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Cytochrome P-450 Monooxygenase System

Localization in Smooth Muscle of Rabbit Aorta

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SUMMARY

Cytochrome P-450 monooxygenase isozymes and NADPH-cytochrome P-450 reductase were detected in the microsomal fraction of rabbit aorta by immunoblotting and by enzymatic activity. The monomeric molecular weights of aortal proteins that cross-reacted with antibodies to cytochrome P-450 forms 2 or 6 and reductase were identical to those of the proteins purified from the liver. The induction of form 6 immunoreactive protein and *O*-deethylation of 7-ethoxyresorufin (a reaction catalyzed by form 6) was observed in aorta following treatment of rabbits with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or β-naphthoflavone. The amount of reductase protein (equivalent to 22.4 ± 3.2 activity units/mg of protein) correlated with the cytochrome *c* reductase activity (18.3 ± 1.8 units/mg) and was the same for both treated and untreated rabbits. Consistent with immunoblot data, the amount of form 2 was insufficient for detection of activity (*N*-demethylation of benzphetamine). Significantly, removal of the endothelium, which was confirmed by light microscopy and by scanning electron microscopy, reduced by only 8 to 32% the specific enzymatic activity or content of immunoreactive proteins; only traces of protein or activity were recovered in the endothelial fraction. In studies of the vasculature, the potential of this metabolic pathway for the activation or detoxication of mutagens, carcinogens, toxins, or drugs and metabolism of endogenous substrates warrants consideration, especially in regard to the mutational events reported to be involved in the formation of atherosclerotic plaques.

INTRODUCTION

Steroids, fatty acids, and drugs as well as numerous carcinogens, mutagens, and other toxins are substrates for the cytochrome P-450 monooxygenase system. The wide range of activity is due in great part to the multiplicity of cytochrome P-450 isozymes that is associated with this membrane-bound system (1). Generally, the isozymes can be distinguished by monomeric molecular weight, epitopes, substrate selectivity, and inducibility by exogenous compounds. These parameters must be used in combination rather than individually to differentiate among isozymes because of some similarities in monomeric molecular weights, immunoreactivities, or enzymatic activities (1–5). The high concentration of cytochrome P-450 in the liver has facilitated the extensive study of the hepatic microsomal isozymes. However, the elucidation of extrahepatic distribution is essential for delineating the association between localized metabolic activation and tissue-selective toxicity, as well as for discovering the physiological roles of this apparently ubiquitous enzyme system. In the case of vasculature, where atherosclerotic plaques develop monoclonally (6), presumably subsequent to a mutational event in smooth muscle, the role of cytochrome P-450-dependent activation of circulating promutagens or procarcinogens may be of toxicological relevance.

The major differences among the abilities of various tissues to metabolize xenobiotics appear not to be due to unique cytochrome P-450 isozymes but rather to different proportions and concentrations of isozymes and reductase, and different responses to exogenous inducers. Therefore, antibodies elicited against isozymes purified from one tissue have proven to be important for examining other organs, particularly those in which the enzyme concentrations are low. Immunohistochemical staining of the vasculature, reportedly the endothelium (7), metabolism of BP[1] by aortic homogenates (8) or cultured smooth muscle cells from human fetal aorta (9), and the isolation of a microsomal aortic hemoprotein exhibiting the spectral characteristics of cytochrome P-

[1] The abbreviations used are: BP, benzo(*a*)pyrene; βNF, β-naphthoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; SEM, scanning electron microscopy; 7-ERF, 7-ethoxyresorufin; reductase, NADPH-cytochrome P-450 reductase.

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450 (10) suggest the existence of a vascular cytochrome P-450 monooxygenase system. It should, however, be noted that even monoclonal antibodies may cross-react with functionally unrelated proteins (11), that the metabolism of BP or 7,12-dimethylbenzanthracene is not exclusively via monooxygenation (e.g., co-oxidation by prostaglandin H synthase) (12), and that the isolated aortic hemoprotein, reported to be a prostacyclin and thromboxane A_2 synthase, exhibits no monooxygenase activity and is not reduced by NADPH-cytochrome P-450 reductase in spite of its apparent P-450-type spectrum (10).

In this investigation, we have determined the immunoreactivity and monomeric molecular weights (immunoblotting) and catalytic activity of individual cytochrome P-450 monooxygenase components, cytochrome P-450 forms 2 and 6 and NADPH-cytochrome P-450 reductase (EC 1.6.2.4) in microsomes from rabbit whole aorta or aorta stripped of endothelium. Removal of the endothelium to yield smooth muscle was confirmed by microscopic analysis. The effect of treatment with the inducers βNF or TCDD was examined. Further, for comparison, similar data obtained from striated muscle of the leg are presented.

