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THE PIG AS A MODEL FOR STUDYING AH RECEPTOR AND OTHER PAH-BINDING PROTEINS IN MAN

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Summary. In this report we compare the three major binding proteins existing in hepatic cytosols of pig and human. Many data suggest that for the AH receptor, which mediates the biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a structural as well as a functional variability exists across species. Similar conclusions can be drawn from the interspecies characterization of benzo(a)pyrene binding proteins, namely the 4S protein and the 8S protein. Using fractionation procedures such as sucrose gradient sedimentation and gel permeation chromatography we obtained enriched fractions containing each of these binding proteins. By saturation experiments and analysis of data by Scatchard and Woolf plots we determined binding characteristics and we conclude that pig and human AH receptors were closely related proteins since their K_d (18 ± 0.4nM) were found quite similar. On the other hand, 4S proteins from pig (K_d = 16.7 nM; B_{max} = 5.5 pmol/mg) and from human (K_d = 14nM; B_{max}= 4.5 pmol/mg) as well as 8S proteins (K_d ≈300 nM) also exhibit remarkable similarities.

Species differences in drug metabolism and more especially in cytochrome P-450 function and regulation are now well documented (1-3). These differences, which could be the result of the gene evolution due to an animal-plant warfare, are an important cause of large variations in the disposition, the pharmacological and toxicological effects of xenobiotics (4). For pharmaceutical industry and regulatory agencies, this species differences are a source of difficulties in the extrapolation of pharmacology and toxicity data from the laboratory animals to human clinical medicine.

The domestic pig is becoming an animal of choice in certain areas of biomedical research (5). This popularity stems from the many anatomical and functional similarities of pig and human but, at the present time, the lack of data precludes the

Abbreviations: TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; AHR, Aryl hydrocarbon receptor; PAH, polycyclic aromatic hydrocarbon; TCDF, 2,3,7,8-tetrachloro-dibenzofuran; BP, benzo(a)pyrene; BNF, β-naphthoflavone; 3-MC, 3-methylcholanthrene.

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effective utilization of this species. As large differences in the ability of TCDD to elicit toxic and biological responses in various species have been reported (6,7), it was of a prime interest to discover an animal species in which the regulatory protein mediating these responses, the AHR, would be, if not identical, at least very close to that existing in human. Up to now, when the specific binding of [^{3}H]TCDD to hepatic cytosol was examined in various species, the human AHR appears as quite different from those of the other animal species studied (8).

In this communication, examining, in the pig liver, some properties of the AHR as well as those of other PAH-binding proteins, namely 4S protein and 8S protein, we conclude that these proteins are remarkably similar, in their binding characteristics, to those present in human.

MATERIALS AND METHODS

Liver samples. One human liver sample was obtained from a lobectomy resected for a hepatic adenoma on a 24 year-old woman. The use of this sample was authorized by the French National Ethics Committee in the case where the liver could not be used for transplantation. Pig liver was obtained from a female (50 kg) purchased from the stock-farming Labatut, Lombez, France.

Chemicals. [^{3}H]TCDD (35 Ci/mmol) and TCDF were purchased from Chemsyn Science Lab. (Lenexa, TX). [^{3}H]BP 80 Ci/mmol) was obtained from Amersham Corp. (Buchs, England). BNF, 3-MC and other chemicals were from Sigma (Saint Louis, MO).

Preparation of cytosol and separation of binding proteins. Cytosol was prepared from human and pig livers as described (9) by centrifugation of homogenates at 9,000g for 20 min in HEDGM buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, 20 mM sodium molybdate and 10% glycerol (v/v, pH 7.6. The supernatants were centrifuged at 100,000g for 1 h to yield supernatant cytosol fractions. Enriched 9S fraction was prepared by sedimentation of cytosol on a 5-20% sucrose gradient for 2 h 40 min at 372,000g on a VTi vertical rotor. Fractions 15-22 were pooled and concentrated in CF25 centriflo membrane cones (Amicon). Separation of 4S and 8S proteins was carried out by gel permeation chromatography of liver cytosols to Sephacryl S-300 HR columns as described previously (10).

