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Preliminary Communication

Fatty acid-binding site environments of serum vitamin D-binding protein and albumin are different

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Abstract

Vitamin D-binding protein (DBP) and albumin (ALB) are abundant serum proteins and both possess high-affinity binding for saturated and unsaturated fatty acids. However, certain differences exist. We surmised that in cases where serum albumin level is low, DBP presumably can act as a transporter of fatty acids. To explore this possibility we synthesized several alkylating derivatives of ^{14}C-palmitic acid to probe the fatty acid-binding pockets of DBP and ALB. We observed that *N*-ethyl-5-phenylisooxazolium-3′-sulfonate-ester (WRK-ester) of ^{14}C-palmitic acid specifically labeled DBP; but *p*-nitrophenyl- and *N*-hydroxysuccinimidyl-esters failed to do so. However, *p*-nitrophenyl ester of ^{14}C-palmitic acid specifically labeled bovine ALB, indicating that the micro-environment of the fatty acid-binding domains of DBP and ALB may be different; and DBP may not replace ALB as a transporter of fatty acids.
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1. Introduction

Group specific component (Gc) or vitamin D-binding protein (DBP) is a sparsely glycosylated and polymorphic serum protein. The two major phenotypes are Gc1 and Gc2, differing from each other by four (4) amino acids in the primary structure as well as structure of attached polysaccharide. Gc1 is further divided into two subtypes differing in primary structure as well as structure of the attached carbohydrates [1–3].

DBP is a multi-functional protein [4]. Its binding of vitamin D and its metabolites has been studied extensively leading to the understanding that DBP is responsible for the stepwise activation of vitamin D_3 to 25-hydroxyvitamin D_3 (25-OH-D_3) and finally to its physiologically most active metabolite, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). It is also involved in the transportation of these small molecules to organs and cells wherever they are required. In addition DBP plays an integral role in the circulating actin-scavenging system in plasma. Plasma gelsolin severe filaments of F-actin, and DBP binds to actin monomer (G-actin) with high affinity, thus preventing G-actin to polymerize and clog arteries during cell-injury and lysis [5,6]. Presence of actin–DBP complex in the sera of human and animals sustaining injuries/inflammation, e.g. trophoblastic emboli, severe hepatitis, acute lung injury, etc. positively implicates DBP in thrombosis and heart attack [7]. DBP also binds chemotactic agents such as C5a and C5a des Arg, thus enhancing complement activation on neutrophil chemotaxis [8,9]. Furthermore, a post-translationally modified form of DBP (DBP-macrophage activating factor, DBP-*maf*) has been shown to have strong macrophage- and osteoclast-activating [10–14] and anti-angiogenic and anti-tumor properties [15,16].

In addition to above properties of DBP and its derivative (DBP-*maf*), DBP binds saturated and unsaturated fatty acids with high affinity ($K_d = 10^5$–$10^6\ M^{-1}$), similar to plasma ALB [17,18]. However, certain differences do

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exist. For example, Ena et al. demonstrated that molar ratio of fatty acids, bound to human DBP to DBP is 0.4 compared with 1.8 for human ALB [19]. Furthermore, majority of DBP-bound fatty acids are mono-unsaturated or saturated, and abundance of poly-unsaturated fatty acids is less than 5% (of the total bound fatty acids) [19]. Another interesting observation includes competition between vitamin D sterols and fatty acids in terms of binding to DBP. For example, it was reported that poly-unsaturated fatty acids, such as arachidonic or linoleic acid, strongly compete with 25-OH-D_3 and 1,25$(OH)_2D_3$ for binding to DBP, in sharp contrast with saturated fatty acids e.g. palmitic acid, which offer no significant competition [19,20]. Furthermore, Bouillon et al. observed that addition of human ALB in a physiological ALB:DBP ratio did not impair the inhibitory effect of linoleic acid towards DBP-25-OH-D_3-binding [20].

We hypothesized that this apparent anomaly between DBP and ALB in terms of fatty acid-binding might be related to the actual binding process between these proteins and fatty acids, which, in turn, might be related to the micro-environment of the fatty acid-binding pockets of these proteins. In order to evaluate this possibility we synthesized several reactive esters of ^{14}C-palmitic acid as potential affinity labeling reagents for DBP and ALB. Results of these studies and their probable physiological implications are discussed in this report.

2. Materials and methods

Purified human DBP was obtained from commercially available pooled human serum (American Red Cross, Dedham, MA) by a ligand affinity chromatographic method developed in our laboratory [21]. Defatted bovine serum ALB (BSA) and all chemicals were purchased from Sigma–Aldrich, Milwaukee, WI, except 1-^{14}C-palmitic acid (specific activity 56 mCi/mmol) which was a product of NEN-DuPont, Boston, MA.

