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Changes in endoplasmic reticulum associated with β-naphthoflavone-inducible cytochrome P450 in murine liver: a quantitative analysis

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Abstract

Cytochrome P450s are found associated with membranes of endoplasmic reticulum where they catalyse the oxidation and biotransformation of a wide range of substances. It is therefore expected that their induction and catalytic activities would correlate with the ultrastructure of the cell. In this study, we compared the structure of the hepatocytes of C57BL/6 mice at the basal level in the untreated control animal, and followed induction with the β-naphthoflavone in the experimental animal. Qualitative analysis of liver sections from control, corn oil-treated mice, and the induced, β-naphthoflavone-treated mice were essentially similar with no obvious differences between the two groups.

Direct morphometry was utilized to quantify the amounts of smooth and rough endoplasmic reticulum in the hepatocytes. A computerized Bioquant Image Analysis System was used to determine the areas of endoplasmic reticulum in the hepatocytes. Morphometric analysis showed 1.004-fold increase in the amount of smooth endoplasmic reticulum and 1.018-fold increase in amount of rough endoplasmic reticulum following treatment of C57BL/6 mice with β-naphthoflavone. These increases were not significantly different from amounts in control hepatocytes (P). In conclusion, induction of cytochrome P450 by β-naphthoflavone does not appear to elicit increased proliferation of the endoplasmic reticulum.

Résumé

Les cytochrome P 450s sont associés aux membranes du reticulum endoplasmique où ils catalysent l'oxidation et la biotransformation d'une grande variété de substances. Il est donc à supposer que leur induction et leurs activités catalytiques seraient correlatives à l'ultrastructure de la cellule. Dans cette étude, nous avons comparé les hépatocytes des souris C57BL/6 au niveau basal chez un animal témoin non-traité, et l'induction avec la β-naphthoflavone dans l'animal expérimental. L'analyse qualitative des `sections de foie prises du cas témoin, du souris traité à l'huile de ma'i's, et de l'animal expérimental induit et traité à β-naphthoflavone étaient essentiellement similaires.

La morphométrie directe a été utilisée pour évaluer la quantité des reticulum plasmiques lisse et rougeux dans les hepatocytes. Le "Bioquant Image Analysis System" a été utilise pour déterminer les endroits de réticulum endoplasmique dans les hépatocytes. L'analyse morphométrique a montré des augmentations de 1.004 fois dans la quantité de réticulum endoplasmique lisse et de 1.018 fois dans la quantité de réticulum endoplasmique rougeuse après le traitement des souris C57BL/6 avec β-naphthoflavone. Ces augmentations n'étaient pas considérablement différentes des quantités trouvées dans les cas témoins hepatocytes ($P<0.15$). Pour conclure, l'induction de cytochrome P450 avec β-naphthoflavone ne provoque pas de proliferation augmentée du reticulum endoplasmique.

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Introduction

The cytochrome P450 gene superfamily encodes numerous related enzymes which are assigned into families and subfamilies on the basis of amino acid and nucleotide sequence. They differ but overlap in their species and tissue distribution, physiochemical properties, inducibility and substrate specificity [1-3]. Cytochrome P450 enzymes are distributed. widely in nature and occur in microorganisms, plants and animals. In mammalian tissues, cytochrome P450s are found associated with cellular organelles and are particularly abundant in membranes of the rough endoplasmic reticulum (rER), smooth endoplasmic reticulum (sER) and mitochondria [4,5].

To date, different cytochrome P450s have been identified, isolated and characterized mainly from hepatic tissue. However, many nonhepatic tissues also possess the enzymes [6]. The liver is the major site of metabolism of environmental chemicals, xenobiotics and various endogenous substances. Numerous studies have therefore been carried out to determine its role in pharmacological responses to drugs. The liver is uniquely situated to take up substances delivered to it via the blood stream. The cytochrome P450s located especially on the sER of the hepatocytes can then catalyse the oxidation and biotransformation of the substances.

A widely accepted hypothesis in modern cell biology is that the activity of some enzymes is strongly correlated with the amount of certain organelles. Such structure-function relationship appears to indicate the ability of the organism to react to and adapt to changes in its environment such as drug administration [7]. Since cytochrome P450 enzymes are membrane-bound, their activities would correlate with the ultrastructure of the cell during metabolism. Various exogenous and endogenous stimuli can regulate and moderate the expression of specific P450 isozymes. A major class of cytochrome P450 is the 3-methylcholanthrene (3-MC-) inducible forms, namely CYP1A1 and CYP1A2. Previous investigations on changes in hepatic sER following treatment of an animal with 3-MC-type agents are limited and the results appear to be inconclusive and conflicting. While some studies showed that induction of cytochrome P450 with 3-MC-type agent did not produce proliferation of hepaticsER [8-10], other investigators reported that rats treated with 3-MC or β-naphthoflavone (βNF) showed proliferation of the sER in hepatocytes as observed by electron microscopy [11]. It was reasoned that quantification by morphometric analysis might be required in order to obtain further clarification of these discrepancies.