Binding experiments and velocity sedimentation on sucrose gradient. Separated protein samples (0.4 ml) were incubated with [^{3}H]BP or [^{3}H]TCDD in the absence or presence of competitors. The radioligands and chemicals used as competitors were added in 5 µl dimethyl sulfoxide. Samples were analyzed by density gradient centrifugation. 300 µl were loaded on either 5-20% or 10-30% sucrose gradients and centrifuged for 2 h at 372,000g in a VTi vertical rotor. Twenty-two fractions (282 µl; 8 drops per fraction) were collected and analyzed for radioactivity in a scintillator counter.

RESULTS AND DISCUSSION

When enriched 9S fractions of human and pig liver cytosols were incubated with [^{3}H]TCDD and analyzed on a sucrose density gradient, large peaks of radioactivity occured in the 9S region. These peaks were almost completely eliminated when the samples were incubated in the presence of a large excess of TCDF (Fig. 1). The same experiments carried out with crude liver cytosols did not allow to detect such specific

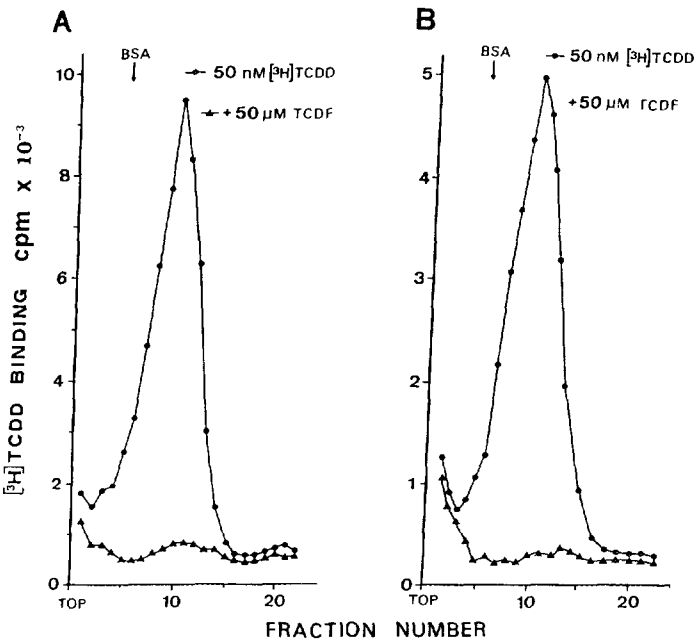

Figure 1. [^{3}H]TCDD binding to the enriched 9S fractions from human and pig livers. Samples (0.4 ml) of enriched 9S fractions from human (2.5 mg protein/ml) (A) and pig (3.4 mg protein/ml) (B) livers, separated from cytosol by sucrose gradient sedimentation as described under Materials and Methods, were incubated, for 1 h at 4°C, with 50 nM [^{3}H]TCDD in the absence or presence of 50 μM TCDF. The samples, not treated with charcoal-dextran, were examined on 5-20% sucrose density gradients as detailed in Materials and Methods. Duplicate determinations gave values which differed by less than ± 5%.

Table I. COMPARATIVE POTENCY OF VARIOUS COMPETITORS FOR [^{3}H]TCDD BOUND TO HUMAN AND PIG HEPATIC AHRs

COMPETITORS	DOSE μM	[^{3}H]TCDD BINDING[a] percent of control	
		Human	Pig
TCDF	50	4.1	0
3-MC	100	6.2	5.0
BNF	50	54.3	56.0
	100	35.0	38.2
	250	16.3	14.7

[a] Samples (0.4 ml) of human and pig enriched 9S fractions (0.375 mg protein/ml), prepared from liver cytosols as described in Materials and Methods, were incubated, for 2 h at 4°C, with 50nM[^{3}H]TCDD in absence or presence of compound tested. Specific binding was determined by sucrose gradient sedimentation as detailed in Materials and Methods. The control values (100 %) were 47 and 20 pmol [^{3}H]TCDD/mg protein, respectively, bound to the human and pig AHRs. Duplicate determinations gave values which differed by less that ± 10 %.