MATERIALS AND METHODS

Preparation of aortal microsomes. The aortal segment between the heart and kidneys was removed from adult (15- to 25-week-old) male New Zealand White rabbits. For rabbits treated with βNF or TCDD, the intraperitoneal dosage was 80 mg or 10 μg/kg of body weight, respectively, in corn oil 72 to 96 hr prior to death. Twelve or more aortas were cleaned and pooled, frozen in liquid nitrogen, and ground in a mortar to permit adequate homogenization in 5 parts of 1.15% potassium chloride/phosphate, pH 7.4, at 4°. The washed microsomal fraction (>100,000*g* pellet) was obtained by standard differential centrifugation (13) and the protein content was estimated by the method of Lowry *et al.* (14). In order to prepare aortal smooth muscle, aortas were filled with 0.2% collagenase (EC 3.4.24.3) in Dulbecco's balanced salt solution and incubated for 45 min at 37°. The aortas were then rinsed and cut open, and the lumen was scraped firmly with the edge of a glass slide and again rinsed. The microsomal fraction was obtained from the scraped aortas as described above. Samples of the tissue were fixed in formalin and processed to make paraffin-embedded sections for light microscopy or were critical point dried and gold coated for SEM.

Immunoblotting. The microsomal fractions were electrophoresed on 7.5% acrylamide gels in the presence of sodium dodecyl sulfate (15), electrophoretically blotted onto nitrocellulose sheets that were then exposed to specific antibodies (IgG fraction), and stained by the bridged immunoperoxidase (peroxidase-antiperoxidase/diaminobenzidine) method (16) that was modified as described (17). The stain was quantitated by densitometry with a soft laser. Similarly, a pulmonary microsomal sample was analyzed using pure P-450 forms 2 and 6 and the reductase as standards (17), and an aliquot was used in each experiment for comparison.

Enzymatic assays. Freshly isolated microsomes were used for the determination of NADPH-cytochrome *c* reductase activity (18) and the direct fluorometric determination of *O*-deethylation of 7-ethoxyresorufin (19) at 30°. Benzphetamine *N*-demethylation at 37° was determined by formaldehyde production (20). The conditions used were optimal for both hepatic and pulmonary enzymes.

Microscopy. Formalin-fixed aorta embedded in paraffin was sectioned (6 μm thick) and stained with hematoxylin and eosin or with Verhaeff's elastin stain. For SEM, tissue was dehydrated in ethanol, critical point dried, and gold coated by standard procedures.

Equipment and supplies. Antibodies (IgG fractions) to pulmonary form 2, hepatic reductase, and form 6 were raised in goats and characterized as previously described (21, 22). Pure form 6 and anti-form 6 were gifts from Dr. E. Johnson (Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, CA).

RESULTS

Microscopy. Scanning electron microscopy revealed the surface of the lumen of the aorta with the endothelium intact (Fig. 1A) and after treatment with collagenase and scraping to remove the endothelium (Fig. 1B). Incubation of the aorta in buffer without collagenase for 45 min did not appear to damage the endothelium when compared to tissue fixed immediately upon removal from the rabbit (not shown). In cross section, the luminal surface of the digested and scraped aorta (Fig. 2B) exhibited loss of smooth muscle and layers of connective tissue in addition to the endothelium as compared to whole aorta (Fig. 2A). The treatment with collagenase appeared to cause separation of the connective tissue layers, and some smooth muscle cells exhibited a contracted appearance.