This study was undertaken to determine whether induction of cytochrome P450 after administration of βNF occurred concurrently with significant proliferation of the sER and the rER. We have also morphometrically compared the amount of sER and rER in the liver at the basal level and following treatment of C57BL/6 mice with βNF using the Bioquant Image Analysis System.

Materials and methods

Adult male mice of C57BL/6 strain (20-22g) were obtained from Charles River Canada (St. Constant Quebec, Canada). Animals were housed in group plastic cages over hard wood bedding (Betachip, Northeastern Products Corp., Warrensburg, NY). They were kept in a temperature (25 ± 1°C) and light (6.00a.m.-6.00p.m.) — controlled environment. They were allowed food (Purina Rodent Chow) and water *ad libitum*.

Mice were divided into two groups. The first group was administered βNF (80mg/kg. Ip) (Sigma Chemical Co; St. Louis, MO) in corn oil at 72h and 48h before death. The second group served as controls and were given an equivalent volume of the vehicle.

Excised livers were fixed in Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.01M sodium cacodylate buffer, pH 7.2) by intra-cardiac perfusion. Liver slices were post-fixed in 2% osmium tetroxide in 0.1M sodium cacodylate buffer and stained with uranyl acetate. After dehydration in graded concentrations of acetone, tissues were infiltrated and embedded in Epon. Ultrathin sections were cut from two to four blocks from each mouse with LKB 2188 Nova ultramicrotome using a Dupont diamond knife. Sections were stained with 6% uranyl acetate followed by 0.35% lead acetate and subsequently examined with a Hitachi H-7000 electron microscope. Centrilobular hepatocytes were examined because previous studies have shown that constitutive levels of 3-MC-inducible cytochrome P450s were predominantly found in centrilobular hepatocytes [12, 13]. A regional difference in the distribution of sER within the liver of untreated rats has also been reported [14]. Centrilobular hepatocytes have the

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highest concentration of sER, followed by the mid-zonal, and the periportal cells have the least.

For morphometric analysis, at least 50 micrographs of centrilobular hepatocytes (at 15,000x or 20,000x magnification) from 6 mice were used. The area occupied by sER and rER in each cell was measured by computerized morphometry using a Bioquant Image Analysis System. The membranes of the sER and rER were traced with a "mouse" connected to the computer, in order to determine the area in μm^2. The cell area was measured likewise. The amounts of sER and rER in the hepatocyte were expressed as the ratio of sER area or rER area to the cell area. All data were calculated as mean ±SD, and comparisons between groups were made with the student's t-test. A p value < 0.05 was considered to be significant.

Results

The general architecture of the liver was well preserved and there were no observable differences between the livers from corn oil-treated and βNF-treated mice at the light microscopic level. Plates of the hepatocytes were arranged in cords radiating from the central vein. The ultrastructure of hepatocytes from the two groups was essentially similar under the electron microscope (figs 1a and b). No obvious changes in the amounts or distribution of the sER and rER were detected by qualitative examination. Using the quantitative morphometric analysis, there was a 1.004-fold and 1.018-fold increase in the amounts of sER and rER in the hepatocyte following treatment of mice with βNF. These increases were not statistically significant (P <0.15) when compared with the amounts of sER and rER in non-induced mice (table 1).

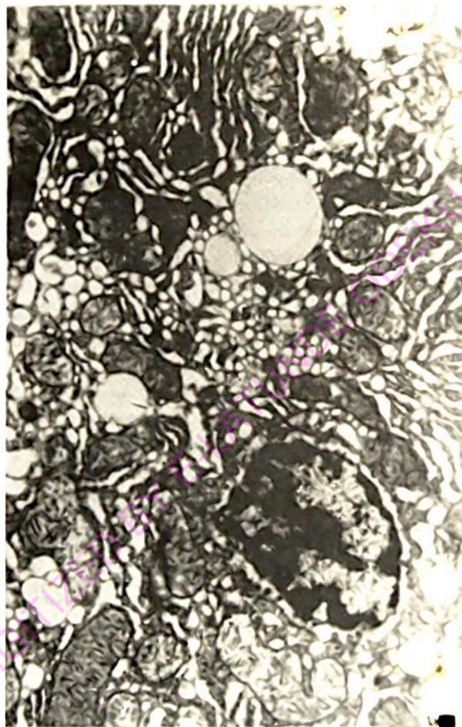

Fig. 1a: Ultrastructure of centrilobular hepatocyte of C57BL/6 mice; hepatocyte from control mouse treated with corn oil. Smooth endoplasmic reticulum (sER), rough endoplasmic (rER), mitochondria (m), (15,500x).

