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LACK OF EVIDENCE FOR A HEPATIC PEROXISOME PROLIFERATOR RECEPTOR AND AN EXPLANATION FOR THE BINDING OF HYPOLIPIDAEMIC DRUGS TO LIVER HOMOGENATES

MARK N. MILTON,* CLIFF R. ELCOMBE,† GEORGE E. N. KASS‡ and G. GORDON GIBSON*§

*University of Surrey, Department of Biochemistry, Division of Pharmacology and Toxicology, Guildford, Surrey, GU2 5XH, and †I.C.I. p.l.c., Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, U.K.

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Abstract—The existence of a postulated hepatic receptor responsible for the peroxisomal proliferation induced in rodents by hypolipidaemic drugs has been investigated. [^{3}H]-nafenopin and [^{3}H]-ciprofibrate were used as labelled ligands and two competitive binding assays, using either a charcoal-dextran or a hydroxylapatite method, were developed to investigate potential binding. In both assay systems, specific displaceable binding of either nafenopin or ciprofibrate to whole homogenate, microsomal and cytosolic fractions of rat liver could not be detected in a variety of buffer systems. A positive control of ligand binding to bovine serum albumin indicated the validity of the binding assays used. In addition, both nafenopin and ciprofibrate exhibited displaceable binding to serum albumin using the hydroxylapatite binding assay and a Scatchard analysis of the binding of [^{3}H]-nafenopin to fatty acid free rat serum albumin yielded a dissociation constant of 5.2×10^{-7} M and 86 pmol of ligand bound per mg protein. Taken collectively, our data strongly argues against the existence of a specific hepatic peroxisome proliferation receptor and indicates that the peroxisome proliferating hypolipidaemic drugs bind to serum albumin and possibly to other cellular proteins not involved in the activation of genes necessary for peroxisome proliferation.

Many structurally diverse hypolipidaemic agents have been shown to increase both hepatic peroxisome numbers and certain associated enzyme activities when administered to rodents, particularly the induction of enzymes involved in the peroxisomal β-oxidation of long-chain fatty acids [1, 2]. As well as inducing certain peroxisomal enzymes hypolipidaemic agents, such as clofibrate, ciprofibrate and nafenopin, also induce the levels of an isoenzyme of cytochrome P-450 (cytochrome P-452), which has a high specificity for the ω-hydroxylation of lauric acid [3, 4]. In addition, chronic peroxisome proliferation is of toxicological interest because it has been associated with an increase in the incidence of hepatocellular tumours in rodents [5]. The mechanism of carcinogenesis of these hypolipidaemic agents is as yet unknown but it is widely held that they act through a non-genotoxic mechanism. Furthermore, peroxisome proliferation has been extensively studied both *in vivo* and *in vitro* and appears to be a species-specific phenomenon restricted to rodents [6–13], although some researchers have managed to induce peroxisome proliferation in higher species (including monkeys), albeit only at very high and pharmacologically unrealistic doses [14, 15].

An explanation has been sought to account for species, tissue and sex differences in peroxisome proliferation, and for the mechanism of induction of the enzymes of peroxisomal β-oxidation and microsomal ω-oxidation of fatty acids. One proposed mechanism involves the existence of one or more specific hepatic cytosolic receptors for the hypolipidaemic agents [16], analogous to the Ah receptor for TCDD [17]. The presence or absence of these receptors could ostensibly account for the tissue, sex and species differences in the observed responses. A nafenopin binding-protein has previously been tentatively detected in rat liver cytosol [16] and has recently been semi-purified [18]. Another postulated mechanism rationalising the above hepatic responses involves a substrate overload of fatty acids which then induces the peroxisomal β-oxidation and microsomal ω-oxidation enzymes, leading to peroxisome proliferation [18, 19].

In this paper we have attempted to reproduce the binding of hypolipidaemic drugs to the postulated receptor and our results demonstrate the failure to detect specific binding to hepatic homogenates, and therefore cast considerable doubt on the existence of such a receptor. In addition, we offer a possible explanation to account for the observed "non-specific" binding.

‡ Current address: Karolinska Institute, Department of Toxicology, Stockholm, Sweden.

§ Author for correspondence.

