Uptake and degradation of vitamin D binding protein and vitamin D binding protein–actin complex *in vivo* in the rat

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We have labelled the rat vitamin D binding protein (DBP), DBP–actin and rat albumin with ^{125}I -tyramine–cellobiose (^{125}I -TC). In contrast with traditional ^{125}I -labelling techniques where degraded radioactive metabolites are released into plasma, the ^{125}I -TC moiety is trapped intracellularly in the tissues, where the degradation of the labelled proteins takes place. By using this labelling method, the catabolism of proteins can be studied *in vivo*. In this study we have used this labelling technique to compare the tissue uptake and degradation of DBP, DBP–actin and albumin in the rat. DBP–actin was cleared from plasma at a considerably faster rate than DBP. After intravenous injection of labelled DBP–actin complex, 48 % of the radioactive dose was recovered in the liver after 30 min, compared with 14 % when labelled DBP was administered. Only small amounts of DBP–actin complex were recovered in the kidneys. In contrast with the results obtained with DBP–actin complex, liver and kidneys contributed about equally in the uptake and degradation of DBP determined 24 h after the injection. When labelled DBP was compared with labelled albumin, the amount of radioactivity taken up by the liver and kidneys by 24 h after the injection was 2 and 5 times higher respectively. In conclusion, liver and kidneys are the major organs for catabolism of DBP in the rat. Furthermore, binding of actin to DBP enhances the clearance of DBP from circulation as well as its uptake by the liver.

INTRODUCTION

In mammals, vitamin D_3 and its metabolites are mainly transported in blood bound to an α -globulin [1] with an M_r of 52 000–58 000 [1,2]. This protein is named the binding protein for vitamin D and its metabolites (DBP). The levels of DBP in rat and human plasma are 300–500 $\mu\text{g}/\text{ml}$ (6–10 μM) [3,4]. Under physiological conditions, only 1–3 % of DBP is occupied by vitamin D_3 or its metabolites [3,4].

In addition to binding to vitamin D_3 and its metabolites, DBP also has high-affinity binding sites for actin [5], and during tissue homogenization, DBP–actin complex is formed [5,6]. The DBP–actin complex is rapidly removed from the circulation *in vivo* [7,8]. Plasma from healthy humans contains no significant amount of the complex. In contrast, plasma from patients with acute hepatic necrosis [9] or from pregnant women [10] contains a considerable amount of DBP as DBP–actin complex.

It is not known whether DBP also has a function in the delivery or uptake of vitamin D_3 and its metabolites in different organs and cell types. So far, no receptor for DBP has been demonstrated.

In this paper, we describe the accumulation of DBP in different organs after intravenous injection of ^{125}I -tyramine–cellobiose (^{125}I -TC)-labelled DBP or DBP–actin complex. The ^{125}I -TC-labelled DBP was bound to actin *in vitro* and the complex was injected into rats. The tissue distribution and clearance of the labelled was compared with that of labelled DBP.

MATERIALS AND METHODS

Materials

Carrier-free ^{125}I - and 25-hydroxy[26(27)-methyl- ^3H]cholecalciferol (sp. radioactivity 22 Ci/mmol) were purchased from Amersham International, Amersham, Bucks., U.K. CNBr-

activated Sepharose, Agarose A, Polybuffer exchanger PBE 94 and Polybuffer 74 for chromatofocusing were from Pharmacia, Uppsala, Sweden. Gel-filtration material AcA-44 was obtained from LKB, Bromma, Sweden. Bovine muscle actin was purchased from Sigma, St. Louis, MO, U.S.A. All other chemicals were high-purity grade.

Animals

Adult rabbits (about 3 kg) were used for production of antisera against rat DBP and rat albumin. Male Wistar rats (150–250 g) were fed on an ordinary laboratory chow.

Purification of rat DBP

Rat DBP was prepared as described previously [11] and applied to a chromatofocusing column (1.8 cm $\times$ 30 cm) with 0.025 M-histidine, pH 6.2, as starting buffer. The protein was eluted with Polybuffer 74 (diluted 1:10), pH 4.0. The purity of DBP was checked by 0.1 % SDS/PAGE (10 % gels) and for binding activity for [^3H]25-hydroxyvitamin D_3 . The amount of DBP was quantified by single radial immunodiffusion [3].

