ABSTRACT:

In the study of tissues that contain several forms of one cytochrome P450 subfamily, it is useful to develop immunoblotting techniques so that the various individual members of the family can be distinguished. This paper describes improvements in the immunoblotting technique to distinguish members of the rat cytochrome P450 4A subfamily, 4A1, 4A2, and 4A3, as they are present in Sprague-Dawley rat liver microsomes. This procedure was used to investigate differences in the cytochrome P450 4A forms observed under various conditions such as: untreated versus peroxisome proliferator treated rats, Sprague-Dawley versus Fischer 344 male versus female rats, and liver versus kidney microsomes. In liver microsomes of male Sprague-Dawley rats, forms 4A1, 4A2, and 4A3 were induced by the peroxisome proliferators, clofibrate, di-(2-ethylhexyl) phthalate, dehydroepiandrosterone, aspirin, and ibuprofen. Expression of the 4A forms shows strain specificity. A comparison of the cytochrome P450 4A forms in male Sprague-Dawley and Fischer 344 rats treated with peroxisome proliferators demonstrated that three distinct protein bands are visible on immunoblots of liver microsomes of Sprague-Dawley rats, whereas only two distinct protein bands are detectable in liver microsomes of Fischer 344 rats. The two protein bands in liver microsomes of male Fischer 344 rats migrate in positions corresponding to the 4A2 and 4A3 bands in male Sprague-Dawley rats. There did not appear to be a protein band corresponding to the 4A1 band of Sprague-Dawley rats. Expression of the 4A forms also shows gender specificity. In liver microsomes of female Sprague-Dawley rats, expression of the P450 4A2 form was not observed after treatment with a peroxisome proliferator. Expression of the 4A forms also shows tissue specificity. In kidney, 4A2 is the major protein band in male Sprague-Dawley rats with minor amounts of the 4A3 protein, whereas two prominent protein bands (4A2 and 4A3) are seen in male Fischer 344 rats.

Hydroxylation of fatty acids, such as lauric and arachidonic acids, at the omega carbon atom is catalyzed by members of the cytochrome P450 (CYP) family. There are four CYP4A forms that have been identified, 4A1, 4A2, 4A3, and 4A8. The first three were identified in liver and kidney microsomes of male Sprague-Dawley and Fischer 344 rats treated with peroxisome proliferators. We have previously reported the separation and immunodetection of various isoforms in the CYP4A subfamily. There has been some difficulty in consistently obtaining clear separation of the three 4A forms in immunoblots, and recent studies have reported 1, 2, or 3 protein bands in rat liver microsomes. In this paper we report a SDS-PAGE and immunoblotting technique that is derived from modifications (21) of the discontinuous PAGE procedure of Laemmli (22) and that permits consistent resolution of the three CYP4A forms in liver microsomes of the male Sprague-Dawley (S/D) and Fischer 344 (F344) rats, differences were found in their separation patterns. These differences may help to explain the varying results that have appeared in the literature concerning the CYP4A subfamily.

Materials and Methods

Materials. Chemicals used in these studies were electrophoresis grade, when available, or reagent grade. Water for reagents was deionized and then passed through a ModuLab ModuPure water system (Continental Water Systems Corp., San Antonio, TX). A 30% acrylamide/Bis solution (30% T:1% Bis), TEMED, ammonium persulfate, BME, HMW prestained standards, and AP color development kit (AP Conjugate Substrate Kit) were purchased from Bio-Rad (Richmond, CA). Glycine, EDTA, SDS (sodium dodecyl sulfate), Tween 20, BSA (bovine serum albumin, fraction V), Trizma HCl and Trizma base, glyceral, clofibrate (2-(p-chlorophenoxy)-2-methyl-propanoic acid ethyl ester), aspirin (ASA), and ibuprofen (IBU) were purchased from Sigma Chemical Co. (St. Louis, MO). The steroid, DHEA, was purchased from Steraloids.
aspirator for 10 min while stirring. Polymerization was initiated by addition of EDTA, 0.1% SDS (w:v), pH 6.8). The gel solution was degassed with a water blower (BDH Ltd. (Poole, UK) and obtained from Hoefer Sci. Inst. (San Francisco, CA). Instant nonfat dry milk, applesauce, and Hain sawflower oil in a glass container were obtained from a local grocer. Pure nitrocellulose (NC) transfer membranes (BA85, pore size 0.45 μm) and gel blot paper (GB003, used to dry blots after developing) were obtained from Schleicher & Schuell (Keene, NH). Chromatography paper (1 Chr, used in the transfer “sandwich”) was obtained from Whatman (Clifton, NJ). The plasticizer DEHP was obtained from Aldrich Chemical Company (Milwaukee, WI).

