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Apolipoprotein B binds ferritin by hemin-mediated binding: evidence of direct binding of apolipoprotein B and ferritin to hemin

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Abstract Apolipoprotein B (apoB) is known to be a ferritin-binding protein. Here we show that apoB binds to ferritin through hemin-mediated binding. Human apoB bound to bovine spleen, horse spleen, and canine liver ferritins, but did not bind to bovine apoferritin, even after incorporation of iron into it. Incubation of apoferritin with hemin resulted in apoB binding with apoferritin at the same level as with holoferritin. In contrast, hemin inhibited binding of apoB to ferritin. Bovine spleen apoferritin bound biotinylated hemin, and hemin inhibited the binding between the apoferritin and biotinylated hemin, suggesting that ferritin binds hemin directly. ApoB and LDL containing apoB bound biotinylated hemin, and their bindings were also inhibited by hemin, but not protoporphyrin IX. These data demonstrate that binding of apoB to ferritin is mediated through ferritin's binding to hemin, and also that apoB binds hemin directly.

Keywords Apolipoprotein B · Apoferritin · Ferritin · Hemin · LDL

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Introduction

Apolipoprotein B (apoB) is the principal apolipoprotein constituent of chylomicrons, very low density lipoproteins (VLDL), and low-density lipoproteins (LDL) and plays an important role in the transport and metabolism of plasma triacylglycerol (TG) and cholesterol (Kane et al. 1980; Chan 1992; Segrest et al. 2001). ApoB-100 is a large, hydrophobic protein with molecular mass of 512 kDa, is primarily synthesized by mammalian livers and secreted with cholesterol and TG in VLDL, the precursor of LDL (Law et al. 1986; Segrest et al. 2001). ApoB-48, the N-terminal 48% of ApoB-100, is expressed via a posttranscriptional modification of the apoB-100 mRNA and is essential for the formation and secretion of chylomicrons (Chan 1992). In humans, apoB circulates in various forms, apoB-100 of chylomicrons, VLDL, and LDL, apoB-74 and apoB-26 of LDL, and apoB-48 of chylomicrons (Kane et al. 1980).

Ferritin, an iron-binding protein, is a ubiquitous protein in all living organisms, from microorganisms to mammals (Theil 1987; Andrews et al. 1992). It is a 24-mer composed of heart (H) and liver (L) subunits, with molecular masses of 21 and 19 kDa, respectively (Theil 1987; Andrews et al. 1992). In mammals, ferritin circulates in relatively low concentration (1 μg ml^{-1}) as well as tissues (Addison et al. 1972). Ferritin is identified as a

fusion protein that binds apoB directly (Rashid et al. 2002) and can post-translationally down-regulate apoB secretion (Hevi and Chuck 2003). LDL has been reported to recognize hemin probably through apoB (Camejo et al. 1998). In atherosclerotic lesions, the ferritin gene is expressed, indicating that the iron-binding protein, ferritin, plays a role in the pathogenic effects of the disease (Pang et al. 1996). This data provides insight into the metabolic relationships between iron storage and transport of TG and cholesterol.

Bacterial ferritins are clearly different in their heme-binding sites from those of mammalian ferritins (Kadir and Moore 1990). However, it has been demonstrated that horse spleen apoferritin possesses a hemin-binding site for demetallating hemin and metallating protoporphyrin IX (Crichton et al. 1997), suggesting that mammalian ferritins bind heme similar to bacterial ferritins.

In this study, we demonstrate that ferritin binds directly hemin, and that apoB binds ferritin by hemin-mediated binding.

Materials and methods

Chemicals

Human apoB, hemin (ferriprotoporphyrin IX chloride), and horse spleen ferritin were purchased from Sigma, St. Louis, MO, USA. Protoporphyrin IX was from Frontier Scientific Inc., Logan, UT, USA. NeutrAvidin with alkaline phosphatase (ALP) conjugated, EX-Link biotin-PEO_2-amine, and 1-ethyl–3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were from Pierce Chemical Company and Hyclone Inc., Rockford, IL, USA. Goat anti-human apoB–100 polyclonal antibody was from Academy Bio-Medical Company Inc., Houston, TX, USA. ALP labeled-rabbit anti-goat IgG(Fc) antibody was from American Qualex International Inc., Calle Negocio San Clemente, CA, USA. Immuno Plate Maxisorp F96 was from Nunc, Roskilde, Denmark. Bovine serum albumin was from Roche Diagnostics, Mannleria, Germany. Avidin was from EY Laboratories Inc., San Mateo, CA, USA. Sephadex G10 was from Pharmacia, Uppsala, Sweden. Microcon YM–100 was from Millipore Corporation, Bedford, MA, USA. BlockAce was from Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan. Other reagents used were of analytical grade.

Ferritin and apoferritin preparation

Horse spleen ferritin monomers were purified from commercial horse spleen ferritin as described previously (Orino et al. 1993). Bovine spleen and canine liver ferritin monomers were purified from pieces of frozen bovine spleen and canine liver, respectively, as described previously (Kakuta et al. 1997; Orino et al. 2004, 2006). Bovine spleen apoferritin was prepared by dialysis with 100 mM thioglycollic acid in 100 mM acetate buffer (pH 5.5) followed by phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM sodium phosphate, pH 7.2).