‖ Abbreviations used: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; RSA, rat serum albumin; EDTA, ethylenediamine tetra-acetic acid; HEDG/KCl buffer, 25 mM HEPES buffer, pH 7.4, containing 1.5 mM EDTA, 1 mM DTT, 0.4 M KCl and 10% (v/v) glycerol; PMSF, phenylmethylsulfonylfluoride; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

MATERIALS AND METHODS

Materials. [^{3}H]-nafenopin (13 Ci/mmole) and [^{3}H]ciprofibrate (8.04 Ci/mmole) were kind gifts from Dr P. Bentley, Ciba Geigy, Basle, Switzerland, and Dr F. Bonner, Sterling-Winthrop, Alnwick, U.K. respectively. HEPES, DTT, BSA, RSA, fatty acid free RSA and Tween 80 were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Activated charcoal, dextran, KCl, EDTA and sucrose were obtained from BDH Chemicals (Poole, Dorset, U.K.). Hydroxylapatite was obtained from Bio-Rad (Richmond, CA), and glycerol and LKB optiphase liquid scintillant from Fisons (Loughborough, Leicestershire, U.K.). All other chemicals were obtained from commercial sources and were of the highest purity available.

Preparation of subcellular fractions. Untreated male Wistar rats (University of Surrey Breeders) weighing approximately 150 g, were killed by cervical dislocation, their livers excised, washed and perfused with ice-cold HEDG/KCl buffer and all subsequent steps were carried out at 0–4°. The livers were finely minced in HEDG/KCl buffer to give a 33% (w/v) homogenate and subcellular fractions were obtained by differential centrifugation. The homogenate was centrifuged at 1000 g_{av} for 10 min (JA-17 rotor) and the supernatant removed. The pellet was resuspended in 20 ml of buffer and centrifuged at 1000 g_{av} for 10 min. This procedure was repeated, and the final pellet containing nuclei and cellular debris was resuspended in 15 ml of HEDG/KCl buffer.

Cytosolic fractions were obtained by centrifuging the rat liver homogenate at 9000 g_{av} (JA-17 rotor) for 15 min, the supernatant removed and centrifuged at 106,000 g_{av} for 60 min (60 Ti rotor). The supernatant represented the cytosolic fraction and the pellet, containing the microsomes, was resuspended in HEDG/KCl buffer. All liver fractions were stored at −80°. Protein concentrations were determined by the method of Lowry *et al.* using crystalline bovine serum albumin as the standard.

Binding assays. Dextran-coated pellets were prepared by dissolving dextran T70 (4.5 g) in 200 ml of HEDG/KCl buffer and adding activated charcoal (45 g). The activated charcoal was resuspended using a magnetic stirrer and 1.8 ml aliquots (containing approximately 400 mg charcoal and 40 mg dextran) were transferred to tubes. The tubes were then centrifuged at 2000 g_{av} for 15 min and the supernatant discarded. Hydroxylapatite was washed with the appropriate buffer until the pH of the washes remained at 7.4 and subsequently resuspended in two volumes of the same buffer at 0–4°.

For the binding assays the subcellular fractions were dialyzed overnight, against 2 × 1 litre of the required buffer, prior to use. Two millilitres of sample (0–20 mg protein) was added to tubes containing [^{3}H]-nafenopin or [^{3}H]-ciprofibrate with or without unlabelled ligand (500 nmoles in 10 μl of ethanol). The tubes were vortexed and incubated at 4° for 80 min. Aliquots were then removed for determination of binding by either the charcoal-dextran method or the hydroxylapatite method. For method development a fixed concentration of 250 pmoles/ml was used.

For the charcoal-dextran assay, 500 μl of the incubation mixture was added to a tube containing a charcoal-dextran pellet, the tube vortexed and incubated for 11 min at 4°. The charcoal-dextran was then removed by centrifugation at 3500 g_{av} for 20 min, the supernatant removed and transferred to a plastic beta vial, containing 4.5 ml of optiphase liquid scintillation cocktail, for the determination of radioactivity. Alternatively, for the hydroxylapatite assay, an aliquot of the incubation mixture (200 μl) was added to a tube containing 0.25 ml of hydroxylapatite suspension and incubated for 30 min at 4°, with gentle shaking every 10 min. At the end of this time ice-cold buffer (1.0 ml, as indicated in the relevant tables) containing Tween 80 (0.5% v/v) was added, the tubes vortexed and subsequently centrifuged at 1500 r.p.m. for 5 min. The supernatant was removed, the hydroxylapatite pellet resuspended in absolute ethanol (2 ml) and transferred to a plastic beta vial, containing optiphase liquid scintillation cocktail (4.5 ml), for the determination of radioactivity.

For both the charcoal-dextran and hydroxylapatite assays, total and non-specific binding was determined by measuring the activity bound in the presence or absence of a 500-fold molar excess of unlabelled hypolipidaemic drug. In separate experiments, the binding of nafenopin and ciprofibrate to BSA served as a positive control.