2.1. Synthesis (Fig. 1)

The *N*-hydroxysuccinimido- and *p*-nitrophenyl-esters of palmitic acid were synthesized by dicyclohexylcarbodiimide (DCC)-coupling of palmitic acid with *N*-hydroxysuccinimide, or *p*-nitrophenol in the presence of a catalytic amount of *N*,*N*′-dimethylaminopryridine (DMAP) in anhydrous dichloromethane. Synthesis of WRK-palmitate was carried out by treating palmitic acid with *N*-ethyl-5-phenyl-isooxazolium-3′-sulfonate (Woodward's reagent K) and triethylamine in acetonitrile. Product from each reaction was purified by preparative chromatography on silica plates (Analtech, Vineland, NJ), and each product was characterized by NMR. Radioactive synthesis was carried out exactly the same way except palmitic acid was replaced with ^{14}C-palmitic acid. Products from the radioactive reaction were isolated by TLC matching with corresponding unlabeled compounds.

2.2. Affinity labeling studies of bovine serum ALB and DBP with N-hydroxy-succinimido-^{14}C-palmitate (A), p-nitrophenyl-^{14}C-palmitate (B), and WRK-^{14}C-palmitate (C)

Twenty-microgram samples each of BSA and DBP in 20 μl of TEST buffer (50 mM Tris–HCL, 150 mM NaCl, 1.5 mM EDTA, 0.1% Triton X-100, pH 8.8) were treated with *N*-hydroxysuccinimdo-^{14}C-palmitate (A), *p*-nitrophenyl-^{14}C-palmitate (B), or WRK-^{14}C-palmitate (C) (each 20,000 cpm) at 25 °C for 20 h. Parallel samples of BSA and DBP containing additional sodium palmitate (1 μg in 10 μl of buffer) were also treated the same way. At the end of the experiment all the samples were analyzed on a 7.5% SDS–polycarylamide gel, followed by drying the gel and scanning of radioactivity in a Biosan phosphorimager.

3. Results and discussion

There is a remarkable structural homology among ALB, DBP, α-feto protein (AFP) and afamin, members of the albumin gene family. All these proteins have modular structures with three domains (domains I–III) and high cysteine-content [22]. In the case of DBP all the Cys residues (total 28) are oxidized to form 14 disulfide bonds. In contrast, ALB contains several free sulphydryl groups in its primary structure. Furthermore, DBP has a shorter domain III than ALB. These structural differences may explain gross functional differences between DBP and ALB. For instance, vitamin D sterols- and G-actin binding and related functions are unique to DBP. On the other hand, DBP possesses relatively weaker binding for fatty acids compared with ALB. Furthermore, DBP contains a single high-affinity fatty acid-binding site compared to ALB which contains several low- and high-affinity binding sites [18]. In ALB these binding sites are distributed among various domains of the protein, although high-affinity-binding sites are located in domain III [23]. Moreover, as described earlier, DBP, in contrast with ALB, discriminates between saturated and unsaturated fatty acids in terms of binding.

All the above observations point to difference in the nature of binding between ALB and DBP and fatty acids, which in turn may be related to the fatty acid-binding pocket structure of these proteins. Affinity and photo-affinity labeling techniques have been used widely to probe binding pockets and catalytic active sites of receptors and enzymes, respectively [24]. Our laboratory has used these techniques, and others to probe the vitamin D and actin-binding domain structures of DBP, leading to crystal structure of the DBP–actin complex [25–35].

In the current study we synthesized radiolabeled versions of three reactive esters of palmitic acid to probe the fatty acid-binding pockets of DBP and ALB. We chose palmitic acid, a saturated fatty acid as model because DBP has a propensity to bind saturated and mono-unsaturated fatty acids stronger that poly-unsaturated fatty acids [19,20].

