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Detection and localization of CYP1A1/CYP1A2 in murine liver: electrophoresis, immunoblotting and immunocytochemistry

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Abstract

Multiple forms of cytochrome P450 exist some of which are selectively inducible by exposure of the organism to a variety of foreign compounds. In this study, a monoclonal antibody specific for 3-methylcholanthrene-inducible cytochrome P450, Mab 1-7-1, was used to detect, localize and quantify CYP1A1/CYP1A2 in livers of C57BL/6 mice. Mab 1-7-1 recognized a faint band in the range between 45- 66 Kd in Western immunoblots of liver microsomes from control mice, and a strong band in the same range, in liver microsomes from β-naphthoflavone-treated mice. Microsome from control liver contained minimal levels of CYP1A1/CYP1A2; pretreatment with β-naphthoflavone caused an increase in their expression. Immunoelectron microscopy was used to demonstrate the cellular localization and quantification of these isozymes in the liver. The immunolabeling procedure confirmed the endoplasmic reticulum as the primary site of CYP1A1/CYP1A2 induction in hepatocytes. This organelle showed the highest labeling density after treatment with β-naphthoflavone. Increase in CYP1A1/CYP1A2 was 33.4-fold by morphometric analysis in induced hepatocytes in comparison to non-induced cells. In conclusion, CYP1A1/CYP1A2 is highly induced by β-naphthoflavone in C57BL/6 mouse liver, and the cellular site of expression is the endoplasmic reticulum.

Résumé

Des formes multiples de cytochrome P450 existent dont quelques unes peuvent être induites d'une fagon sélective en exposant l'organisme a une variété de composés étrangers. Dans cette étude, un anticorps monoclonal spécifique pour 3-methylchlolanthrène — cytochrome inductible P450, Mab 1-7-1, a été employé pour détecter, localiser et quantifier CYP-1A1/CYP1A2 dans des foies de C57BL/6 souris. Mab 1-7-1 a repéré une bande faible chez le groupe 45 — 66 Kd dans les immunoblots occidentaux des microsomes de foie pris des souris — témoin, et une bande forte dans le meme groupe, dans des microsomes de foie qui viement des souris traites avec β-naphthoflavone.

Les microsomes pris de foie du cas témoin contenait des niveaux minimaux de CYP1A1/CYP1A2; le traitement préalable avec β-naphthoflavone a causé une augmentation leur expression. La microscopie immunoélectron est employée pour démontrer la localisation céllulaire et la quantification de ces isozymes dans le foie. La procédure de l'immunoétiquetage a confirmé que le reticulum endoplasmique est le site primaire de l'induction de CYP1A1/CYP1A2 dans les hépatocytes. Cette organelle a montré la plus haute densité de l'étiquetage après le traitement avec β-naphthoflavone. Par le biais de l'analyse morphometrique de hepatocytes induitsi, on voit que l'augmentation de CYP1A1/CYP1A2 et de 33.4 fois en comparaison avec des cellules non-induitsi. Pour conclure, CYP1A1/CYP1A2 est tres induit par β-naphthoflavone dans le foie de souris C57BL/6, et le site cellulaire de son expression est le reticulum endoplasmique.

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Introduction

Cytochrome P450 is a group of hemoproteins which belong to the mixed function oxidase system. They act as terminal mono oxygenases in the biotransformation of a wide range of exogenous and endogenous substrates including drugs, carcinogens, steriods, fatty acids and cholesterol [1,2]. The net effect of their activity may result in chemical detoxification or activation and toxicity. The ability of cytochrome P450 to metabolize such a diverse range of substrates is achieved by multiple forms, each having either distinct or overlapping substrate specificities [3, 4]. Many cytochrome P450 isozymes have been isolated and purified in a number of tissues from different animal species. Monoclonal antibodies (Mabs) have been developed to several cytochrome P450s, and they serve as reliable probes for the detection, immunopurification and DNA analysis of immunochemically related cytochrome P450s [5-7].