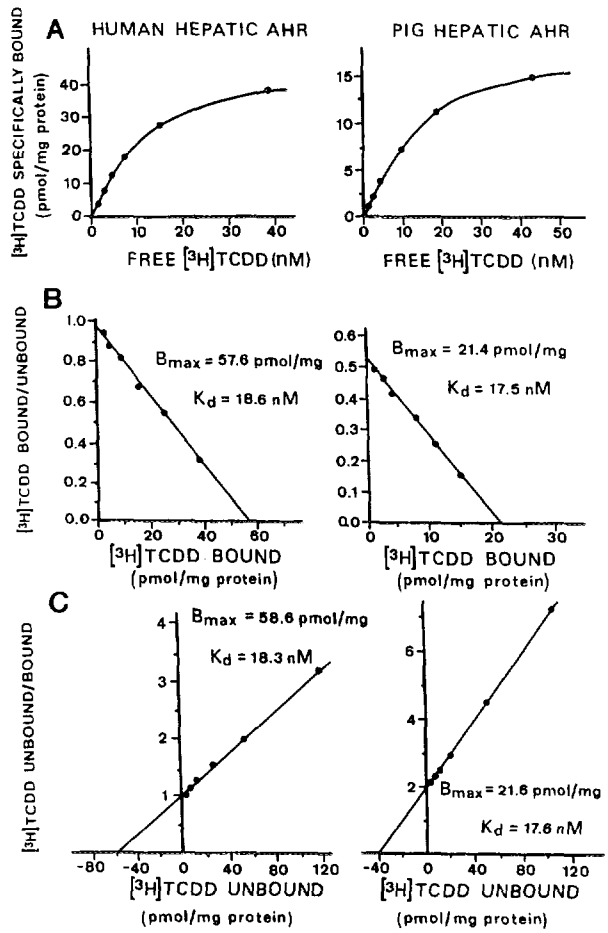

Figure 2. Saturation analysis and determination of apparent affinity of binding of [^{3}H]TCDD in enriched 9S fractions from human and pig liver. Samples (0.4 ml) of enriched 9S fractions from human (2.5 mg protein/ml) and from pig (3.4 mg protein/ml), prepared from liver cytosols as described in Materials and Methods, were incubated, for 2 h at 4°C, with [^{3}H]TCDD at concentrations ranging from 1.5 to 50 nM. Specific binding in the 9S peak was determined for each sample by sucrose density gradient analysis. The Scatchard plots (B) and Woolf plots (C) were derived from data shown in the saturation plots (A). Duplicate determinations gave values which differed by less than ± 5%.

binding peaks (not illustrated). Other ligands (3-MC, BNF), known to be agonists for the AHR, also displaced the [^{3}H]TCDD from the binding peaks in the 9S region of the sucrose gradient. The remarkable quantitative similarity of these displacements for the human and pig AHRs (Table I) suggested that the two binding entities could be closely related proteins. Such an assumption has been investigated by the estimation of their respective binding affinity for TCDD. Incubating the enriched 9S fractions with a wide range of radioligand concentrations (1.5 to 50 nM), then determining the specific binding in the 9S region by sucrose gradient analysis for each sample, we observed, as shown Fig. 2A, that about 40 nM [^{3}H]TCDD was necessary to saturate both the

