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DETECTION OF A NAFENOPIN-BINDING PROTEIN IN RAT LIVER CYTOSOL ASSOCIATED WITH THE INDUCTION OF PEROXISOME PROLIFERATION BY HYPOLIPIDEMIC COMPOUNDS

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SUMMARY: [^{3}H]nafenopin, a known inducer of liver peroxisomal enzymes, was shown to bind to a specific, saturable pool of binding sites in cytosols from rat liver and kidney cortex. Tissue levels of this binding protein (liver > kidney cortex; not detectable in myocardium, skeletal muscle) were seen to correlate with the ability of nafenopin to induce peroxisomal enzymes in these organs. Clofibrate and ciprofibrate, which are structurally similar to nafenopin, competitively blocked the specific binding of [^{3}H]nafenopin. Phenobarbital, a non-inducer of peroxisomes, and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid and 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio(N-β-hydroxyethyl)acetamide, which are structurally unrelated peroxisome proliferators, did not compete for the specific [^{3}H]nafenopin binding sites. The [^{3}H]nafenopin binding protein is proposed as a mediator of the drug-induced increase in peroxisomes and associated peroxisomal enzymes.

Exposure of rodents to any of a group of structurally-dissimilar compounds, such as nafenopin[1], which are capable of lowering plasma triglyceride levels, is uniformly associated with a marked proliferation of peroxisomes in hepatic parenchymal cells (1,2). These cytoplasmic organelles contain catalase and several enzymes involved in the β-oxidation of long-chain fatty acids (3). The activities of these enzymes in livers are elevated in association with peroxisome proliferation (4), and chronic peroxisome proliferation due to ingestion of these drugs is associated with the development of hepatocellular tumors (5).

To date, no clear explanation has emerged to explain the mechanism by which these drugs can induce the levels of peroxisomal enzymes. The tissue specificity

[1]Abbreviations used are: nafenopin, 2-methyl-2-p(1,2,3,4-tetrahydro-1-naphthyl) phenoxypropionic acid; clofibrate, ethyl-α-p-chloro-phenoxyisobutyrate; ciprofibrate, 2-(4-(2,2-di-chlorocyclopropyl)phenoxy)-2-methyl propionic acid; BR-931, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio (N-β-hydroxyethyl)acetamide; Wy-14,643, (4-chloro-6-(2,3-xylidino)-2-pyrimidnylthio)acetic acid; I_{50}, concentration of compound which inhibits specific binding by 50%.

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of this response, as well as the appearance of increased levels of peroxisomal enzyme mRNAs in drug-treated livers (6), both suggest the possible role of a tissue-specific receptor which can bind these drug molecules and mediate the subsequent enhanced expression of the battery of peroxisomal enzymes.

In this paper, we report the presence of a protein in hepatic cytosol and at a lower level in kidney cortex cytosol which can stereospecifically bind a representative ligand, (^{3}H)nafenopin. The presence of nafenopin binding protein in these tissues is highly correlated with the ability of the drug to induce peroxisomal enzymes.

MATERIALS AND METHODS

(^{3}H) nafenopin (13 Ci/mmole) was custom synthesized by Amersham, Arlington Heights, IL; (1-^{14}C) palmitoyl-CoA was also obtained from Amersham. The hypolipidemic drugs used as competitive ligands were obtained from the following sources: nafenopin, Ciba-Geigy, Summit, NJ; clofibrate, Ayerst Laboratories, New York, NY; ciprofibrate, SterlingWinthrop Research Institute, Rensselaer, NY; BR-931, LPB Istituto Farmaceutico, Milan, Italy; and Wy-14,643, Wyeth Laboratories, Radnor, PA. HEPES, palmitoyl-CoA and CoA were obtained from Sigma Chemical Company, St. Louis, MO. Activated charcoal (PX-21) was a generous gift from Amoco Research, Chicago, Illinois.

Livers from untreated F344 male rats (Charles River, Madison, WI) were perfused in situ with HEDG/KCl (4°) buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.4 M KCl, pH 7.4), and then homogenized (2 vols) and centrifuged as previously described (7). Cytosol fractions from these livers were either frozen (-80°) or used directly to determine (^{3}H)nafenopin binding capacity.