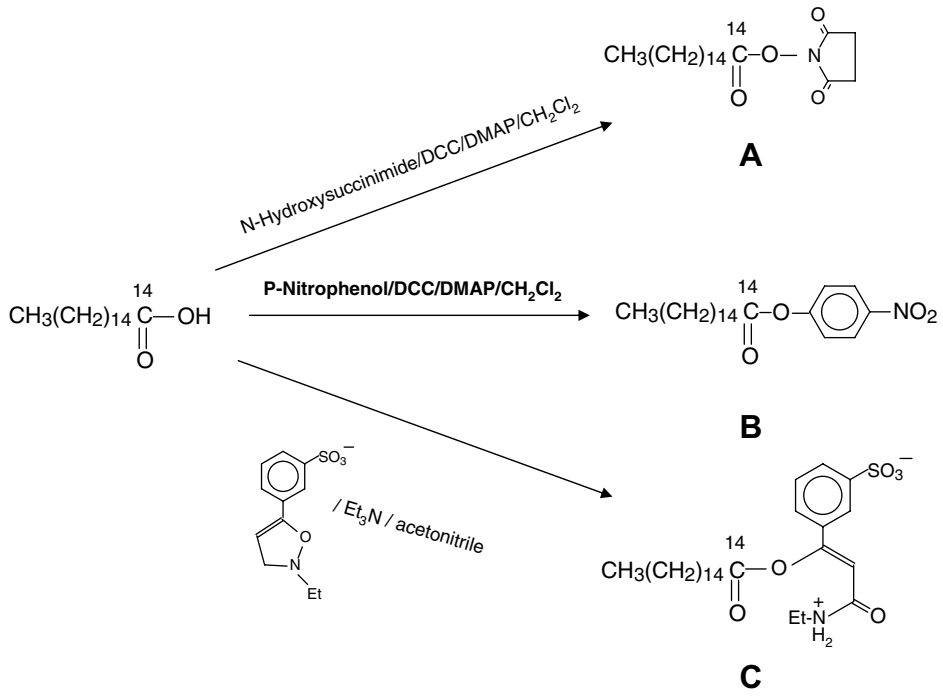

Fig. 1. Scheme for the synthesis of *N*-hydroxy-succinimido-^{14}C-palmitate (A), *p*-nitrophenyl-^{14}C-palmitate (B), and WRK-^{14}C-palmitate (C).

Reed employed WRK-^{14}C-palmitate (C) to affinity label the fatty acid-binding pocket/s of bovine serum ALB [36]. In our case, incubation of a sample of human serum DBP (hDBP) with WRK-^{14}C-palmitate (C) covalently labeled the protein as determined by autoradiography (Fig. 2, lane 1). When the incubation was carried out in the presence of an excess of sodium palmitate, labeling was completely obliterated (Fig. 2, lane 2). These results strongly indicated that WRK-^{14}C-palmitate (C) specifically labeled the palmitic acid-binding pocket in hDBP. These results also suggested that structure and chemical environment of the fatty acid-binding pockets of DBP and ALB are similar.

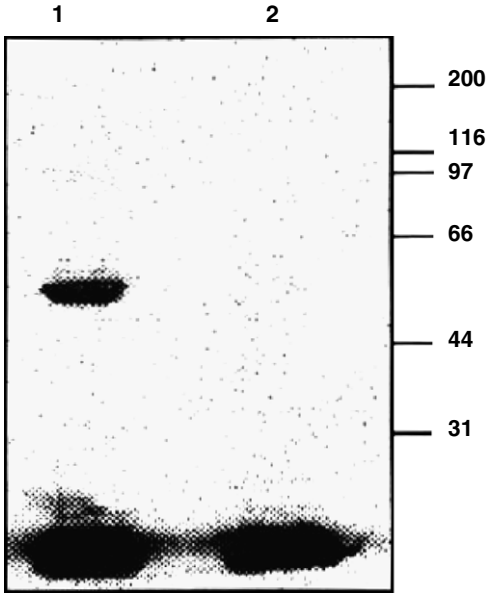

Fig. 2. Affinity labeling of hDBP with WRK-^{14}C-palmitate (C): samples of hDBP were incubated at 25 °C with WRK-^{14}C-palmitate (C) alone (lane 1), or in the presence of an excess of sodium palmitate (lane 2). The samples were electroporesed on a SDS gel and exposed to a phosphorimager. Positions of the standard molecular weight markers are denoted on the right.

Surprisingly other activated esters of palmitic acid i.e. *N*-hydroxysuccinimidyl-^{14}C-palmitate (A) and *p*-nitrophenyl-^{14}C-palmitate (B) failed to label DBP in the presence or in the absence of an excess of sodium palmitate. In the case of BSA, *N*-hydroxysuccinimidyl-^{14}C-palmitate (A) failed to label this protein. But, *p*-nitrophenyl-^{14}C-palmitate (B) labeled BSA, and labeling was significantly reduced in the presence of an excess of palmitic acid, denoting specific labeling of the fatty acid-binding pocket (results not shown).

Collectively the above results suggest that chemical/electronic environments of the fatty acid-binding pockets of DBP and ALB are different, so that ALB can tolerate a hydrophobic (*p*-nitrophenyl) as well as a hydrophilic (Woodward K reagent) head group at the carboxy terminal of palmitic acid. But, fatty acid-binding site of DBP can only accommodate a polar and Zwitterionic head group (Woodward K reagent).

Analbuminemia is a rare hereditary disease in which the afflicted individuals have very low or negligible amount of circulating serum ALB [37–39]. We surmised that since both ALB and DBP bind fatty acids with high affinity, DBP may replace ALB in carrying fatty acids, particularly saturated and mono-unsaturated fatty acids in the cases of low or negligible amount of circulating ALB. However,

results of the study delineated in this communication suggest that chemical and electronic environment of the fatty acid-binding pockets of DBP and ALB might be different. As a result binding and transportation of various fatty acids might be different. Thus, DBP may not replace ALB in terms of fatty acid scavenging and transportation.

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