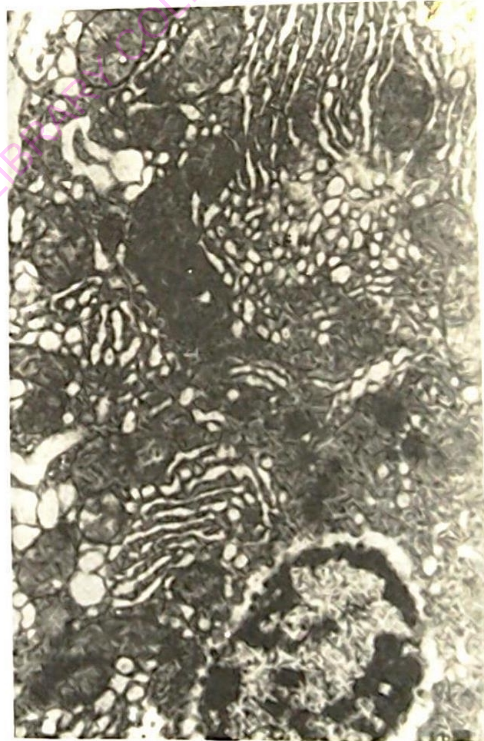

Fig. 1b: Ultrastructure of centrilobular hepatocyte of C57Bl/6 mice; hepatocyte from βNF-treated mouse. Smooth endoplasmic reticulum (sER), rough endoplasmic (rER), mitochondria (m), (15,500x).

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Table 1: Morphometric analysis of the smooth endoplasmic reticulum (sER) and rough endoplasmic reticulum (rER) in corn oil-treated and βNF-treated C57BL/6 mice (surface area in $\mu m^2 \pm SD$)[a]

Treatment	Cell area[a]	sER area/Cell[a]	sER area/ Cell area ($x10^{-2}$)	rER area/ Cell	rER area/ Cell area ($x10^{-2}$)
Corn oil	22.62 ± 1.95	1.54 ± 0.09	6.81 ± 0.47	0.73 ± 0.14	3.23 ± 0.43
βNF	23.10 ± 2.01	1.58 ± 0.10	6.84 ± 0.38[b]	0.76 ± 0.19	3.29 ± 0.60[b]

[a] Values are means ± SD derived from 50 representative sections of 6 mice
[b] $P < 0.15$ when compared to control values

Discussion

3-MC-inducible cytochrome P450s primarily localized in the sER and significant proliferation of this organelle following treatment with 3-MC or βNF has been reported by some authors [11]. Others reported that 3-MC of βNF did not cause proliferation of hepatic sER [7]. Since analysis and interpretation of qualitative findings invariably includes subjective components, there is a need to seek for an objective means of measuring and quantifying morphometric structures and their alterations. Morphometric measurement is not only objective, it is reproducible and it helps to correlate morphology to function or to other morphologic parameters. The areas of the endoplasmic reticulum and cell were measured directly in the test field with the aid of the computer. Although this method is relatively expensive, it offers greater accuracy and it is less time-consuming than other indirect stereological methods. The results obtained from the present study showed no statistically significant differences in amounts of sER and rER in both the corn oil-treated and βNF-treated mice. The induction of cytochrome P450 by βNF did not occur concurrently with proliferation of hepatic endoplasmic reticulum. It is interesting to note that some other categories of inducers such as phenobarbital, not only causes an increase in the level of CYP2B1 and CYP2B2, it also produces increased proliferation of sER and increased liver weight [15,16]. Within 16h of administration of phenobarbital to rats, there is 25-100-fold increase in the level of mRNA specific for phenobarbital- inducible forms compared with untreated rats [17].

The induction of 3-MC-inducible P450s is also mediated by increased transcription of the mRNA. 3-MC type inducers are lipid soluble and they enter the cell by diffusion across the plasma membrane. The inducer binds to the Ah receptor within the cytosol [18] and the complex formed is transferred into the nucleus where it binds with the specific gene region [19,20]. The net effect of this is to increase the rate of CYP1A1/CYP1A2 gene transcription and synthesis of specific mRNA[22,22]. The mRNA is transported from the nucleus back into the cytosol where translocation occurs for the selective synthesis of CYP1A1/CYP1A2. The prosthetic heme group derived from the mitochondria is inserted and the isozyme becomes incorporated into portions of the sER, where it is catalytically active.

Since the mechanism of induction of the 3-MC and phenobarbital-inducible categories of P450 is similar (i.e. by genetic transcription with *de novo* protein synthesis), why would there be sER proliferation in one group and none in the other? The reason for proliferation of sER following phenobarbital treatment is not exactly known. Since cytochrome P450s are intrinsic membrane proteins of the endoplasmic reticulum, it may be that the proliferation of the sER offers a greater membrane surface for accommodation of newly synthesized enzymes. Over the years however, the concept of P450 membrane topology has changed from one in which it is predicted to occupy large segments of the membrane. The recent hypothesis is that P450s are bound to the endoplasmic reticulum by one or two transmembrane peptides located at the amino terminus, and that the active sites are a portion of the cytoplasm [23,24]. This orientation is structurally suitable for the chemical interaction of cytochrome P450 enzyme with both hydrophobic and hydrophilic substrates, and for their association with other enzymes in the mono-oxygenase pathway. Bacterial

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models have been used in conjunction with X-ray crystallographic methods to elucidate the three-dimensional structure and function of cytochrome P450s [25]. Similar models have been predicted for other P450s, especially mammalian ones [26]. Establishment of the membrane topology of cytochrome P450s by X-ray crystallography, no doubt, is quite demanding. Nonetheless, it would help us understand the membrane insertion and conformation of cytochrome P450s better.

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