Purification of rat albumin

Rat albumin was isolated by an anti-(rat albumin) column as described for DBP [11], except that albumin was eluted from the column by 3.5 M-ammonium thiocyanate.

Iodination of purified rat DBP and rat albumin

Purified rat DBP and rat albumin were labelled with ^{125}I -TC by the method described by Pittman *et al.* [12]. Purified rat DBP or rat albumin (10 nmol) were incubated with 8 nmol of ^{125}I -TC (0.8 mCi). Unconjugated ^{125}I -TC was removed by dialysis against 4 litres of 0.1 M- NH_4HCO_3 and then 10 litres of 20 mM-potassium phosphate buffer (pH 7.4)/0.9 % NaCl (PBS). More than 95 %

Abbreviations used: DBP, the binding protein for vitamin D and its metabolites; ^{125}I -TC, ^{125}I -labelled tyramine–cellobiose; $t_{1/2}$, plasma half-life.

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of the radioactivity was trichloroacetic acid-precipitable. The ^{125}I -TC-labelled proteins were purified on an AcA-44 column (2.6 cm $\times$ 30 cm) with 10 mM-potassium phosphate/0.9 % NaCl, pH 7.4. The purity of ^{125}I -TC-labelled DBP and albumin was confirmed using 0.1 % SDS/PAGE (10 % gels). The radioactivity had the same mobility as purified rat DBP and rat albumin respectively. ^{125}I -TC-DBP reacted with antiserum against rat DBP when tested by single radial immunodiffusion [3].

Incubation of ^{125}I -TC-DBP with actin

Actin was dissolved in PBS. In a buffer with the ionic concentration of PBS, monomeric actin will polymerize spontaneously into filamentous actin [13]. Polymerization of actin is an indication of its native properties [14]. The formation of filamentous actin was confirmed by gel filtration on an AcA-44 column as described above. Labelled DBP and actin were incubated in molar ratios of DBP/actin of 1:1 and 1:3. The ^{125}I -TC-DBP-actin complex formed during the incubation was separated from uncomplexed ^{125}I -TC-DBP and actin by gel filtration on an AcA-44 column. This procedure was undertaken to ensure that only native actin was injected as a DBP-actin complex.

Intravenous injection of labelled proteins

^{125}I -TC-DBP, ^{125}I -TC-DBP-actin or ^{125}I -TC-albumin (about 300 pmol) in PBS was injected via the femoral vein. After different periods of time (5 min to 24 h), 20–120 μl of blood was collected. The rats were killed at 30 min, 5 h or 24 h after injection and blood and different organs were collected. The amount of radioactivity recovered in different tissues was calculated by assuming that blood amounts to 6 %, plasma to 3.2 %, liver to 4 %, muscle to 45.5 % and adipose tissue to 7.2 % of total rat body weight [15].

Trichloroacetic acid precipitation

Livers and kidneys were rinsed with PBS and specimens were then homogenized in 0.25 M-sucrose and a 50 % ice-cold trichloroacetic acid solution was added. The final trichloroacetic acid concentration was 12.5 %. The radioactive material was separated into trichloroacetic acid-precipitable (protein-bound) and trichloroacetic acid-soluble material (degraded products) by centrifugation [16].

Other methods

^{125}I radioactivity was counted in a Kontron Automatic Gamma Counter System MR 252.

RESULTS

When ^{125}I -TC-labelled rat DBP was injected intravenously, the half-life ($t_{1/2}$) of DBP during the initial rapid phase (5 min to 12 h) was about 3 h (Fig. 1). When labelled proteins are injected intravenously, the radioactivity is initially cleared from plasma at a rapid rate. This phase is due both to equilibrium of labelled protein with unlabelled protein in the exchangeable compartments of the body and to catabolism of the labelled protein. After this initial phase, DBP disappeared from the circulation at a slower rate, with a $t_{1/2}$ during this second period of about 10 h. This second phase of the decay curve is thought to be due to tissue uptake and catabolism of the protein. At 30 min after the injection of ^{125}I -TC-DBP, about 40 % of the administered dose was recovered in serum, decreasing to 10 % at 5 h and 2 % at 24 h (Fig. 2). When ^{125}I -TC-labelled DBP-actin complex was given by the intravenous route, only 7 % of the injected radioactivity was recovered in serum 30 min after the administration (Fig. 2). After 24 h, the amount of radioactivity in serum had dropped to 0.3 %