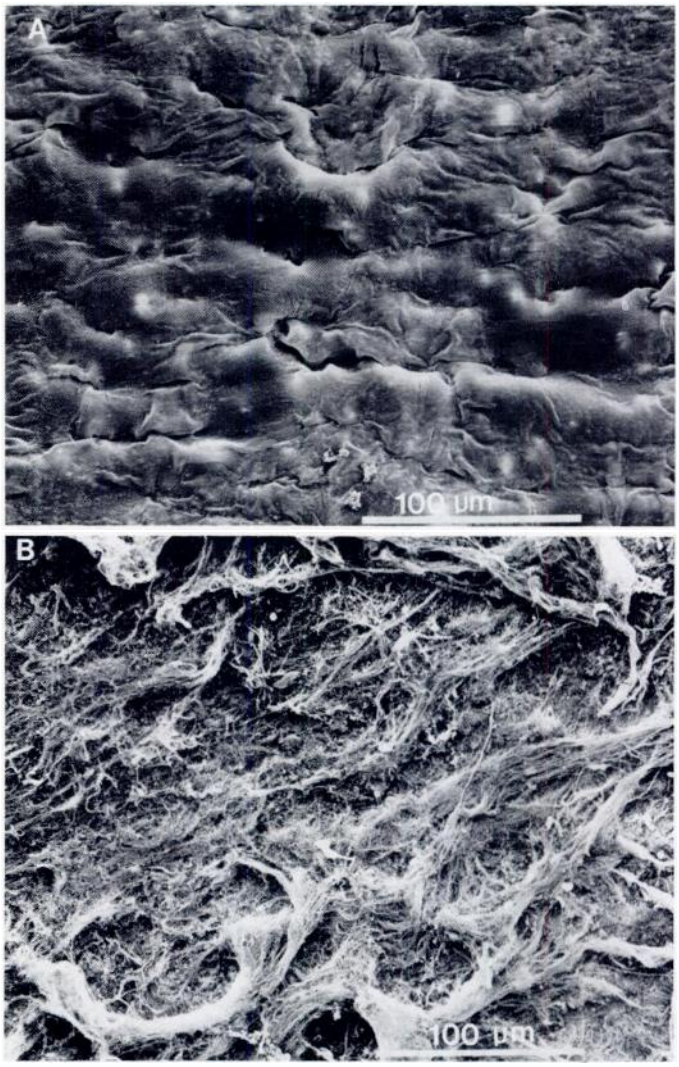

FIG. 1. *Scanning electron microscopy of the lumen of rabbit aorta*
A, endothelium intact; incubation for 45 min at 37° with Dulbecco's balanced salt solution. B, endothelium removed; incubation for 45 min at 37° with 0.2% collagenase followed by scraping with a glass slide.