Exogenous and endogenous stimuli can regulate and moderate the expression of many forms of cytochrome P450 [8]. The phenomenon of induction and the isozyme profiles expressed are unique to specific systems and vary from tissue to tissue. The 3-methycholanthrene-(3-MC-) inducible cytochrome P450s are CYP1A1 and CYP1A2, both having 75% homology in their amino acid sequence [9,10]. The two isozymes are also inducible by β-naphthoflavone (βNF) and various environmental pollutants [11]. They have been implicated in the activation of promutagens into carcinogens [12,17]. Mab 1- 7-1 is a reliable probe for CYP1A1/CYP1A2 in tissues [18].

The mechanism of 3-MC induction of cytochrome P450 has been studied extensively at biochemical and molecular levels, especially in subcellular fractions [4,19]. The distribution of the isozymes within the hepatocytes at the basal level and following induction with 3-MC-type agents has been demonstrated at the light microscopic level [20] and in ultrathin cryosections [21]. However, previous immunohistochemical studies [20] failed to provide quantitative data on the degree of induction. Furthermore, attempts at studying 3-MC induction of cytochrome P450 at the immunoelectron microscopy level have to date yielded poor results. Usually, the conditions of tissue fixation was less than optimal for good ultrastructural preservation of membranes, thus limiting precise immunolocalization.

In the present study, we have used immunoelectron microscopy in conjunction with a specific monoclonal antibody, Mab 1-7-1, to investigate the intracellular sites of expression of 3-MC-inducible cytochrome P450, CYP1A1 and CYP1A2, during the course of induction in murine liver. Localization and visualization of the antigenic sites by immunogold labeling techniques has offered a means of quantification of the isozymes on ultrathin sections by morphometry. Western blotting technique also enabled us to verify the molecular weights of the isozymes in subcellular fractions thereby confirming the specificities of the reactions.

Materials and methods

Chemicals and reagents

Monoclonal antibody (Mab 1-7-1) was a generous gift from Park and Gelboin (Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, Maryland). Goat anti-mouse IgG conjugated to colloidal gold (12nm) was purchased from Bio/Can Scientific (Mississauga, Ontario). Goat anti-mouse IgG (H + L) horseradish peroxidase conjugate, avidin-horseradish peroxidase conjugate, SDS-Page standards, biotinylated SDS-Page standards were from Bio-Rad Labs (Richmond, California). β-naphthoflavone was obtained from Sigma Chemical Co. (St. Louis, Missouri); Tris-HCl from ICN Biomedicals (Cleveland, Ohio); acrylamide and sodium deodecyl sulphate (SDS) from BDH Chemicals (Toronto, Ontario); Lowicryl K4M Kit from J.B. Em (Ponte-Clare, Dorval, Quebec); Enhanced Chemiluminesence (ECL) Western blotting detection system and Hyperfilm-ECL from Amersham Radio-chemicals (Arlington Heights, Illinois); Immobilon P-Transfer membrane was from Millipore Ltd. (Mississauga, Ontario). All other chemicals were of reagent grade and were obtained from standard commercial suppliers.

Treatment of animals

Young adult male C57BL/6 mice weighing 20-22g (Charles River Canada, St. Constant, Quebec) were housed in group plastic cages over hard wood bedding (Betachip). They were kept under a controlled environment at a temperature of 25 ± 1°C with 12h light/dark cycle. They were given food (Purina rodent Chow) and tap water *ad libitum*. The mice were allowed to acclimatize under laboratory conditions for at least 5 days before commencing the experiments.

For induction studies, mice were administered βNF (80mg/kg body weight intraperitoneally) at 72h and 48h before they were sacrificed. The vehicle was corn oil and mice received an injection volume of 0.08ml/10g body weight. Control mice received only the vehicle.