Fig. 1. Clearance of radiolabelled rat DBP from the circulation

^{125}I -TC-labelled DBP was injected intravenously into rats, and blood samples were collected between 5 min and 26 h after the injection. The calculation of radioactivity recovered in blood was based on the assumption that blood amounts to 6 % of rat body weight. The amount of radioactivity recovered after 5 min was taken as 100 %. This amount corresponded to a mean of 45.2 % of the injected dose. The results are given as a percentage of the initial amount of radioactivity recovered in blood. Data were pooled from four rats.

of the administered dose. Rat albumin was cleared from plasma at a slower rate than DBP, and 5 h after the injection a mean of 26 % of the injected radioactivity was recovered in the circulation. The amount of radioactivity recovered decreased further to 7 % at 24 h after administration (Fig. 2).

Fig. 2. Recovery of radioactivity in plasma after injection of ^{125}I -TC-DBP, ^{125}I -TC-DBP-actin and ^{125}I -TC-albumin

The percentages of the injected doses of ^{125}I -TC-DBP ($\square$), ^{125}I -TC-DBP-actin ($\blacksquare$) or ^{125}I -TC-albumin ($\hatched$) recovered in plasma are shown at 30 min, 5 h and 24 h after injection of the labelled proteins. The results are given as total radioactivity recovered in plasma as a percentage of injected dose. The calculation is based on the assumption that plasma amounts to 3.2 % of rat body weight. Data are given as means $\pm$ S.D., with numbers of animals in each group in parentheses above bars.

Fig. 3. Recovery of radioactivity in liver after injection of ^{125}I -TC-DBP, ^{125}I -TC-DBP-actin and ^{125}I -TC-albumin

Total radioactivity (a) and trichloroacetic acid-soluble radioactive material (b), recovered in liver are shown at 30 min, 5 h and 24 h after injection of ^{125}I -TC-DBP (□), ^{125}I -TC-DBP-actin (■) or ^{125}I -TC-albumin (▨). The results are given as percentage of injected dose. The calculation is based on the assumption that liver amounts to 4% of rat body weight. Data are given as means $\pm$ S.D., with numbers of animals in each group given in parentheses above bars.

Of the radioactivity recovered in serum after injection of ^{125}I -TC-DBP, only $2.7 \pm 0.2\%$ ($n = 5$, mean $\pm$ S.D.) was detected as degraded material (trichloroacetic acid-soluble) after 30 min. At 5 h and 24 h after the intravenous administration, trichloroacetic acid-soluble material in serum accounted for $3.8 \pm 0.4\%$ ($n = 6$) and $7.7 \pm 2.7\%$ ($n = 6$) respectively of the recorded radioactivity. When ^{125}I -TC-albumin was injected intravenously, $1.9 \pm 0.7\%$ ($n = 5$) and $2.5 \pm 0.4\%$ ($n = 4$) of the radioactivity recovered in plasma was trichloroacetic acid-soluble at 5 and 24 h respectively.

A considerable amount of radioactivity was recovered in the liver from 30 min to 24 h after injection of both ^{125}I -TC-DBP and ^{125}I -TC-DBP-actin complex (Fig. 3a). A mean of 14% of the injected dose of DBP was found in the liver at 30 min after administration, and the amount of radioactivity remained at about 15–17% for up to 24 h (Fig. 3a). Intravenous injection of DBP-actin complex resulted in a markedly greater amount of radioactivity recovered in liver compared with DBP. Mean values of 48 and 43% of the injected radioactivity were detected at 30 min and 24 h respectively after administration (Fig. 3a). In comparison, about 13 and 9% of labelled albumin was recovered in the liver at 5 h and 24 h respectively after intravenous administration (Fig. 3a).