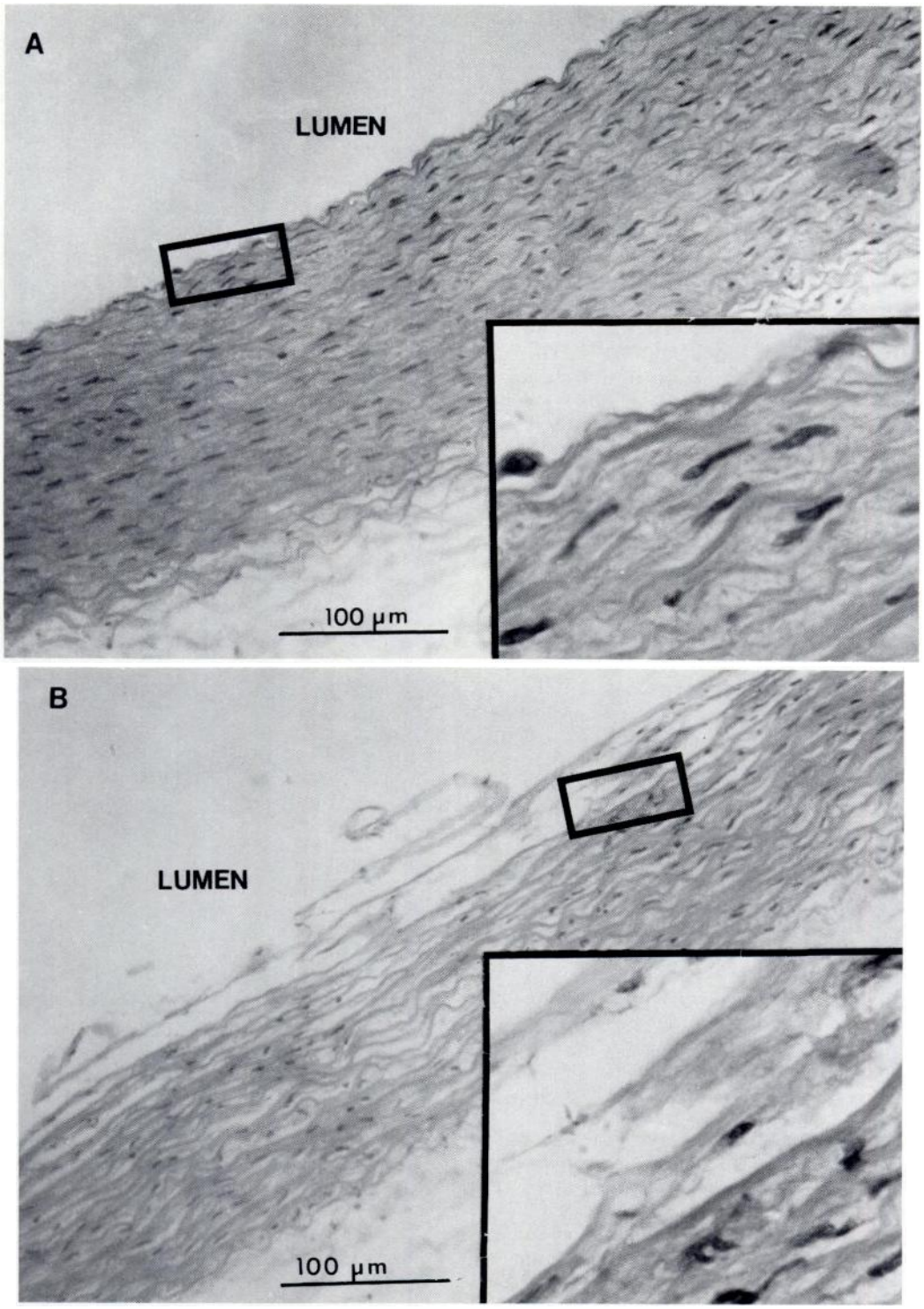

FIG. 2. *Light micrographs of cross section of rabbit aorta*
Paraffin-embedded tissue was sectioned and stained with hematoxylin and eosin. A, endothelium intact. B, endothelium removed as described in the legend to Fig. 1.

Microsomal yield. Grinding the frozen aortas facilitated subsequent homogenization and contributed to consistency in microsomal yield. This modification was shown to have no effect on pulmonary or hepatic microsomal yields or enzymatic activities. Table 1 shows that, on the average, the yield from aortal smooth muscle was essentially the same as that from whole aorta, and that a similar yield was obtained from striated muscle of the leg.

Immunoblots. Immunoblots of microsomes from whole aorta or aortal muscle revealed a 72-kd protein that cross-reacted with the antibody to the reductase; the density of the band from aortal muscle (Fig. 3, *m*) was similar to that of whole aorta (*a*); the pulmonary micro-

TABLE 1

Microsomal yield from aorta, aortal muscle, and striated muscle

Mean ± SD. Microsomes from whole aorta or aortal muscle were prepared from pools of 12 or more aortas; microsomes from striated muscle were from individual rabbits. Numbers of preparations are in parentheses.

Tissue	Microsomal yield
	mg protein/g tissue
Whole aorta	1.5 ± 0.1 (4)
Aortal muscle	1.3 ± 0.4 (3)
Ratio of aorta/muscle	87%
Striated muscle	1.6 ± 0.8 (4)

somes (*l*) were immunoblotted for comparison and about 10-fold less protein was required to obtain band densities similar to those of the aortal samples (e.g., 5 versus 50 μg, respectively). For pure reductase or pulmonary microsomes, as little as 25 fmol (0.1 unit of activity) yielded a distinct band (data not shown). Treatment with βNF or TCDD did not appear to alter the aortal content of immunoreactive reductase protein. Similarly, cytochrome P-450 form 2 (52 kd) was detected in 50 μg of microsomal protein from whole aorta (Fig. 3B) or in aortal muscle (data not shown), and no alteration due to treatment of rabbits with βNF or TCDD was observed. In contrast, these compounds appeared to significantly induce form 6 (60 kd); 20 μg of microsomal protein from either whole aorta or aortal muscle from βNF-treated rabbits yielded a band of density comparable to that from 2 μg of pulmonary microsomes. The form 6 content was determined to be 3.2, 2.9, and 28 pmol/mg of protein for whole aorta, aortal muscle, and lung, respectively (Fig. 4A). There was difficulty in immunoquantitation of form 6 in microsomes from untreated rabbits because of the amount of protein per track necessary for adequate band density; the resolution and uniformity of the bands were compromised when more than 50 μg of protein was