The vertical slab gel electrophoresis chambers (SE 400 and SE 600), tank electro-transfer unit (TE 42), and power supply (PS 500XT) were purchased from Hoefer. A rocking platform (Nutator, Clay Adams, Becton Dickinson, Sparks, MD) and a variable speed rotator platform (R4140, American-Dade, Miami, FL) were also used.

Animals. Male and female S/D and F344 rats were purchased from Simonen Lab. (Giley, CA). Treatments were started when the animals were 6 weeks old. Rats were given (by oral intubation) peroxide-free (21) sawflower oil (1 ml/kg body weight per day) in control studies or sawflower oil combined with DEHP (1.2 g/kg per day) (24) or clofibrate (0.2 g/kg per day) (25). Food was withdrawn 15 hr prior to sacrificing the rats. Rats were sacrificed under pentobarbital anesthesia 24 hr after administration of the final dose of agent. Rats were treated with DHEA (100 mg/kg per day for 4 days, by oral intubation in peroxide-free sawflower oil) (19). Rats were treated with aspirin (500 mg/kg per day for 9 days) (8) or ibuprofen (250 mg/kg per day for 9 days) (9) mixed with powdered chow and applesauce.

Antibodies. Primary antibodies used were as follows: (A) We prepared a polyclonal antibody in rabbit against CYP4A1 which we purified from livers of male S/D rats treated with DEHP (1.2 g/kg per day for 3 days as described above) (26). (B) We purchased a polyclonal antibody from Gentest (Woburn, MA) prepared in goat against CYP4A1 purified from livers of S/D rats treated with clofibrate. AP (alkaline phosphatase) conjugated, affinity purified secondary antibodies were purchased as follows: (A) AP-goat anti-rabbit IgG (Zymed Lab., South San Francisco, CA) and (B) AP-rabbit anti-goat IgG (Sigma Chemical Company).

Methods. Washed microsomes were prepared from rat liver and kidney cortex as previously described (15). Protein concentrations were determined by the procedure of Lowry et al. (27) with microsomes diluted in 0.5 N NaOH to ensure complete disruption of membrane structures. Rat CYP4A1 was purified and antibody prepared as previously described (28).

SDS-PAGE Sample Preparation. Samples were diluted in appropriate buffer and 1 vol of sample mixed with 4 vol of Reducing Sample Buffer [60 mM Tris, pH 6.8, 2 mM EDTA, 2% SDS (w:v), 20% glycerol (w:v), 0.025% bromophenol blue (w:v), with 5% BME (v:v) freshly added]. These samples were boiled for 5 min, vortexed, cooled to room temperature, and spun (2,000 rpm for 2 min) before a 30 μl aliquot containing the desired amount of protein was loaded onto the SDS-PAGE gel. Prestained standards were handled as suggested by the manufacturer; 15 μl (1.5 times the suggested amount) was applied to allow easier viewing of the standards during electrophoresis.