The ^{125}I -radioactivity recovered in the liver at different times

Fig. 4. Recovery of radioactivity in kidneys after injection of ^{125}I -TC-DBP, ^{125}I -TC-DBP-actin and ^{125}I -TC-albumin

Shown are total radioactivity (a) and trichloroacetic acid-soluble radioactive material (b) recovered in kidneys at 30 min, 5 h and 24 h after the injection of ^{125}I -TC-DBP (□), ^{125}I -TC-DBP-actin (■) or ^{125}I -TC-albumin (▨). The results are given as percentage of the injected dose. Data are given as means $\pm$ S.D., with numbers of animals in each group given in parentheses above the bars.

after injection of ^{125}I -TC-DBP, ^{125}I -TC-DBP-actin or ^{125}I -TC-albumin was separated into trichloroacetic acid-precipitable and soluble material. All the proteins studied were taken up and also degraded in the liver as reflected by the amount of trichloroacetic acid-soluble radioactivity recovered in this organ. At 30 min after administration, about 4 and 22% of the injected doses of labelled DBP and DBP-actin complex respectively were recovered in the liver as trichloroacetic acid-soluble materials. At 24 h after the injection, 7 and 19% respectively of the given doses of labelled DBP and DBP-actin complex were recovered as trichloroacetic acid-soluble radioactivity in the liver (Fig. 3b). In comparison, between 3 and 7% of the injected dose of labelled rat albumin was recovered as trichloroacetic acid-soluble material in liver between 30 min and 24 h after administration (Fig. 3b).

Radioactivity also accumulated in the kidneys when ^{125}I -TC-DBP was injected intravenously. At 30 min after administration, a mean of 6% of the injected dose was recovered in this organ. The amount of radioactivity recovered in the kidneys increased to 11% after 5 h and 12% after 24 h (Fig. 4a). Of the radioactivity recovered in kidneys after different periods of time, there was an increase in the percentage detected as degraded material with increasing time after injection of labelled DBP. About 2% of the injected dose of labelled DBP was recovered as trichloroacetic acid-soluble material in kidneys at 30 min after administration.

Table 1. Tissue distribution of ^{125}I radioactivity after intravenous injection of ^{125}I -TC-DBP

Effect of time on percentage of injected dose recovered in different tissues. The radioactivity was determined in tissues from 6–9 rats at each time point. The values represent means $\pm$ s.d. The calculation was based on the assumption that muscle amounts to 45.5 % and adipose tissue to 7.2 % of total body weight [5].

Tissue	Radioactivity recovered (% of injected dose) after:		
	0.5 h	5 h	24 h
Skeletal muscle	5.4 $\pm$ 0.6	7.4 $\pm$ 2.9	5.6 $\pm$ 2.2
Adipose tissue	1.5 $\pm$ 0.3	2.4 $\pm$ 1.5	1.2 $\pm$ 0.5
Spleen	1.3 $\pm$ 0.2	1.3 $\pm$ 0.3	1.4 $\pm$ 0.4
Testes	0.5 $\pm$ 0.7	0.9 $\pm$ 0.1	0.6 $\pm$ 0.1
Lung	1.2 $\pm$ 0.1	0.6 $\pm$ 0.1	0.3 $\pm$ 0.1
Heart	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1
Thyroid/parathyroid/ thymus	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1
Adrenals	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0	0.04 $\pm$ 0.01
Brain	0.1 $\pm$ 0.0	0.03 $\pm$ 0.06	0.01 $\pm$ 0.0
Skin	0.14 $\pm$ 0.03*	0.33 $\pm$ 0.27*	0.18 $\pm$ 0.04*
Small intestine	0.20 $\pm$ 0.07*	0.52 $\pm$ 0.40*	0.21 $\pm$ 0.10*
Large intestine	0.12 $\pm$ 0.03*	0.41 $\pm$ 0.29*	0.19 $\pm$ 0.08*
Bone (tibia)	0.12 $\pm$ 0.02*	0.10 $\pm$ 0.03*	0.19 $\pm$ 0.11*

* Radioactivity is given as percentage of injected dose recovered per g of tissue.

The amount of degraded material recovered in this organ increased to 7 % of the given dose at 24 h after the injection (Fig. 4b).