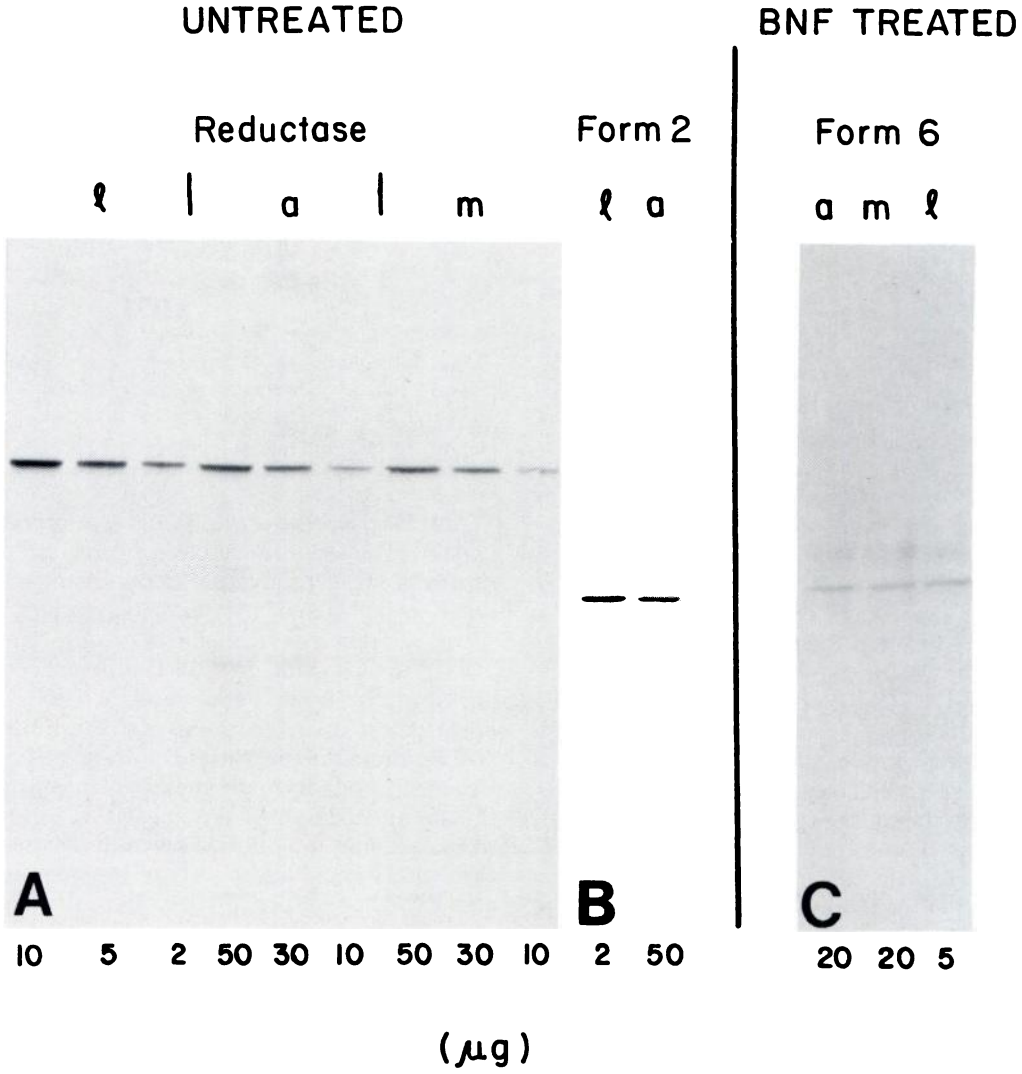

FIG. 3. *Immunoblots of microsomes from aorta (a) or aortic muscle (m) or lung (l) stained to show NADPH-cytochrome P-450 reductase or cytochrome P-450 forms 2 or 6 from untreated or β-naphthoflavone-treated rabbits*

The amount (μg) of microsomal protein per track is as indicated. The techniques for blotting and immunoperoxidase staining with diaminobenzidine are as described previously (10).

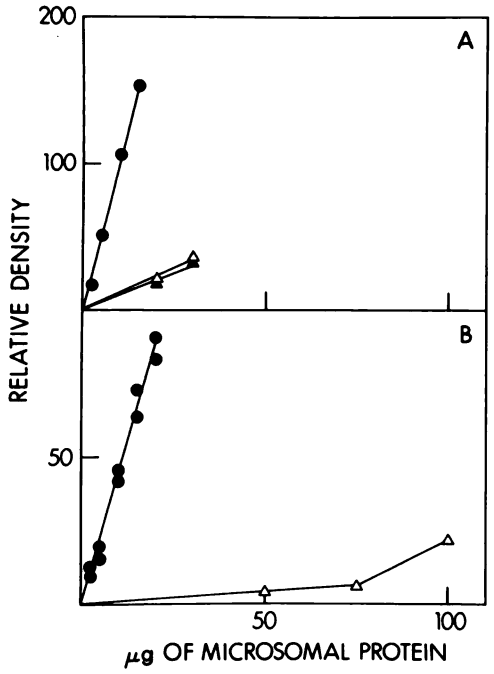

FIG. 4. *Densitometric quantitation of immunoblots of microsomal proteins from whole aorta, aortal muscle, or lung*

