is not reproducible from one laboratory to another. In addition to the surgical stress *per se* it should originate from a number of distinct contributions including nature of anesthetics, duration and techniques of the operation, quality of recovery of the animals which in turn will affect their nutritional status, strain of the animals, etc. Indeed some of these contributions are able to affect significantly the level of P-450. However, our and other's results [5, 6] show that these contributions remain low and could even occasionally be negligible. We therefore conclude that the observed changes in P-450 level and/or expression after HPX do indeed result from changes in gene expression resulting from adaptation of the cell to the regeneration program.

On the other hand the hypothesis of Yoshida *et al.* [5] requires to be reconsidered in the light of our results. These authors claimed that the strong increase in heme oxygenase activity they observed after HPX was responsible for the deficiency of spectrally detectable cytochrome P-450. The results reported in this paper provide, as documented in the previous paragraph, a different interpretation, which in turns allows us to explain the enhanced heme oxygenase activity. Specially relevant in this respect is the observation that mRNA and apoproteins level sharply falls to a minimum 12–24 hr after HPX whereas, according to Yoshida *et al.* heme oxygenase reaches a maximum between 24 and 72 hr after the operation (no data were reported at 48 hr). It is currently admitted that during degradation of cytochromes P-450 the heme moiety is transferred to the hepatocyte heme pool whose concentration is the result of a balance between heme synthesis, exchange between P-450 forms and degradation [26–28]. In this respect heme is a natural inducer of heme oxygenase. Under conditions of strong inhibition of expression of cytochrome P-450 genes where the balance between P-450 *de novo* synthesis and degradation is displaced towards degradation, it is reasonable to expect a concomitant strong increase of the heme pool and consequently of the heme oxygenase level. We therefore suggest that the enhanced heme oxygenase activity observed by Yoshida *et al.* [5] during liver regeneration is the *consequence rather than the cause* of the P-450 cytochromes deficiency.

In addition to the regenerating liver after HPX, it has been repeatedly shown that drug metabolism activities and cytochrome P-450 were repressed in highly proliferating tissues: this is the case in the fetal liver where cytochromes P-450 are absent except shortly before birth [29–30] and in liver nodules and/or hepatomas [31–33].

Interestingly, Stout and Frederick [34] recently observed that the decrease in P-450 (and other hemoproteins) occurring in spontaneous liver mouse tumor was accompanied by an increase in heme oxygenase. Although no kinetics or quantitative analyses were carried out at the specific mRNA and/or apoproteins levels it is not unreasonable to suspect that, here again, heme oxygenase increase could simply result from heme release consecutive to repressed expression of P-450 genes in highly proliferating hepatocytes.

The reasons for this are not yet clearly understood but they are likely to result from genomic alterations which could possibly take place under the influence of either "toxohormones", which have been suspected to be released from hepatomas [35, 36], or hepatotrophic factor such as EGF in the case of regeneration [37]. Future studies directed towards the characterization of physical and/or chemical modifications of P-450 genes (increased methylation, masking of hypersensitive sites, etc.) during liver regeneration or tumor development would provide critical information on these points. Whatever the origin of this effect it must be kept in mind, from a pharmacological point of view, that medication of patients during the liver regeneration period after HPX should be carefully scheduled in order to avoid any adverse reaction due to this transient deficit in drug metabolizing capacity of the liver.

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