RESULTS AND DISCUSSION

In preliminary experiments, no displaceable binding of [^{3}H]-ciprofibrate to whole homogenate, microsomes or cytosolic fractions was observed in the

Table 1. Binding of hypolipidaemics to rat liver fractions in the charcoal–dextran assay system

[^{3}H]-ligand	Buffer system	Binding (pmol [^{3}H]-ligand/mg protein)*				
		Cytosol		Microsomes		BSA
		Total	Non-specific	Total	Non-specific	Total
Ciprofibrate	TEDGP/KCl†	0.62 ± 0.11	0.89 ± 0.27	1.42 ± 0.52	1.31 ± 0.39	0.73 ± 0.44
Ciprofibrate	HEDGP/KCl	0.81 ± 0.16	0.70 ± 0.04	1.07 ± 0.30	1.26 ± 0.66	1.53 ± 0.88
Nafenopin	TEDGP/KCl	0.50 ± 0.08	0.52 ± 0.06	0.60 ± 0.92	0.65 ± 0.20	0.96 ± 0.44
Nafenopin	HEDGP/KCl	0.37 ± 0.06	0.54 ± 0.05	0.51 ± 0.05	0.55 ± 0.44	1.12 ± 0.28

* Results are expressed as mean ± SD of replicate samples.
† P indicates the presence of PMSF (50 μM).

hydroxylapatite assay system, even although the *identical* assay system of Lalwani *et al.* [16] was employed (data not shown). This lack of binding was not a feature of the buffer system used (or the dialysed preparation) as specific binding was absent in the presence of HEDG, HEDG/KCl, 50 and 100 mM KPO_4 and 50 and 100 mM Tris buffers (all buffers were pH 7.4 and contained 10% glycerol v/v). Total binding observed was of the range of 1–5 pmoles/mg protein. However, in the positive control experiment, [^{3}H]-ciprofibrate did bind to BSA up to the extent of 130 pmol/mg protein in the HEDG/KCl buffer system, indicating the validity of this particular assay, in as much as the hydroxylapatite did bind BSA as a positive control and presumably would also bind a receptor protein. The hydroxylapatite-binding assay is based upon the assumption that any "receptor" present is insoluble in the presence of hydroxylapatite. The validity of this assumption, however, can only be resolved by studying the binding of pure "receptor" to hydroxylapatite. As a valid binding assay would be an integral part of the purification of the "receptor" it is extremely difficult to determine whether the "receptor" is soluble in the presence of hydroxylapatite. We therefore believe that there is no evidence to suggest that the "receptor" is not being detected due to its inability to bind to hydroxylapatite.

In order to obviate the possibility of the receptor being denatured by proteolysis PMSF (50 μM) was included into the buffer systems. Tables 1 and 2 show a comparison of the binding of [^{3}H]-ciprofibrate and [^{3}H]-nafenopin to rat liver fractions using both the hydroxylapatite and charcoal-dextran assay systems. In general, there does not appear to be significant reproducible, displaceable binding of either [^{3}H]-ciprofibrate or [^{3}H]-nafenopin to any of the liver fractions. The low amount of specific binding in certain cases in Table 2, although statistically significant, represents only a vanishingly low level of binding.

From this data it appears that the hydroxylapatite binding assay consistently gives higher figures for total and non-specific binding than that of the charcoal-dextran method. In view of the fact that the previously reported receptor [8, 10] was identified by the charcoal-dextran method, it is informative to note several features of this assay which may influence interpretation of the data. The charcoal-dextran binding assay suffers from several inherent difficulties, including stripping of the [^{3}H]-ligand from the ligand–protein complex, the need to optimise the assay conditions for each batch of buffer used, the long spin time required to separate the charcoal-dextran bound ligand from the protein-bound ligand and the inability to analyse nuclear fractions for binding. This inability to analyse the nuclear fractions is due to the globular nature of the fraction which results in nuclear fractions being spun down along with the charcoal-dextran bound ligand, thus underestimating the amount of binding present. As any cytosolic receptor will need to translocate to the nucleus in order to alter the transcription of the genes for the induced proteins, then this inherent flaw in the technique may be very limiting. Problems may also arise when studying the nature of the bind-

Table 2. Binding of hypolipidaemics to rat liver fractions in the hydroxylapatite assay system