Immunoblots were stained for form 6, and the density as determined by laser scan is plotted versus the amount of microsomal protein applied. ●, values obtained for pulmonary microsomes; △, for whole aorta microsomes; ▲, for aortal muscle. Panel A shows the values for whole aorta or aortal muscle from rabbits treated with βNF, and Panel B shows values from untreated rabbit.

applied. As shown in Fig. 4B, the density increased nonlinearly between 50 and 100 μg of applied aortal muscle microsomes, and below 50 μg the relative density was near the detection limit of the densitometer; from the apparently linear segment, the form 6 content was calculated to be about 0.5 pmol/mg. The postmitochondrial supernatant (S-9) fractions were also examined by immunoblotting and the relationship between the immunoreactivity of whole aorta and aortal muscle was similar to that of comparable microsomal fractions. Fig. 5 shows a blot of 75 and 40 μg of whole aorta and aortal muscle (S-9) fraction stained for reductase. The density per microgram of protein of the band for aortal muscle was 83 and 75% of that of whole aorta in the S-9 fractions and the microsomal samples, respectively. Blots of microsomes from striated muscle indicated the presence of form 2 and reductase in untreated rabbits and form 6 when the rabbits had been treated with βNF or TCDD (data not shown).

Comparison of enzymatic activity with immunoreactivity. The microsomal cytochrome *c* reductase activity of aortal muscle was 13.7 ± 2.7 ($n = 3$) versus 18.3 ± 1.8 ($n = 3$) units/mg for whole aorta (Table 2). The enzymatic activity predicted from the immunoblots, assuming that the specific activity and immunoreactivity is the same as the pulmonary or hepatic reductase, was 15.3 versus 22.4 units/mg (68%), respectively (Table 2). The activities were determined from freshly prepared microsomes, were stable upon storage at −70°, and were totally inhibited by the antibody to the reductase.

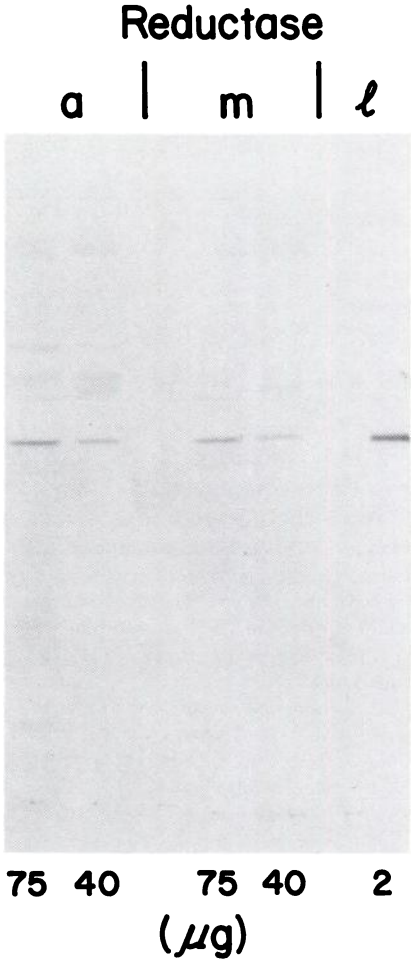

FIG. 5. *Immunoblots of postmitochondrial supernatant (S-9) fractions of whole aorta and aortal muscle from βNF-treated rabbits*

The amount of S-9 protein applied is an indicated for whole aorta (*a*) and aortal muscle (*m*); pulmonary microsomes (*l*) are shown for comparison. The blot was stained for reductase.

Aortal muscle and whole aortal microsomes from βNF-treated rabbits *O*-deethylated 7-ERF at 2.2 and 2.4 pmol/min/mg, respectively, and the corresponding form 6 contents were 4.0 and 4.7 pmol/mg, respectively (Table 2). Thus, microsomes from aortal muscle contain 92% of the 7-ERF activity and 85% of the form 6 content of whole aortal microsomes. Trace rates of 7-ERF metabolism by aortal microsomes from untreated rabbits were observed (<0.6 pmol/min/mg) (data not shown). Relatively high concentrations of microsomal aortal protein (400 μg/ml) in the incubations were necessary. However, when a small amount of pulmonary microsomes was added to the aortal incubations, there was an increase in rate equal to that observed with the pulmonary microsomes alone, indicating the absence of enzymatic inhibition or quenching of the fluorescent product. The activity was completely inhibited by the addition of antireductase. When aortal S-9 fractions were used, no activity was observed. The addition of the product, resorufin, to the S-9 fraction resulted in a rapid decrease in fluorescence, indicative of the presence of quinone reductase (DT-diaphorase, EC 1.6.99.2) (23).