SDS-PAGE. All electrophoresis buffers were prepared by combining the appropriate amounts of Tris base and Tris base to obtain the correct pH as measured at room temperature. A resolving gel solution of acrylamide (8% T) was prepared containing Resolving Gel Buffer [95 mM Tris HCl, 280 mM Trizma base, 2 mM EDTA, and 0.1% SDS (w:v), pH 8.8]. The gel solution was degassed with a water aspirator for 10 min while stirring. Polymerization was initiated by addition of a 20% (w:v) ammonium persulfate solution (0.0025 v:v of gel solution) and TEMED (0.001 v:v). The gel solution was gently poured into the gel frame with the comb inserted to form 0.8-cm wells. The stacking gel was allowed to polymerize at least 30 min. The comb was gently removed and the wells washed gently and filled with Stacking Gel Buffer. The gel running apparatus was assembled with Chamber Buffer (5.1 mM Trizma HCl, 44.9 mM Trizma base, 384 mM glycine, 2 mM EDTA, and 0.1% SDS (w:v), pH 8.3). The gel was prerun at 4°C, 25 mA/Agel for 6 hr. Samples were loaded in 50 μl volume per well and the gel was run at 4°C, 25 mA/Agel for 9−12 hr until the prestained standards were at the desired position in the gel. The electrophoresis was stopped when the 120 kDa protein band (β-galactosidase in the prestained standards) had migrated to a position 8 cm from the bottom of the gel (the resolving gel being a total of ~12 cm in length). We found that this indicated that the CYP4A proteins had run to the desired portion of the gel (within an area 0.5 cm wide that is 85−91% towards the bottom of the gel as measured on the NC membrane after immunostaining).

Transblotting. At the end of the electrophoresis run, the gel frame was opened, the stacking gel removed, and the resolving gel equilibrated for 20 min in Transfer Buffer [2.5 mM Trizma HCl, 22.5 mM Trizma base, 192 mM glycine, pH 8.3, 20% ethanol (v:v)]. The NC membrane was prerun in water; NC and blottter papers were presoaked in Transfer Buffer. The transfer “sandwich” was prepared in Transfer Buffer using one 1/8 inch foam sponge (purchased with the electro-transfer cassette) and one sheet of presoaked Whatman 1 Chr paper on each side of the “sandwich.” Quantitative transfer of proteins from the polyacrylamide gel to the NC membrane was accomplished in the tank electro-transfer unit at 370 mA at 4°C for 4 hr with stirring, using a maximum of two cassettes at a time in the center positions.

At the end of the transfer process, the NC membrane was removed and washed in 100 ml TBS (Tris Buffered Saline, 6 mM Trizma HCl, 4 mM Trizma base, 150 mM NaCl, pH 8.0) for 5 min with gentle agitation on the rotating platform (as for all subsequent steps until the membrane was dried after staining). The membrane was blocked for 2 hr in 100 ml of Blocking Solution (2.5% nonfat dry milk (w:v), 2.5% BSA (w:v) in TBS, prepared on the rocking platform). The membrane was washed three times in 100 ml TBST-20 for 5 min per wash. The membrane was incubated with the primary antibody diluted in TBST-20 with 1% BSA (w:v) for 8 hr or overnight. The membrane was washed three times in 100 ml TBST-20 for 5 min per wash and then incubated with AP-conjugated secondary antibody diluted in TBST-20 with 1% BSA (w:v) for 2 hr. The membrane was washed three times in TBST-20 followed by three washes in TBS. The immunoblot was stained with the AP color development kit as per the manufacturer’s instructions. The membrane was allowed to air dry and was photographed. The primary antibody solution was saved and was re-used 3−5 times. The secondary antibody solution was made fresh each time. Identification of the CYP4A1 protein band on immunoblots was previously determined using the purified protein isolated from liver microsomes of DEHP-treated rats (15). Identification of the putative CYP4A2 and CYP4A3 protein bands on the immunoblots was made based on studies of Kimura et al. (4) and Sundseth and Waxman (5) on the expression of these mRNA and proteins in kidney and liver of female and male rats. The immunoblot patterns shown in this study for the different microsomal samples are representative of a minimum of four different immunoblots performed with liver microsomes from four to six individual rats. Kidney cortex microsomes from three to six individual rats were pooled for use in this study.

The following factors appear to be important in obtaining separation of the CYP4A forms of male S/D rats by this procedure: 1) Standardization of the buffer components: We consistently use the same brand of chemicals and have worked out precise amounts of each chemical to weigh out, making sure to use consistent amounts of Trizma base and Trizma-HCl to achieve the desired pH. It is very important in working out the components of the electrophoresis buffers to check the pH of the Tris buffers used in this procedure at room temperature. 2) Standardization of the gel composition with respect to acrylamide: We use a premade acrylamide solution which has been stabilized to minimize precipitation or degradation of the acrylamide within the shelf life of the product. We have only used the Bio-Rad product and have not attempted to achieve separation with other brands of premade acrylamide solutions. 3).
In Panel A, the Gentest antibody was used to detect CYP4A bands in microsomes from male S/D rats as follows: Lane 1: kidney, control. Lane 2: liver, control. Lane 3: liver, 1 day treatment with clofibrate. Lane 4: liver, 3 days treatment with clofibrate. In Panel B, the antibody prepared in our laboratory was used to detect CYP4A bands in microsomes from male S/D rats as follows: Lane 1: kidney, control. Lane 2: liver, control. Lane 3: liver, 1 day treatment with DEHP. Lane 4: liver, 3 days treatment with DEHP. 2 μg of liver microsomal protein was loaded per lane; 5 μg of kidney microsomal protein was loaded.