One ml of rat liver cytosol (0-10 mg protein) was typically added to tubes containing (^{3}H)nafenopin (50-500 pmoles) with or without unlabeled nafenopin (500 nmoles); the ligand was dissolved in a total volume of 0.06 ml of ethylene glycol. The incubation was for 1 hr at 4°. The radioligand-treated cytosols were then transferred to tubes already containing dextran-coated charcoal pellets (10 mg charcoal + 1 mg dextran pelleted from HEDG/KCl). The vortexed mixtures were incubated for 15 min at 4°, and the dextran charcoal was then removed by centrifugation at 4000 x g for 15 min. Aliquots of cytosol before and after dextran-coated charcoal treatment were taken for the determination of radioactivity.

For studies of peroxisomal enzyme induction, F344 male rats were fed for 6 weeks with either normal chow or chow containing 0.1% (w/w) nafenopin. The animals were then sacrificed and 10% homogenates of rat liver, kidney, heart or skeletal muscle were prepared in 0.25 M sucrose/0.1% ethanol (4°). For heart and skeletal muscle, ground glass homogenizers were used. The homogenates were assayed for the specific activity of peroxisomal catalase and the oxidation of (1-^{14}C) palmitoyl CoA as previously described (8).

RESULTS AND DISCUSSION

Specific binding of (^{3}H)nafenopin to rat liver cytosol

The binding of (^{3}H)nafenopin to the 105,000 x g supernatant fraction from rat liver was resistant to stripping with dextran-coated charcoal; control

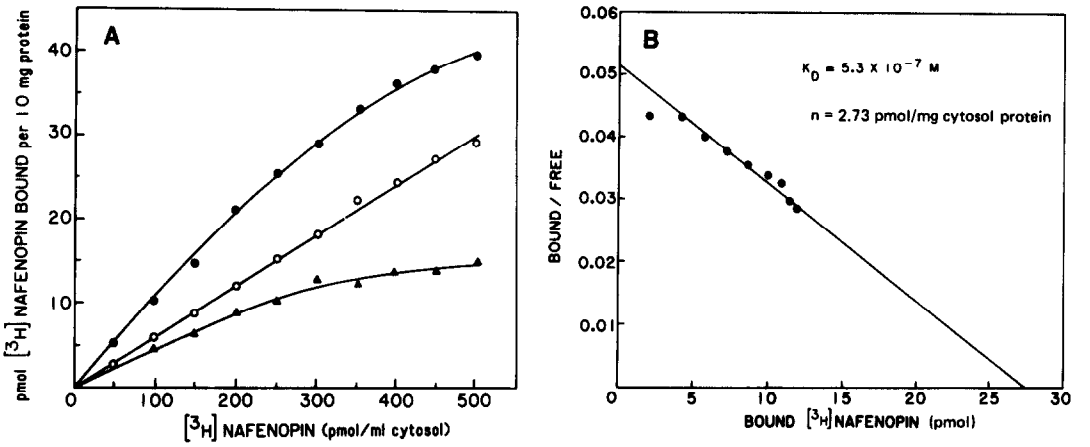


Figure 1 The binding of (^{3}H)nafenopin to liver cytosol from F344 male rats. A, The specific binding of (^{3}H)nafenopin (▲) is calculated as the difference between the total binding (●) and the non-specific (nondisplaceable) binding (o). The figure shows the concentration of (^{3}H)nafenopin bound versus the total (^{3}H)nafenopin added per ml cytosol. One ml incubations contained 10 mg cytosol protein. B, Scatchard plot of the specific binding data from A.