Microsomes from striated muscle of rabbits treated

TABLE 2

The relative content of P-450 form 6 and NADPH-cytochrome P-450 reductase in microsomes from aorta or aortal muscle from rabbits treated with β-naphthoflavone

Mean ± SD. Each *n* in parentheses represents a pool of 12 or more aortas.

Microsomes[a]	Reductase		Form 6	
	Cytochrome *c*[b]	Blot[c]	7-ERF[d]	Blot[c]
	units/mg		*pmol/min/mg*	*pmol/mg*
Aortal muscle	13.7 ± 2.7 (3)	15.3 (2)	2.2 (2)	4.0 ± 1.2 (3)
Whole aorta	18.3 ± 1.8 (3)	22.4 ± 3.2 (3)	2.4 (2)	4.7 ± 0.8 (3)
Ratio muscle/aorta	75%	68%	92%	85%

[a] Treatment was with β-naphthoflavone. Microsomal fractions were prepared from aortas or aortic muscle.

[b] Reduction of cytochrome *c* was measured as previously described (8); 1 unit = 1 nmol of cytochrome *c* reduced/min.

[c] The amount of immunoreactive proteins was determined from the density of the immunoblots: for reductase, the activity was calculated from the amount of immunoreactive protein assuming the specific activity to be the same as that of hepatic or pulmonary reductase.

[d] The rate of *O*-deethylation of 7-ethoxyresorufin was determined fluorometrically as previously described (9).

TABLE 3

The content and enzymatic activity of NADPH-cytochrome P-450 reductase and P-450 form 6 in microsomes from striated muscle

See legend of Table 2 for experimental procedures.

Microsomes	Reductase		Form 6	
	Cytochrome *c*	Blot	7-ERF	Blot
	units/mg		*pmol/min/mg*	*pmol/mg*
Untreated	1.8	3.8	<0.2	<0.3
Treated[a]	2.2	3.8	1.0	1.8

[a] Rabbits were treated with 10 μg of TCDD/kg of body weight 96 hr prior to death.

with βNF or TCDD *O*-deethylated 7-ERF at the rate of 1 pmol/min/mg and had a form 6 content estimated to be 1.8 pmol/mg (Table 3). The reductase content and activity were not altered by treatment; the measured activity was about 2 units/mg, and the activity predicted from the immunoblots was 3.8 units/mg (Table 3).

In 1-hr incubations, no *N*-demethylation of benzphetamine was detected in incubations of 1 mg of aortal microsomal proteins, which was consistent with the form 2 content (<3 pmol/mg). The proportion of the total cytochrome P-450 content that forms 2 and 6 comprise is unknown as the sensitivity of the spectral assay for cytochrome P-450 is inadequate for aortal microsomes.

DISCUSSION

We have identified three components of the vascular microsomal cytochrome P-450 monooxygenase system by immunoblotting: cytochrome P-450 forms 2 and 6 and NADPH-cytochrome P-450 reductase. Enzymatic activities consistent with the estimated concentrations of form 6 and reductase and the induction of form 6 and 7-ERF *O*-deethylation activity by βNF (or TCDD) were also observed. Significantly, the majority, if not all, of this vascular activity and corresponding enzyme protein, is associated with the smooth muscle layers of the aorta.

It would appear that removal of the endothelium results in a greater loss of reductase protein and activity (32 and 25%) than of form 6 protein and activity (15 and 18%). This might be attributed to differential distributions of these enzymes between the endothelium and the smooth muscle. However, the disparity may be due to a difference between the susceptibility of the proteins to proteolysis that may occur during the incubation with collagenase. Histological sections indicate that the smooth muscle cells are disrupted by this treatment, and intracellular release of lysosomal protease may occur. Also, upon homogenization of the tissue, the microsomes may encounter residual collagenase or any protease impurities present in the collagenase preparation that may partially digest the reductase, but not the cytochromes which may be more protected by the microsomal membrane. Thus, it is more likely that we are underestimating rather than overestimating the fraction of monooxygenase components present in the aortal muscle.