SDS-Page and Western Blotting

Mice were sacrificed by cervical dislocation and liver microsomal proteins were prepared by differential centrifugation [22, 23]. Excised livers were rinsed in ice-cold buffered 0.15M KCl, pH 7.4, and were homogenized in the same buffer in a Potter-Elvehjem homogenizer. The homogenates were centrifugated at 9000g at 4°C for 20min and the resulting supernatants were centrifugated at 105,000g at 4°C for 1h (L8-60m Beckman ultracentrifuge using a Ti-50 rotor). The microsomal preparations were resuspended in 0.01M potassium phosphate buffer, pH 7.4, and stored in aliquots at -70°C. The protein concentrations of microsomes were determined by Lowry's method [24]. Microsomal proteins and molecular weight standards were subjected to SDS-PAGE as discribed by Laemmli [25] using 8% separating and 4.5% stacking polyacrylamide gel. The electrophoresis was carried out (Mini-Protean II Kit, Bio-Rad) at 150 volts until the dye front had reached the bottom. The separated proteins were transferred to immobilon - P-transfer membrane using a Hoefer Transphor apparatus at a setting of 1.0amp for 1h as described by Towbin *et al* [26]. Non- specific protein binding sites were blocked by incubating the blots in 5% skimmed milk in 20mM Tris Buffered Saline (TBS) containing 0.1% Tween 20. Blots were incubated in the primary antibody Mab 1-7-1. Protein that bound the Mab was visualized by incubating the membrane with goat anti-mouse IgG horse radish peroxidase conjugate and then reacting with ECL western blotting detection system, according to the manufacturers instructions.

Tissue preparation

The control and βNF-treated mice were anaesthetized with sodium pentobarbital (0.12mg/g). The liver was perfused with 0.9% NaCl through aorta with a hydrostatic pressure of 15-20cm, and the perfusate was allowed to escape from an incision in the right atrium. To determine the best fixative for our study, the latter step was followed by perfusion for 10min with any one of the following fixatives: 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4; 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4; 0.2% glutaradehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer pH 7.4; 0.4% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4. The livers were then excised, cut into blocks, and immersed in the same perfusion fixative for 2h at 4°C. Each batch of tissue sections were kept overnight in 0.1M sodium cacodylate buffer at 4°C. The tissues were then rinsed three times in cacodylate buffer wash containing 10% sucrose and subsequently, reactive aldehyde groups were saturated with 0.15M glycine and buffered saline, pH 7.4. Some authors have shown that post-fixation of tissue in reduced osmium tetroxide results in better preservation of cytoplasmic details [27,28]. To obtain better resolution of cellular ultra-structure, half of the tissue from each mouse was post-fixed in 0.5% osmium tetroxide that had been reduced in 1.5% potassium ferrocyanide. Thereafter, tissues were embedded in lowicry k4M according to previously described methods [29].

Ultrathin (60nm) sections were obtained from the centrilobular region of the liver with a LKB 2188 Nova Ultramicrotome using freshly made glass knives. The sections were mounted on formvar-coated nickel grids.

Immunoelectron microscopy

Osmicated tissue sections were reacted for 2h with saturated sodium metaperiodate to expose the antigen binding sites. Both the osmicated and non-osmicated sections were incubated for 5min. with 1% ovalbumin in buffered saline to block nonspecific binding sites. The sections were then incubated for 2h with Mab 1-7-1 at dilutions ranging from 1:10 to 1:100 to determine the concentration for optimal labeling. Tissue sections from the same livers were used for both experimental and control incubations, which were conducted simultaneously so that conditions of labeling were optimally standardized. Negative control incubations were performed with omission of the primary antibody. Sections were then reacted with affinity-purified goat anti-mouse IgG conjugated to colloidal gold 12nm (1:40). Sections were stained in 3% aqueous uranyl acetate and were examined with a Hitachi H-7000 electron microscope. All electron photomicrographs were exposed at a magnification of 12,000x.