were examined; however, neither 10% T nor 15% T acrylamide gels achieved separation of the 4A1 and 4A2 bands of male S/D rats. 4) Standardization of electrophoresis run conditions: We found the most important consideration is to run the electrophoresis until the CYP4A proteins have migrated to a standardized portion of the gel so that the CYP4A proteins are within an area 0.5 cm wide that is 85–91% towards the end of the gel (as measured on the NC membrane after immunostaining). Since the CYP4A protein bands cannot be visualized while the SDS-PAGE gel is running, we depend on visualizing the 120 kDa protein (β-galactosidase) in the prestained standards. When this relatively sharp band has migrated ~33% (8 cm from the bottom) of the length of the gel (a total of ~12 cm), the CYP4A proteins are in the desired portion of the gel. 5) Selection of the proper amount of protein: We found that it was very important to determine the proper amount of protein to be loaded for each type of sample. Using too much protein resulted in diffuse bands which could not be distinguished from each other. 6) Selection of the proper induction protocol: Additionally, it was important to choose the proper induction protocol; distinct bands can easily be distinguished in liver microsomes from male rats given one dose of DEHP, but it is very difficult to obtain clearly distinct bands following the 3-day or longer dosing regimens which are customarily used to obtain maximal induction with DEHP or other peroxisome proliferators without adjusting the amount of protein applied.

Results

By using the immunoblot procedure described above in Methods, we were able to consistently obtain separation of the protein bands for CYP4A1 (band 1), 4A2 (band 2), and 4A3 (band 3) in male S/D rats treated with various peroxisome proliferators, as shown in figs. 1 and 2. This separation is optimal in samples such as the one shown in lane 3, fig. 1, Panel A, containing 2 μg of liver microsomal protein from a male S/D rat given one treatment of clofibrate. As can be seen in lane 4, upon further treatment with clofibrate (3 days) and thus further induction of the enzymes, the protein bands may become too thick to allow optimal separation of the 4A1 and 4A2 bands (bands 1 and 2) at 2 μg of protein per lane. Similar results are obtained with DEHP-treated rats as shown in fig. 1, Panel B. The three protein bands are present in samples from untreated animals, but the amount of each band varies, particularly for 4A2, making visualization of the three distinct bands difficult (lanes marked 1, fig. 1, Panels A and B). In most instances, band 2 (fig. 1, lanes marked 2 and fig. 2, lane CON) is not detected when liver microsomes from control S/D rats are immunoblotted. The utility of the technique is clearly illustrated in fig. 1, as it can be seen that the samples in lanes marked 1 (male S/D kidney microsomes) contain predominantly 4A2. The immunoblot depicted in Panel A of fig. 1, showing induction of CYP4A forms by clofibrate treatment of male S/D rats, was developed using the polyclonal antibody available commercially from Gentest. The immunoblot depicted in Panel B, showing induction of CYP4A forms by DEHP treatment of male S/D rats, was developed using the polyclonal antibody made in our laboratory to CYP4A1 isolated from DEHP-treated rats.

Fig. 2 shows induction of CYP4A4 forms in liver microsomes of male S/D rats treated with various peroxisome proliferators. The length of treatment with each agent (other than DEHP) was chosen to maximize peroxisome proliferation and lauric acid hydroxylase activity. Induction of all three forms of CYP4A4 above the control level (CON) can be seen for DEHP, dehydroepiandrosterone (DHEA), aspirin (ASA), and ibuprofen (IBU). Samples of male S/D kidney microsomes (KID) are included to illustrate the position of CYP4A2 on the blot.