We attempted to determine the content of cytochrome P-450 components in the tissue removed by collagenase digestion and scraping. Immunoblotting of the small amount of tissue obtained indicated a trace of cross-reactivity that may have been due to contamination by smooth muscle (data not shown). As the endothelial layer is only 1 cell thick, in contrast to 20–25 layers of smooth muscle cells, its contribution to the whole aortal monooxygenase content may be quite small even if the cellular concentration is the same in both cell types. Thus, sufficient numbers of pure, freshly isolated endothelial cells are requisite for determining their monooxygenase capacity. In a preliminary examination of bovine endothelial cells that were purified by successive passages in culture, no cross-reactivity with the antibody to the reductase was detected. This is not surprising, however, as monooxygenase enzyme content is frequently diminished during culture (24), making this readily available source of endothelial cells potentially unsuitable.

Therefore, our results, which are unambiguous with respect to the smooth muscle fraction, are not clear in the case of the endothelium. In contrast, Dees *et al.* (7), who used immunohistochemistry and fluorescence microscopy, have concluded that cytochrome P-450 form 6 is present in the endothelium, but not the smooth muscle layer, of the pulmonary vasculature of rabbits treated with TCDD. There are several possible explanations for this apparent difference. First, localization of form 6 in aorta and pulmonary vasculature may differ. Second, immunohistochemical techniques may not detect form 6 in smooth muscle due to low sensitivity or antibody inaccessibility. Third, form 6 in endothelium, if present, may be sufficiently concentrated to allow for immunohistochemical detection and still account for only a small fraction of the vascular total. Fourth, localization of antigens in specific cells cannot be firmly established by immunofluorescence because of the lack of positive iden-

tification of the *in situ* epitopes and the poor resolution of cell types in the dark field. The difficulty of discerning the endothelium of the pulmonary vasculature, except by electron microscopy, has been noted by Ryan (25).

In comparing the enzymatic protein content with the enzyme activity, quite a good correlation was observed for the reductase, assuming that the specific activity of aortal reductase is 50,000 units/mg reductase (i.e., same as the pure enzyme and the pulmonary microsomal reductase). However, it is somewhat more difficult to predict the microsomal enzymatic rate for cytochrome P-450-mediated activities using the specific activity of pure cytochrome because of the many variables that affect these rates. For form 6, the specific activity for *O*-deethylation of 7-ERF has been reported to be 0.4 mol/min/mol form 6 (19); turnover in pulmonary microsomes used in this study was 0.95 mol/min/mol form 6. It has been observed[2] that induction of form 6 by TCDD or βNF results in a decrease in pulmonary specific activity corresponding to a decrease in the ratio of reductase to form 6. Similarly, we previously observed that the specific activity for *N*-demethylation of benzphetamine by form 2 in microsomes from lung and liver from untreated or phenobarbital-treated rabbits varied with a qualitative correspondence between the specific activities and the ratios of reductase to form 2; the specific activity of form 2 purified from these tissues did not vary (26, 27). The aortal and leg muscle specific activities for 7-ERF were both about 0.5 mol/min/mol form 6 in spite of the 10-fold lower reductase content in the leg sample. Another significant variable in these comparisons, no doubt, is the actual composition of the extrahepatic microsomal fractions, which unlike hepatic fractions, contain a high proportion of sedimenting fragments that are not vesicles of endoplasmic reticulum. Thus, the environment of the cytochrome in purified systems, pulmonary, aortal, and leg microsomal fractions, is likely to be quite different. Therefore, a 2-fold difference in specific activity between pulmonary and muscular microsomes is not inconsistent with form 6 being functionally the same among these tissues. In addition, the inhibition of 7-ERF *O*-deethylation in aortal microsomes by the antibody to the reductase indicates the presence of a typical electron transport system as is the case in the lung and liver. This is in contrast to the lack of enzymatic reduction of the aortic hemoprotein isolated by Ullrich and coworkers (10).

The presence of form 6, which is active in the metabolism of BP (28) in tissues that are not considered to be highly active in xenobiotic metabolism, may be the basis for the activation of [^{3}H]BP to form surprisingly high levels of DNA adducts in leg muscle and brain, i.e., levels similar to that in the metabolically active liver (29). Whether the adducts are present in the vasculature and/or the parenchyma of these tissues requires further investigation. Given the heterogeneity of the vasculature, cytochrome P-450 monooxygenase cannot be assumed to be in all vascular beds.

In addition to identifying vascular components that have the potential to activate promutagens or procarcinogens *in vivo*, our data suggest that the metabolism *in vitro* mediated by homogenates or subcellular fractions of well perfused organs may contain a component of vascular origin.

[2] B. Domin, personal communication.

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