Less than 2 % of the injected DBP-actin complex was recovered in the kidneys at 30 min or 24 h after injection (Fig. 4a). When labelled albumin was injected intravenously, about 3 % of the given dose was detected in this organ between 30 min and 24 h after injection (Fig. 4a). About half of the radioactivity recovered in the kidneys was trichloroacetic acid-soluble material when labelled DBP-actin complex or albumin was injected.

Radioactive material was also detected in organs other than liver and kidneys when ^{125}I -TC-DBP was injected intravenously. About 5–8 % of the administered dose was recovered in skeletal muscle between 30 min and 24 h after the injection, and during the same time period 1–3 % was recovered in adipose tissue (Table 1). About 1 % was recovered in the testes. Less than 1.5 % of the injected activity was detected in each of the following organs: lung, heart, thyroid/parathyroid/thymus and adrenal. In these parenchymal tissues only minor amounts of radioactivity was detected and the radioactivity decreased with increasing time after the injection of labelled DBP. Most of the radioactivity recovered in these organs may therefore be due to blood contamination. There was no effect of time on the small amount of radioactivity detected in the following tissues: spleen, bone (tibia), small intestine and large intestine (Table 1).

DISCUSSION

The ^{125}I -TC and dilactitol- ^{125}I -tyramine labelling techniques are convenient methods with which to study the degradation of proteins *in vivo* as well as *in vitro* [12,17]. With these methods, the degradation products are trapped within the cells. Pittman *et al.* [12] reported that only small amounts of trichloroacetic acid-soluble materials are released into plasma from the tissues in which degradation takes place [12]. When urine was collected for

a 24 h period after intravenous administration of different ^{125}I -TC-labelled proteins, about 3–6 % of the activity was recovered in the urine. When we compared the amount of radioactivity recovered in different tissues at 5 and 24 h after injection, it was found that 94 % of the radioactivity recovered 5 h after intravenous administration remained in the same organs 19 h later (Figs. 3a, and 4a; Table 1). This suggests that the ^{125}I -TC moiety is trapped intracellularly as shown previously by Pittman *et al.* [12]. Degradation in different tissues can therefore be determined by the amount of trichloroacetic acid-soluble material present in these organs. Plasma clearance of the proteins labelled with this method was similar to the clearance obtained when the proteins were labelled by a traditional labelling technique [12]. We have previously reported a $t_{1/2}$ for ^{125}I -labelled rat DBP in the rat of about 8 h [11]. This result has been confirmed by Harper *et al.* [18], who calculated the $t_{1/2}$ in the rat to be 7 h. In both of these studies, rat DBP was labelled with ^{125}I by traditional labelling techniques. In this study, we report a similar plasma $t_{1/2}$ of ^{125}I -TC-DBP, i.e. about 10 h (Fig. 1). In addition to exhibiting similar plasma decay rates, ^{125}I -TC-DBP reacted with antiserum raised against rat DBP, was able to bind actin and had the same mobility on SDS/PAGE as purified DBP. Liver sinusoidal endothelial cells may remove denatured proteins by a scavenger receptor [19]. When ^{125}I -TC-DBP was injected intravenously, less than 20 % of the radioactivity recovered in the liver was in the endothelial cells (results not shown), indicating that the labelled DBP was not denatured. These results suggest that ^{125}I -TC-DBP had biological properties similar to those of DBP in plasma. The TC-labelling method should thus be suitable to study the tissue uptake and degradation of DBP and DBP-actin complex *in vivo*.

In this report we have shown that DBP was taken up and degraded in both liver and kidneys. These two organs contained about equal amounts of trichloroacetic acid-soluble products 24 h after injection, indicating that DBP was taken up and degraded in both organs and that these two tissues contributed about equally to the catabolism of DBP *in vivo* (Figs. 3b and 4b).

In the kidney there was a marked increase in the amount of radioactivity recovered with time (Fig. 4a), whereas in the liver there was only a small increase in activity with time when ^{125}I -TC-DBP was injected intravenously. When albumin labelled by this technique was administered intravenously, there was even a decrease in the radioactivity recovered in the liver from 5 to 24 h after administration (Fig. 3a). This loss of radioactivity in liver with time is in accordance with results published by Pittman *et al.* [12] who reported that about 20–25 % of the radioactivity accumulated in liver was released into the intestine, probably via bile. In the experiments with ^{125}I -TC-DBP, the liver uptake of DBP during the 24 h period was higher than the loss of activity via bile. It remains to be investigated whether there is an enterohepatic circulation of intact proteins.