		Binding (pmol [^{3}H]-ligand/mg protein)*						
		Cytosol		Microsomes		Homogenate		BSA
[^{3}H]-ligand	Buffer system	Total	Non-specific	Total	Non-specific	Total	Non-specific	Total
Ciprofibrate	TEDGP/KCl†	11.61 ± 1.50	12.39 ± 1.24	13.39 ± 1.24	14.85 ± 2.96	19.25 ± 2.34	19.32 ± 2.30	28.31 ± 4.24
Ciprofibrate	HEDGP/KCl	12.67 ± 1.64	12.89 ± 1.63	18.76 ± 1.41	16.07 ± 1.73	20.90 ± 1.22	24.73 ± 1.22	39.25 ± 12.60
Nafenopin	TEDGP/KCl	9.07 ± 1.75	6.61 ± 0.86‡	10.15 ± 1.11	10.33 ± 1.46	13.15 ± 1.55	13.52 ± 1.49	39.75 ± 20.41
Nafenopin	HEDGP/KCl	8.55 ± 1.02	7.70 ± 0.63	13.68 ± 1.38	11.75 ± 1.48§	12.75 ± 1.29	11.62 ± 1.29	66.31 ± 6.67

* Results are expressed as mean ± SD of replicate samples.
† P indicates the presence of PMSF (50 μM).
‡ Significantly different from total binding, $P < 0.02$.
§ Significantly different from total binding, $P < 0.05$.

Table 3. Binding of hypolipidaemics to bovine serum albumin using the hydroxylapatite assay system

	Binding (pmol [^{3}H]-ligand/mg protein)*					
	TEDGP/KCl† buffer			HEDGP/KCl buffer		
[^{3}H]-ligand	Total	Non-specific	Specific	Total	Non-specific	Specific
Nafenopin	86.16 ± 12.61	35.03 ± 11.67‡	51.13	49.28 ± 10.60	21.82 ± 1.40‡	27.46
Ciprofibrate	29.27 ± 4.13	24.13 ± 3.05§	5.14	31.55 ± 8.26	18.58 ± 2.67‡	12.97

* Results are expressed as mean ± SD of replicate samples.
† P indicates the presence of PMSF (50 μM).
‡ Significantly different from total binding, $P < 0.001$.
§ Significantly different from total binding, $P < 0.05$.

ing of the ligand to the cytosol. As increasing ligand concentrations are used the amount of charcoal-dextran required to remove the excess unbound ligand may need to be increased. This change in assay conditions may present additional problems when interpreting the data.

In a previous series of experiments (data not shown) we determined that at low concentrations of charcoal-dextran (10 mg/assay) the unbound [^{3}H]-ligand is not completely removed from solution when an excess of unlabelled ligand is present. This would result in a false estimate of the amount of binding present. At higher concentrations of charcoal-dextran (800 mg/assay) the charcoal appears to strip the ligand from the protein–ligand complex resulting in an underestimation of the amount of binding present. At a ratio of 220 mg charcoal/dextran per mg protein an acceptable balance between the saturation of the charcoal-dextran and stripping of the protein–ligand complex was obtained. Therefore we found that it was not possible to obtain interpretable data using the method of Lalwani *et al.* [16]. The hydroxylapatite binding assay, however, does not appear to suffer from quite as many drawbacks as the charcoal-dextran assay. It relies on the binding of the protein-ligand complex to the hydroxylapatite which is more specific than the removal of excess ligand by the charcoal-dextran method.

In view of the data in Tables 1 and 2, an explanation needs to be found for the fact that any binding occurs at all. It has been known for a long time that clofibrate (and other hypolipidaemic drugs) binds to serum albumin and that this binding is displaceable [22, 23]. Clofibrate is known to compete with fatty acids for binding sites on serum albumin and therefore we decided to look at the binding of [^{3}H]-nafenopin and [^{3}H]-ciprofibrate to serum albumin. Table 3 shows the displaceable binding of the ligands to BSA. As we were able to show displaceable binding of both ligands to BSA it was decided to study the binding of the ligands to rat serum albumin in order to further mimic the binding seen in rat liver cytosol. When RSA was used to obtain a Scatchard plot a non-linear curve resulted (data not shown). Displaceable binding was seen at a range of ligand concentrations but it was not possible to saturate the total binding. As the rat serum albumin used in the above studies was not fatty acid free, the anomalous Scatchard plot could be due to the [^{3}H]-nafenopin and [^{3}H]-ciprofibrate displacing fatty acids from high affinity sites and the fatty acids binding to low affinity sites. When fatty acid free RSA was used a Scatchard plot was obtained from which the number of binding sites per mg protein and the dissociation constant (K_d) for nafenopin were determined (Fig. 1 and Table 4). The dissociation constant obtained is similar to that previously reported by other workers [16] and appears to represent a "receptor" of low affinity and high capacity. The usual criteria for designating protein binding as a "receptor", i.e. low capacity-high affinity binding, identification of an endogenous ligand, correlation with biological response, etc., are not adequately satisfied by the previously reported data [16]. Table 4 shows a comparison between the peroxisome proliferator "receptor" and other hepatic receptors. In addition, the failure to detect the receptor in heart and skeletal muscle [16] is not consistent with the induction of peroxisomal β-oxidation by nafenopin in these tissues [24, 29, 30].