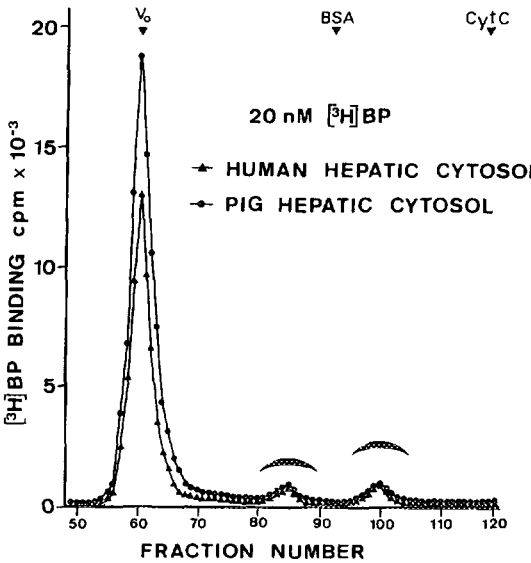

Figure 3. Analysis of human and pig hepatic cytosols by gel permeation chromatography. 7.5 ml-samples of cytosol from human and pig livers (about 25 mg protein/ml) were incubated, for 1 h at 4°C, with 20 nM [^{3}H]BP. Following dextran-coated charcoal adsorption with 75 mg charcoal/7.5 mg dextran pelleted from 7.5 ml HEDGM buffer, the samples were chromatographed on Sephacryl S-300 HR columns (100 x 2.6 cm) equilibrated with HEDGM buffer, then eluted under gravity flow (16.2 ml/h). 2.7 ml fractions were collected. Blue dextran, bovine serum albumin and cytochrome c were used as markers to standardize the columns.

human and pig AHRs. Binding data were further analyzed by Scatchard (Fig. 2B) and Woolf (Fig. 2C) plots and the kinetic parameters K_d and B_{max} are indicated on the respective plots. The values are similar whatever the method used and, if the concentration of specific [^{3}H]TCDD binding sites was about 2.7- fold lower in pig liver (21.5 pmol/mg protein) than in human liver (58 pmol/mg protein), it is noteworthy that the apparent affinity with which [^{3}H]TCDD bound AHR was almost identical (K_d = 18 nM).This relative low binding affinity explains the large stripping effect observed when the samples were treated with the charcoal-dextran (not illustrated). The AHR generally exhibits a high-affinity binding with [^{3}H]TCDD and the K_d values in diverse animal species and mammalian cells in culture are found equal to or lower than 1 nM (11-13). Two notable exceptions exist 1) the human AHRs (K_d = 3 to 12 nM) and 2) the hepatic AHR of the nonresponsive DBA/2J mouse strain which displays an apparent affinity for [^{3}H]TCDD about 10-fold lower (K_d = 16 nM) than that of the responsive C57BL/6J mouse strain (K_d =1.8 nM) (14). Data reported here demonstrate, for the first time, that hepatic pig and human AHRs (K_d =18 nM) belong, as that of nonresponsive mice, to the class of the lowest affinity AHRs.

Fig. 3 shows the chromatographic profiles of crude hepatic human and pig cytosols to Sephacryl S-300 HR. The cytosolic samples have been incubated with [^{3}H]BP then treated wirth charcoal-dextran. The major peak of radioactivity, which is