experiments showed > 99% removal of (^{3}H)nafenopin from incubations containing no cytosol protein. The specific binding is defined as the pool of displaceable binding. As seen in Fig. 1A, the total binding curve represents (^{3}H)nafenopin bound to specific (or displaceable) binding sites plus the non-specific binding. Non-specific binding represents sites which retained (^{3}H)nafenopin even in the presence of a 1000-fold molar excess of nafenopin. The difference between the total and the non-specific curves represents the pool of saturable, specific binding sites. Fig. 1B is a Scatchard plot of the data from Fig. 1A; the equilibrium dissociation constant (K_D) derived from this curve is 0.53 μM, and the total number of binding sites (n) is 2.73 pmol/mg cytosol protein. This K_D suggests a ligand-receptor interaction with an approximately 1000-fold lower affinity than that seen between either TCDD (9) or 17β estradiol (10) and their respective cytosol receptors. This relatively low-affinity, high saturation binding may truly represent characteristics of the binding protein or it may also be related to the ligand which we used. This ligand, (^{3}H)nafenopin, was selected because it was the only peroxisome-proliferating compound which we could obtain at the time with an adequately high specific activity for binding experiments. We have previously shown that rat milk containing only metabolites of nafenopin or metabolites

of Wy-14,643 was capable of inducing multi-fold proliferation of neonate rat liver peroxisomes (8). We are presently purifying these metabolites by HPLC and will determine the affinity with which they occupy this cytosol binding protein relative to the parent compound, nafenopin.

Correlation of receptor content and organ specificity for peroxisomal proliferation

Examination of (^3H)nafenopin binding in extrahepatic tissues led to the detection of a similar specific binding moeity, but of lower capacity, in cytosol prepared from kidney cortex (Table I). There was no displaceable (^3H)nafenopin binding in cytosols from myocardial or skeletal muscle. The rats fed a diet containing 0.1% nafenopin for 6 weeks showed a sizeable increase in liver peroxisomal catalase and palmitoyl-CoA oxidation (Table I); this was characteristically accompanied by a 6-10 fold increase in the density of liver peroxisomes (electron micrographs not shown). Homogenates from kidney cortex showed similar increases in peroxisomal enzymes, but the specific activities were comparatively lower than for liver. Consistent with the (^3H)nafenopin receptor content, only

TABLE I

Comparison of (^3H)Nafenopin Receptor Content and Peroxisomal Enzyme Induction in Different Organs/Tissues of F344 Male Rats

Organ/ Tissue	(^3H)Nafenopin Receptor Content[a]	Peroxisomal Enzyme Activities			
		Catalase[b]		$(1\text{-}^{14}C)$ Palmitoyl-CoA Oxidation[c]	
		Control	Nafenopin-Treated[d]	Control	Nafenopin-Treated[d]
Liver	140	37.8 ± 2.2	67.3 ± 3.2[f]	1.60 ± 0.14	13.2 ± 0.33[f]
Kidney	53	10.0 ± 1.6	29.0 ± 6.4[f]	0.31 ± 0.03	1.31 ± 0.08[f]
Heart	ND[e]	1.2 ± 0.1	1.5 ± 0.2	ND	ND
Skeletal Muscle	ND	ND	ND	ND	ND

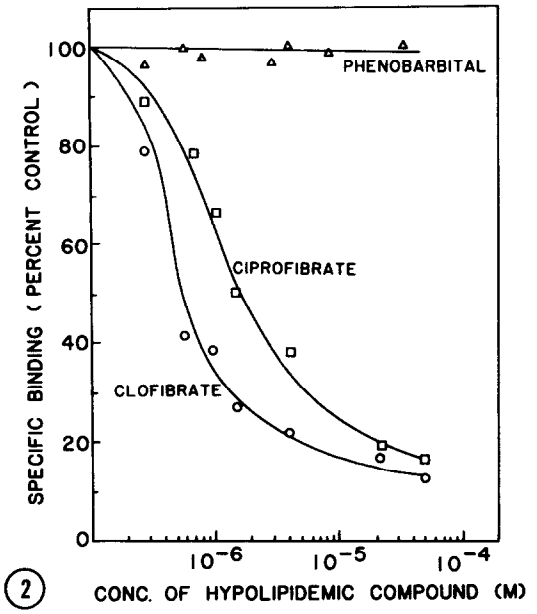
[a]Specific binding in organs/tissues from untreated rats, DPM (^3H)nafenopin bound x 10^{-3}/10mg protein, see Fig. 1A.
[b]Units/mg protein
[c]μmoles/min/gm liver
[d]Nafenopin-pretreated rats received 0.1% nafenopin in rat chow for 6 weeks prior to sacrifice.
[e]Not detectable.
[f]Significantly different from respective control values ($p < 0.005$).