Therefore it seems probable that the protein binding observed earlier is not that of a "receptor–ligand" complex, but due to the ligand binding to other cellular proteins. Such proteins could include albumin (this work [23]), glutathione *S*-transferase [31], and possibly in line with the ability of peroxisome proliferators to mimic fatty acids, fatty acid binding protein [32]. Whether it is possible to completely rationalise the previously reported specific binding

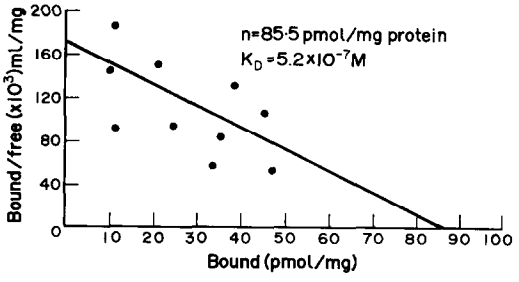

Fig. 1. Scatchard plot of the binding of [^{3}H]-nafenopin to fatty acid free, rat serum albumin. Results are expressed as the mean of triplicate determinations, yielding a regression coefficient of $r = -0.60$.

Table 4. Binding properties of some hepatic receptors

"Receptor"	Ligand	K_D	Capacity (fmol/mg cytosol protein)
"Steroid"	Oestradiol	0.70×10^{-10} M	58
"TCDD"	TCDD	0.27×10^{-9} M	84
"Peroxisome proliferator"	Nafenopin	0.53×10^{-6} M	2730
"Fatty acid free RSA"	Nafenopin	0.52×10^{-6} M	8550
"Fatty acid binding protein"	Fatty acid	0.24×10^{-6} M	1,230,000

Derived from Refs 16, 24–28 and this study.

of [^{3}H]-nafenopin [16] in terms of the contributions of individual proteins remains to be seen, although it is highly unlikely. One drawback to this approach is the need to use fatty acid-free proteins to obtain the best Scatchard plots whereas when present in the cytosol these proteins will be associated with fatty acids.

The question of the existence of this "receptor" has recently been taken one stage further by a report of its semi-purification by affinity chromatography [18]. In this latter study, the hypolipidaemic ligand was attached via the carboxylic acid moiety to the column, cytosol applied and proteins eluted. The major protein eluted had a molecular weight of 70,000 ± 2000. In comparison, the molecular weight of rat serum albumin is 68,000. By varying the ligand bound to the column proteins of different molecular weights were obtained, indicating the presence of at least two distinct receptors. It has also recently been stated that the receptor-binding moiety appears to be the acid form of the hypolipidaemic agents [15]. How this can be rationalised with the hydrophobic protein purified by affinity chromatography is not understood. Similarly, as glucuronidation of the carboxyl moiety of clofibrate is the only known metabolite, it is highly unlikely that this metabolite is involved in receptor binding.

An alternative mechanism for the induction of peroxisome proliferation, which does not invoke the existence of specific receptors, has been postulated [19, 20]. This mechanism involves changes in the fatty acid profile of the cell being responsible for the induction of peroxisomal β-oxidation and microsomal ω-oxidation. This represents a form of "substrate overload". Such a mechanism would give the cell great flexibility in handling short-term changes in lipid metabolism. Alternatively, the induction process may be rationalised by binding of the inducer to a specific, regulatory DNA-binding protein [33], thus providing the proximate biological signal, a concept which is presently being pursued in this laboratory.

It should be emphasised that a recent publication has also challenged the existence of the putative peroxisome proliferator receptor [34]. Using a cDNA probe to the peroxisomal, bifunctional β-oxidation enzyme, these latter workers have followed the time scale of peroxisomal proliferation and concluded that proliferation is inconsistent with a receptor mediated mechanism and that a "multistep cascade mechanism" based on a hepatic lipid overload phenomenon is more consistent with the experimental data [34].

In conclusion, it appears that the previously reported tenative detection of a peroxisome proliferator "receptor" may have to be re-evaluated. A "substrate overload" mechanism appears to be a more likely explanation for the phenomenon of peroxisome proliferation. This mechanism involves the build-up of liver lipids, induction of specific cytochrome P-450-dependent fatty acid hydroxylases with the subsequent formation of long-chain dicarboxylic acids, the latter representing the proximal biological stimulus for peroxisomal proliferation, as mechanistically enunciated elsewhere [35].

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