It was previously reported that liver microsomes from female rats do not have significant levels of CYP4A2 (5). In examining liver microsomes from female S/D rats (fig. 3), only two protein bands were observed in control rats (lane 3) or rats treated with DEHP for 1 (lane 4) or 3 (lane 5) days or clofibrate for 1 day (lane 6). The two protein bands correspond to bands 1 and 3 in liver microsomes of male S/D rats (lane 1) and are identified as forms 4A1 and 4A3. Lane 2 contains kidney microsomes from male S/D rats to indicate the position of the 4A2 band (band 2). Thus, in comparison to liver microsomes from male rats, the 4A2 protein band in liver microsomes of female rats is not induced by DEHP or clofibrate or is present at such low levels that it is undetected by the procedure described in this study.

In fig. 4, a further comparison is made between the Gentest antibody (Panel A) and the antibody which was made in our laboratory (Panel B) using microsomes obtained from S/D and F344 rats. With both antibodies, it can be observed that CYP4A1 (band 1), 4A2 (band 2), and 4A3 (band 3) are induced in liver microsomes of male S/D rats following one treatment with DEHP (lanes marked 2) vs control male rats (lanes marked 1). In both panels, CYP4A2 is identified as the predominant band in male S/D kidney microsomes (lanes marked 3). Although the major constitutive forms of CYP4A4 that are detected in control liver are identified as 4A1 and 4A3 in male S/D rats (lanes marked 1), both antibodies detect a different protein band pattern in liver microsomes of male F344 rats. The major constitutive forms in
Lane 1: male liver, control. Lane 2: male kidney, control. Lane 3, female liver, control. Lane 4, female liver, 1 day treatment with DEHP. Lane 5, female liver, 3 days treatment with DEHP. Lane 6: female liver, 1 day treatment with clofibrate. 2 μg of male S/D liver microsomal protein was loaded per lane; 4 μg of female S/D liver microsomal protein was loaded; 5 μg of male S/D kidney microsomal protein was loaded. The antibody prepared in our laboratory was used.

**Fig. 3.** CYP4A forms in liver microsomes of female Sprague-Dawley rats treated with DEHP or clofibrate.

**Fig. 4.** Comparison of CYP4A forms in male Sprague-Dawley and male Fischer 344 rats.

In Panel A, the Gentest antibody was used; in Panel B the antibody prepared in our laboratory was used. The samples loaded were identical in both panels except for lane 7. Lane 1: S/D liver, control. Lane 2: S/D liver, 1 day treatment with DEHP. Lane 3: S/D kidney, control. Lane 4: F344 liver, control. Lane 5: F344 liver, 1 day treatment with DEHP. Lane 6: F344 kidney, control. Lane 7: S/D liver, control (Panel A) or 1 day treatment with DEHP (Panel B). 2 μg of liver microsomal protein was loaded per lane; 5 μg of kidney microsomal protein was loaded.

Male F344 rat liver (lanes marked 4) correspond to the 4A2 and 4A3 protein bands of the male S/D rat livers (lanes marked 1 and 2). Treatment of F344 rats with DEHP increased the intensity of these two bands (lanes marked 5), but a separation of the protein band pattern into three distinct proteins was not obtained in liver microsomes of F344 rats. Protein bands corresponding to CYP4A2 and 4A3 of S/D rats are identified as the predominant bands in male F344 rat kidney microsomes (lanes marked 6). To further examine the CYP4A protein band pattern of F344 rats, liver microsomes from male versus female F344 rats were studied using the antibody developed in our laboratory (fig. 5). For reference, lanes 1 and 6 illustrate the positions of 4A1, 4A2, and 4A3 in male S/D rat liver microsomes after one treatment with DEHP. Liver microsomes from control male (lane 2) and female (lane 3) F344 rats contain only two observable protein bands. The top band in both male and female F344 rat liver microsomes corresponds to CYP4A3 in male S/D rat liver microsomes; however, the lower bands in the male and female F344 rats do not correspond to each other and do not correspond to the CYP4A1 protein band observed in liver microsomes of male S/D rats (lanes 1 and 6). Notice the positions of the lower bands in fig. 5. The lower band (putatively the 4A1 band) in female F344 rats (lanes 3 and 5) appears to run between the 4A1 and 4A2 bands in male S/D rats (lanes 1 and 6). However, the lower band of the male F344 rat (lanes 2 and 4) corresponds to the 4A2 band in male S/D rats (lanes 1 and 6). Induction of these proteins can be seen after one treatment with DEHP in both males (lane 4 versus lane 2) and females (lane 5 versus lane 3) and the difference in locations of the lower band can still be distinguished, although the lower band in male F344 liver appears to be a doublet in some samples (data not shown).