Our data concerning the liver and kidney uptake of albumin are in accordance with the results published by Strobel *et al.* [17]. The percentage of DBP recovered in liver kidney was 1.3–2 times and 3.4–5.1 times higher respectively than recovery of albumin. Ockner *et al.* [20] have suggested that albumin is taken up via receptor-mediated endocytosis. The even greater accumulation of DBP compared with albumin in liver and kidney suggests that there may be a concentration mechanism (receptor) for DBP in these organs. So far, no receptor for DBP has been isolated, although DBP has been found to be associated with membranes of B-lymphocytes [21] and with a subpopulation of T-lymphocytes [22]. The significant accumulation of DBP in liver (Fig. 3a) and kidneys (Fig. 4a) may suggest the possibility that at least a proportion of vitamin D₃ and its metabolites may be taken up by these organs in association with DBP. Previously published data [23,24] may suggest that vitamin D₃ and its metabolites bound to

DBP are taken up in liver and kidneys; however, the mechanism by which vitamin D₃ and its metabolites are taken up by different tissues and cell types is not known.

The DBP-actin complex was cleared from the circulation more rapidly than was DBP (Fig. 2). These data are in agreement with results reported in the rabbit by Lind *et al.* [7] and Goldschmidt-Clermont *et al.* [8]. In contrast with these findings, Harper *et al.* [18] suggested that DBP and DBP-actin complex are cleared from plasma at similar rates. Our results indicate that ¹²⁵I-TC-DBP-actin cleared from plasma was recovered in the liver, and the rates of hepatic accumulation (Fig. 3a) and degradation (Fig. 3b) of DBP-actin complex were about 3 times higher than for DBP. Our results on liver and kidney accumulation of DBP, as well as liver accumulation of DBP-actin, are also different from the results reported by Harper *et al.* [18]. They recovered less than 2.5 % of the injected doses of both DBP and DBP-actin in liver and kidney 2 h after the injection. The difference between their results and ours concerning liver and kidney accumulation of DBP and DBP-actin complex may be due to different labelling of the proteins. We used the ¹²⁵I-TC technique described by Pittman *et al.* [12]. With this method the degraded material is trapped in the cells in liver (Fig. 3b) and kidney (Fig. 4b). With traditional labelling of protein with ¹²⁵I, as used by Harper *et al.* [18], the degraded material is released from the tissues, where the degradation takes place, into the circulation. The degraded products are then excreted in the urine. In fact, Harper *et al.* [18] reported that, 24 h after the injection of DBP, the major part of the radioactivity recovered was degraded material in the urine.

The rapid plasma clearance and hepatic uptake of the DBP-actin complex may explain why this complex is not detected in plasma under normal physiological conditions but is present in plasma of patients with acute hepatic necrosis [9]. The physiological significance of DBP-actin interaction *in vivo* remains unknown. Gelsolin is responsible for a major part of depolymerization activity in plasma [25]. DBP and actin bind in a molar ratio of 1:1, whereas gelsolin has two binding sites for actin. When monomeric or filamentous actin is injected intravenously, most of the actin recovered in plasma is associated with DBP [7,8]. This binding of actin to DBP *in vivo* is a rapid process [8], although the mechanism by which filamentous actin is bound to DBP in the circulation is unknown. An important function of DBP *in vivo* may be to inhibit the formation of filamentous actin in the circulation by sequestering monomeric actin, as well as to serve as a reservoir of binding capacity for monomeric actin depolymerized by gelsolin.

In conclusion, we have shown that DBP is taken up and degraded in liver and kidneys and that these organs contribute about equally to the catabolism of DBP *in vivo*. Binding of actin to DBP results in markedly enhanced rates of clearance from the circulation, liver uptake and degradation of the complex compared with DBP alone. The mechanism by which the DBP and

DBP-actin complex are taken up by liver remains to be elucidated.

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