Quantitative evaluation of immunolabeling

In ultrathin sections, gold particles are restricted to sectioned surfaces only [29] and therefore, the labeling density is related to the surface area of the intracellular compartment. The number of gold particles within profile areas were counted and this was designated N (gp). A square test grid was randomly superimposed to count the number of test points that fell on organelles, in order to estimate their areas, designated P (org). The numerical labeling density of gold particles per profile area, NA (gp/org) of the different organelles was then calculated using the following formula:

$$NA\ (gp/org) = N\ (gp)/P\ (org) \times m^2/d^2,$$

where m is the final magnification (40,000x) and d is the distance between two test points (1cm). The nonspecific labeling over nuclei and mitochondria, NA(gp/nuc + mito) was subtracted to give actual number of specific gold particles [30, 31]. All data were calculated as means ± standard deviation (SD) and comparisons between groups were made with the student's t test. A p value < 0.05 was considered to be significant.

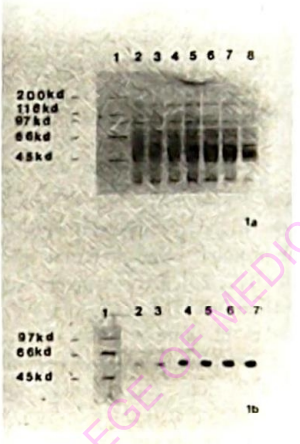
Results

Western immunoblotting

The SDS-PAGE analysis of liver microsomes from control and βNF-treated C57BL/6 mice is shown in Fig. 1a and the Western blots in Fig. 1b. Thus, probing with Mab 1-7-1 revealed single bands in both the corn oil and βNF-treated mice. The band in all corn oil-treated preparations was faint and was approximately in the range between 45-66Kd. The liver microsomes from βNF-treated mice showed a strong band with considerable increase in intensity of the bands. The bands were in the same position as found in the corn oil-treated group and were in the range that corresponded in electro-phoretic mobility of CYP1A1/CYP1A2 [32]. CYP1A1 and CYP1A2 were not resolved separately in this SDS-PAGE system. The results however, confirm the induction of CYP1A1/CYP1A2 by βNF due to the increase in the intensity of the bands.

Localization and quantification of CYP1A1/CYP1A2 by immunoelectron microscopy

Previous studies with light microscopy have shown that CYP1A1/CYP1A2 is highly localized in the centrilobular regions of C57BL/6 mouse liver [20].



Figs. 1a and 1b

(a) 8% SDS-PAGE analysis of liver microsomal proteins from control and BNF-treated C57BL/6 mice. Lane 1 contains molecular weight standard markers (200Kd, 116Kd, 97Kd, 66Kd, and 45Kd); approximately 5 µg protein was loaded into each well. Lanes 2-4 contain liver microsomal proteins from control mice treated with corn oil; lanes 5-8 contain liver microsomal proteins from BNF-treated mice. (Commasie blue stain).

(b) Western immunoblots of liver microsomal proteins from control and BNF-treated C57BL/6 mice. Lane 1 contains molecular weight standard markers (97Kd, 66Kd, and 45Kd); 1 µg protein was loaded into each well. Lanes 2 and 3 contain liver microsomal proteins from corn oil-treated mice; lanes 4-7 contain liver microsomal proteins from BNf-treated mice.

Therefore, sections from the centrilobular regions of the liver were selected for examination. There was good preservation of antigenic sites by the use of a mild fixative, and low temperature embedding in lowicry k4M. Optimization of labeling was obtained by serial dilutions of the primary and secondary antibodies. A dilution of 1:40 of both the primary and secondary antibodies was found to be optimal for this study. Higher concentrations resulted in excessive background immunolabeling.

Fixation of tissue in 4% paraformaldehyde resulted in the highest immunolabeling density but the hepatocyte ultrastructure was rather poor and organelles were not visible. Fixation in 0.1% glutaraldehyde and 4% paraformaldehyde also resulted in excessive vacuolization of the cytoplasm with diffuse background immunolabeling with all dilutions of antibodies. The best fixative for our

study was 0.2% glutaraldehyde with 4% paraformaldehyde and post-osmification of these tissues resulted in better resolution of ultrastructural features especially of the mitochondria and rough endoplasmic reticulum.