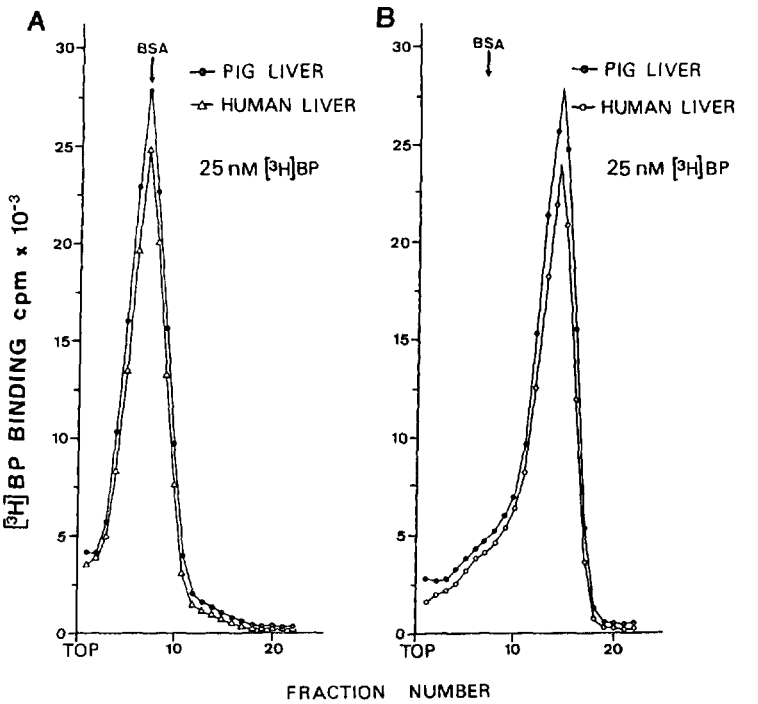

Figure 4. Sucrose density gradient profiles of 95-105 fractions and 80-90 fractions eluted from gel permeation chromatography of human and pig cytosols. Samples (0.4 ml) of 95-105 fractions from pig and from human (4 mg protein/ml) (A) and 80-90 fractions from pig and from human (8 mg protein/ml) (B) were incubated, for 1 h at 4°C, with 25 nM [^{3}H]BP. Samples, not treated with charcoal-dextran, were examined on 5-20% sucrose density gradients as detailed in Materials and Methods. Duplicate determinations gave values which differed by les than ± 5%.

eluted in the void volume are lipoproteins, as it had been previously demonstrated (9). According to our previous reports, and in the same experimental conditions, the various binding proteins endowed with a sufficient high affinity for [^{3}H]TCDD or [^{3}H]BP to retain the radioligand after charcoal treatment, are eluted as following : the AHR was eluted in fractions 70-80 (15), the PAH binding-8S protein (10) and the PAH binding-4S protein (10,15) were eluted in fractions 80-90 and 95-105 respectively. As shown Fig. 3, and as expected, the low-affinity forms of human and pig AHRs are undetectable. 8S protein, as well as 4S protein, appear as small peaks in fractions 80-90 and 95-105 respectively, suggesting that these two proteins display a low binding affinity and/or a low binding capacity for [^{3}H]BP.The fractions containing the 8S protein and the 4S protein were pooled, concentrated in CF25 centriflo membrane cones, then incubated with [^{3}H]BP. The identity of 4S and 8S proteins was attested by their sedimentation in the 4S and the 8S region of sucrose gradients respectively (Fig. 4). Treatment of these [^{3}H]BP-incubated samples with a large excess of nonlabeled BP almost completely eliminated the radioactivity bound to these proteins (not illustrated). It can be noted that the treatment of [^{3}H]BP-incubated samples with charcoal-dextran

removed the radioligand bound to the proteins thereby attesting their low affinity for the radioligand (not illustrated).

For the pig 4S protein, [^{3}H]BP saturation experiments using a wide range of [^{3}H]BP concentrations (5 to 60 nM) indicate, according to the Scatchard and Woolf plot analyses that binding parameters, K_d (16.7 nM) and B_{max} (5.5 pmol/mg protein) are very close to those reported for human hepatic 4S protein (K_d=14 nM; B_{max}= 4.5 pmol/mg protein) (15). In pig as in human, 8S protein, a large binding capacity and low binding affinity protein for [^{3}H]BP (10), were found similar to those characterized in other species with apparent binding affinities (K_d) around 300 nM.

In conclusion, it appears from this work, that human and pig are, in our knowledge, the only one species for which the three major binding proteins for halogenated aromatic hydrocarbons (AHR) and PAHs (4S and 8S proteins) are characterized by a relatively low-affinity for their ligands. Such a similarity should establish the pig as a animal of choice for a wide range of pharmacological and toxicological investigations in this area.

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