a negligible amount of peroxisomal catalase activity was detected in the myocardial homogenate, and no peroxisomal enzyme activities were detectable in skeletal muscle. This demonstrated presence of (^{3}H)nafenopin binding in the two organ sites previousy shown to display drug-induced peroxisome proliferation, and the fact that the saturable receptor levels in liver and kidney mirror both the basal enzyme levels as well as their inducible maxima represent the strongest current evidence implicating the receptor as a mediator of drug-induced peroxisome proliferation.

Receptor competition by nafenopin analogs and other compounds

If the (^{3}H)nafenopin-binding protein mediates the peroxisome-proliferation response, then other known-effective drugs should be expected to compete with (^{3}H)nafenopin for the binding sites; and their relative affinities should roughly correlate with their in vivo potency in inducing rat liver peroxisomal enzymes. The affinity of other known peroxisome proliferators for the receptor was measured by their ability to compete for the specific (^{3}H)nafenopin binding sites (Fig. 2). Both clofibrate (I_{50} = 0.57 μM) and ciprofibrate (I_{50} = 1.7 μM), which are structural derivatives of nafenopin (Fig. 3) and which have known peroxisome-proliferating activity (4), inhibited the specific binding of (^{3}H)nafenopin in a dose-dependent manner. Phenobarbital, which is not a peroxisome proliferator, did not compete with (^{3}H)nafenopin. Wy-14,643 and BR-931, which are known to induce liver peroxisomes to the same extent as nafenopin (4), showed no competition for specific (^{3}H)nafenopin binding sites. This failure of Wy-14,643 and BR-931 to compete with (^{3}H)nafenopin for specific cytosol binding sites raises the possibility of more than one receptor species which can mediate the same biological function, or perhaps, structurally similar metabolites of the hypolipidemic compounds and phthalate ester plasticizers may share a common receptor.

Proliferation of hepatic peroxisomes in rodents persists as long as hypolipidemic compounds are administered, and disappears if drug administration is withdrawn. Recently, we have shown (6) that peroxisomal protein mRNA species are also elevated in livers of hypolipidemic drug-treated rats, and that they drop shortly after stopping drug exposure. The mechanism by which a nafenopin-receptor



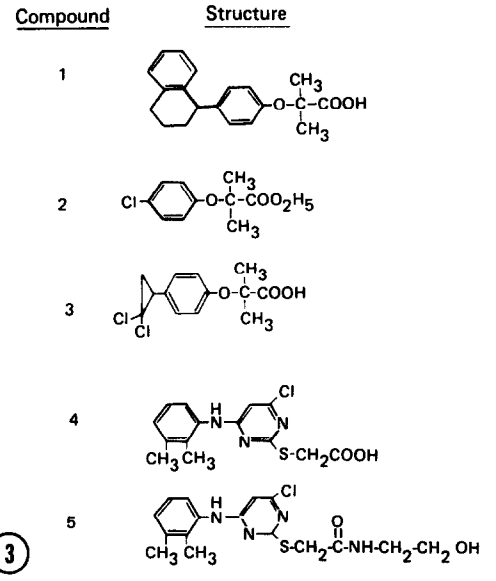


Figure 2 Relative affinity of structurally-similar peroxisome proliferators, clofibrate (o) and ciprofibrate (□) for specific (^{3}H)nafenopin binding sites. Increasing concentrations of peroxisome proliferating compounds were added to 1 ml incubations containing 10 mg cytosol and 300 pmol (^{3}H)nafenopin. The specific binding was measured as described in Materials and Methods. Increasing concentrations of phenobarbital (Δ) or the structurally-dissimilar peroxisome proliferators, BR-931 and WY-14,643 (data not shown) did not compete with the specific binding of (^{3}H)nafenopin.

Figure 3 Structures of hypolipidemic compounds: 1, nafenopin; 2, clofibrate; 3, ciprofibrate; 4, Wy-14,643; and 5, BR-931.

complex could modulate the expression of these peroxisomal protein genes remains to be determined.

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