Immunolabeling was absent in all negative control incubations when Mab 1-7-1 was omitted from the incubation medium. Posivite immunolabeling was observed in liver sections from both corn oil and βNF-treated mice (figs. 2a and 2b). The immunolabeling in the two groups showed lesser density in corn oil-treated mice compared to βNF-treated mice (fig. 3). Immunolabeling was predominantly confined to the cytoplasm of the hepatocytes, especially in the regions of the smooth endoplasmic reticulum (sER) and rough endoplasmic reticulum (rER) while absent or minimal in endothelial lining of sinusoids, epithelial lining of biliary canaliculi and Kuppfer Cells. The outer nuclear membrane and mitochondria showed some immunolabeling although the significance of labeling of the latter structures is unclear. However, for immunoquantification purposes, they were considered to be non-specific labeling.

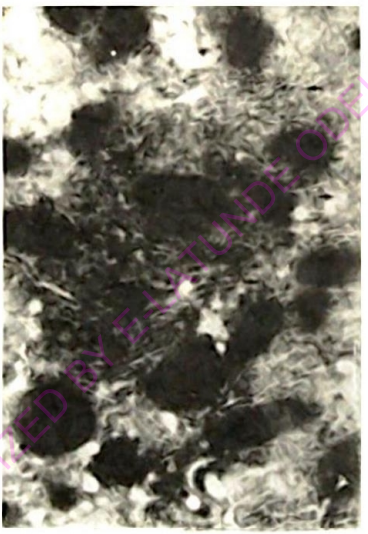


Fig. 2a Immunohistochemical localization of CYP1A1/CYP1A2 in Lowicryl-embedded C57BL/6 mice liver, as detected by Mab 1-7-1 and colloidal gold anti-mouse IgG; (a) tissue from corn oil-treated mouse. Immunogold particles (arrows), rough Endoplasmic reticulum (rER), smooth Endoplasmic Reticulum (sER). (Magnification 40,000X).

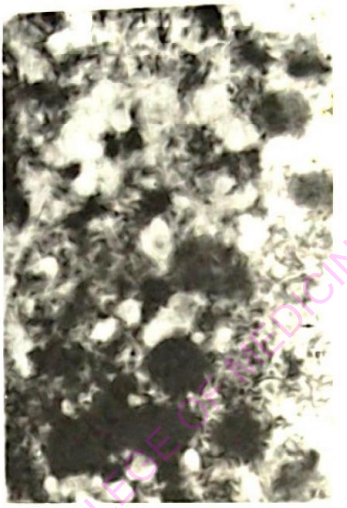


Fig. 2b: Immunohistochemical localization of CYP1A1/CYP1A2 in Lowicryl-embedded C57BL/6 mice liver, as detected by Mab 1-7-1 and colloidal gold anti-mouse IgG; (b) tissue from βNF-treated mouse. Immunogold particles (arrows), rough Endoplasmic Reticulum (rER), smooth Endoplasmic Reticulum (sER). (Magnification 40,000x).

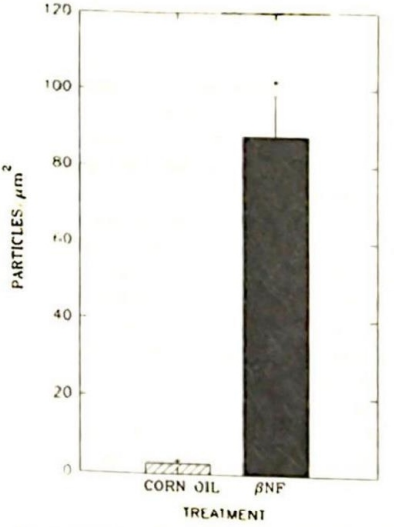


Fig. 3: Immunolabeling of CYP1A1/CYP1A2 in C57BL/6 mice liver by quantitative immunoelectron microscopy. Values denote mean labeling densities ± standard devision in 6 animals over at least 100 representative section. The asterisk denotes a value that is significantly different from control ($P < 0.005$).