**Discussion**

An improved method for performing immunoblotting of CYP4A forms in liver microsomes of S/D rats is reported. This procedure provides a reproducible method to distinctly separate forms 4A1, 4A2, and 4A3 in liver microsomes of male S/D rats treated with a number of peroxisome proliferators, including clofibrate, DEHP, DHEA, aspirin, and ibuprofen. In the studies described in this paper, similar results were obtained using either an antibody preparation purchased from Gentest or a polyclonal antibody which was developed in our laboratory for CYP4A proteins.

CYP4A forms have been characterized in liver and kidney of male and female rats, and it has been demonstrated that there is both gender- and tissue-specific expression of the 4A forms (5,15). The CYP4A2 protein was never observed in immunoblots of liver microsomes of untreated or DEHP- or clofibrate-treated female S/D rats. Female F344 rats also did not appear to have a protein band which corresponded to CYP4A2, but further studies are needed to verify the CYP4A4 forms which are present in liver and kidney microsomes of F344 rats. Our immunoblot studies of kidney microsomes of male S/D rats indicate that 4A2 is expressed constitutively and at much higher amounts than the 4A3 protein which supports the earlier findings of Kimura et al. (4). Similarly, CYP4A2 is expressed constitutively in kidney microsomes of male F344 rats; however, the 4A3 protein was also present at significant levels. A protein band corresponding to CYP4A1 was not observed in kidney microsomes of male S/D and F344 rats.

An unexpected finding of this study was that only two protein bands could be clearly detected in liver microsomes from male F344 rats which were treated with a peroxisome proliferator in comparison with the three protein bands in male S/D rats. Both S/D and F344 male rats had protein bands which correspond to the 4A3 form. Two additional bands are clearly present in microsomes from male S/D rats treated with DEHP whereas only 1 additional band is present in male F344 rats so treated. Using isomeric specific probes which identified untranslated regions at the 3’ end of 4A1, 4A2, and 4A3 mRNA, Sundseth and Waxman (5) concluded that liver of male F344 rats synthesized three 4A proteins which corresponded to forms 4A1, 4A2, and 4A3 identified in S/D rat liver microsomes. It had been suggested that the lower protein band of liver microsomes from male F344 rats is a doublet which contains forms 4A1 and 4A2 (5). Our results
support the findings of Sundseth and Waxman (5) that separation of the lower protein band of F344 rats into distinct 4A1 and 4A2 bands could not be achieved.

Northern blot studies by Sundseth and Waxman (5) demonstrated that high levels of 4A2 mRNA were present in liver of untreated male F344 rats (5), and our immunoblots demonstrate that a band corresponding to the S/D rat 4A2 protein is present in significant amounts in liver microsomes of control male F344 rats in contrast to control S/D rats which had no distinct 4A2 protein band. Significantly less 4A1 mRNA was observed in livers of untreated male F344 rats, (5) and no distinct protein band corresponding to the S/D rat 4A1 protein was observed in our immunoblots. CYP4A2 was reported not to be expressed in livers of female F344 rats (5), and our studies suggest the lower protein band in the female samples does not correspond to the lower band in male F344 liver microsomes. Thus, an explanation for the inability to separate forms 4A1 and 4A2 in liver microsomes of F344 rats is that the 4A1 forms differ between S/D and F344 rats with the band corresponding to the 4A1 band in F344 rats migrating much closer to the 4A2 band.

In summary, this study reports an immunoblot procedure which separates the three CYP4A proteins of S/D rats and should help investigators examine this P450 subfamily. This study also indicates that the 4A proteins are expressed at different levels in S/D, and F344 investigators examine this P450 subfamily. This study also indicates separates the three CYP4A proteins of S/D rats and should help close to the 4A2 band.

References