Treatment of C57BL/6 mice with -NF resulted in a 33.4 fold increase in the number of gold particles with involvement of more hepatocytes (fig. 3). The increase was statistically significant when compared to non-induced levels ($P < 0.0005$). This finding is consistent with the induction of liver microsomal proteins demonstrated by Western blotting (Fig. 1b).

Discussion

In the present study, we have localized and estimated the amount of CYP1A1/CYP1A2 in the liver of responsive C57BL/6 mice, both at the basal level and following treatment with βNF. Mab 1-7-1 which specifically recognizes these isozyme [18,32] was used for labeling in immunoelectron microscopy and in Western blotting. This enabled us to correlate the morphometric data with immunochemical data. Previous immunological studies have shown that Mab 1-7-1 recognizes two antigenically related forms of cytochrome P450 in 3-MC-treated rats, C57BL/6 mice and DBA/2 mice, with MW 57 and 56k [19]. These forms corresponded to CYP1A1 and CYP1A2 respectively.

The use of immunoelectron microscopy technique has only recently been introduced for detection of cytochrome P450s. The objective of the technique is to accurately localize biological substances in relation to cell structure and function. In order to localize the antigen *in situ* at the ultrastructural level, tissue preparation must fix the antigen at its original sites without translocation or inactivation. The successful achievement of this is labour-intensive and time-consuming due to the technical difficulties of tissue preparation and immunolabeling. Nevertheless, an acceptable approach is to compromise and optimize conditions such that there is retention of antigenicity and adequate ultra-structural preservation [29, 33].

In this study, the use of low concentrations of fixative combined with post-fixation with osmium tetroxide and low temperature embedding in Lowicryl yielded optimal results. There was specific labeling over reasonably well preserved organelles with high resolution. Low temperature embedding in Lowicryl was particularly useful for maximal preservation of antigenicity, relatively good ultrastructural preservation and low background labeling. The hydrophobic nature of Lowicryl allowed easy penetration of antibodies. Lowicryl is also useful for evaluating density of immunolabeling because it only reveals antigenic sites on the surface of ultrathin sections [33,34]. Colloidal gold was chosen as a marker for immunolabeling because of its ease of application, its electron density and its high resolution. More importantly, it allows relatively simple quantification of the immunolabeling. Our method not only permitted us to precisely detect and localize CYP1A1/CYP1A2 within the hepatocytes, it also enabled us to quantify their amounts at the constitutive level and following induction with β-NF.

Centrilobular hepatocytes were predominantly examined in view of the findings of previous studies which have shown that constitutive levels of 3-MC-inducible cytochrome P450s are predominantly found in centrilobular hepatocytes and are not detectable in periportal hepatocytes of C57BL/6 mice [18,20]. Our findings show low immunolabeling in non-induced C57BL/6 mouse liver. Since the constitutively expressed isozyme is CYP1A2, it indicates that low levels of this isozyme are present and reside in the centrilobular hepatocytes of non-induced mice. This is further corroborated by our findings in Western blots, where liver microsomal proteins from non-induced mice showed up as weak bands.

Following treatment of C57BL/6 mice with β-NF, there was pronounced immunolabeling with a 33.4-fold increase in density. This increase is statistically significant ($P < 0.0005$) when compared with labeling in non-induced mice. CYP1A1 is the highly inducible isozyme following treatment with 3-MC-type inducer [11]. Other studies also showed that induction resulted in enhanced immunostaining within the hepatic lobule with uniform distribution of positively stained hepatocytes [20]. Therefore, the overall effect of induction with β-NF is increase in number of hepatocytes containing increased amounts of CYP1A1 relative to CYP1A2. The site of induction within the cytoplasm of hepatocytes was mainly in the endoplasmic reticulum.

In conclusion, this study has given a new insight into the *in situ* process of induction of cytochrome P450 by a 3-MC-type agent.

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