Antibodies

Purified antibodies to bovine spleen ferritin were obtained by affinity chromatography of rabbit antisera obtained by immunization of rabbits with ferritin (Kakuta et al. 1997). ALP labeling of antibodies to ferritin was performed according to the method of Avrameas (1969).

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Biotinylated hemin

A solution of hemin was freshly prepared by dissolving 5 mg of hemin in a minimum amount of 0.1 M NaOH. Hemin was biotinylated according to the manufacturer's instructions with soluble EZ-Link Biotin-PEO_2-amine at a molar ratio of EZ-Link Biotin-PEO_2-amine to hemin of 20:1 with EDC as cross-linking reagent. Biotinylated hemin was purified using a Sephadex G10 column (2×35 cm^2) equilibrated with PBS at a flow rate of 11 ml h^{-1}. Hemin concentration was determined using its molar extinction coefficient at 385 nm (εmM = 58.4) (Jung et al. 1997).

Incubation of apoferritin with iron and hemin

One ml of bovine spleen apoferritin (5 μg) in PBS was incubated with ferrous ammonium sulfate (FAS) and hemin at molar ratios of 5,000:1 and 20:1, respectively, to the apoferritin. The mixture was incubated at 37°C for 1 h, and then immediately centrifuged for ultrafiltration using Microcon YM–100 at 14,000 × *g* for 3 min. PBS (0.5 ml) was added to the upper solution, and the tube was re-centrifuged at the same speed. The reservoir was reversed in a new tube and centrifuged at 3,600 × *g* for 3 min. The resulting concentrated sample was adjusted to the initial volume with PBS. As control samples, bovine spleen ferritin and its apoferritin underwent the same treatment. These ferritin samples were used in the binding assay with human apoB as described below.

Binding of apoB and LDL to ferritins and biotinylated hemin

Human apoB and LDL was dissolved in 100 mM Na_2CO_3 (pH 10.0) containing 50 mM NaCl and 10 mM sodium deoxycholate (A buffer) and 0.27 mM EDTA (pH 7.5) containing 150 mM NaCl (B buffer), respectively. 100 μl aliquots of ferritins from bovine spleen, canine liver, or horse spleen (each 1 μg ml^{-1}) in PBS and of human apoB (1 μg ml^{-1}) and LDL (2 μg ml^{-1}) in A and B buffer, respectively, were added to the wells of a Maxisorp F96 microtiter plate, and the plate was kept overnight at 4°C. The protein-coated plate was washed with PBS containing 0.5% Tween 20 (the wash solution), and then masked by incubating with PBS containing 0.1% Tween 20 and 0.1% gelatin (C buffer) or 25% (v/v) BlockAce. After washing, 100 μl aliquots of human apoB (5 μg ml^{-1}), ferritin samples (5 μg ml^{-1}), or biotinylated hemin (47 ng ml^{-1}) in A buffer, PBS, or C buffer, respectively, was added to each well of the plate, and the plate was kept at 37°C for 2 h. After washing, in order to detect the amount of apoB and LDL bound to wells, 100 μl of goat anti-human apoB antibody (1 μg ml^{-1}) in C buffer was added to each well, and the plate was kept at 37°C for 2 h. After washing, 100 μl of ALP-conjugated rabbit anti-goat IgG(Fc) antibody (1 μg ml^{-1}) in C buffer was then added to each well of the plate, and the plate was kept at 37°C for 2 h. For the detection of the amount of ferritin and biotinylated hemin bound to wells, after washing, 100 μl of ALP-conjugated rabbit anti-bovine spleen ferritin antibody (250 ng ml^{-1}) or NeutrAvidin conjugated ALP (2 μg ml^{-1}) in C buffer was added to each well of the plate, and the plate was kept at 37°C for 2 h. The ALP enzyme detection reaction was performed using *p*-nitrophenyl phosphate as described previously (Orino et al. 1993). For the inhibition test, hemin was added such that the resulting solution of ferritin and biotinylated hemin was 2.1–21 μM in NaOH concentration. Protoporphyrin IX was also added such that the resulting solution was 5.7 mM in HC1 concentration.

Direct binding of ferritins with biotinylated hemin

One hundred microliters of avidin (10 μg ml^{-1}) in PBS were added to the wells of a Maxisorp F96 microtiter plate, and the plate was kept overnight at 4°C. The avidin-coated plate was washed with the wash solution, and then masked by incubating with C buffer. After washing, 100 μl aliquots of bovine spleen apoferritin (5 μg ml^{-1}) in PBS, which was incubated with hemin at molar ratios of 20:1 at 37°C for 1 h, was added to each well of the plate, and the plate was kept at 37°C for 2 h. After washing, in order to detect the amount of ferritin bound to wells, 100 μl of ALP-conjugated rabbit anti-bovine spleen ferritin antibody (250 ng ml^{-1}) in C buffer, and the plate was kept at 37°C for 2 h. After washing, The ALP enzyme detection reaction was performed using *p*-nitrophenyl phosphate as described above. For the inhibition test, hemin was added such that the resulting solution of ferritin and biotinylated hemin was 160 μM in NaOH concentration.

Results

Binding of apoB to ferritin

Binding of human apoB to ferritins was conducted on the wells of microtiter plates coated with non-human mammalian ferritins. Human apoB bound strongly to ferritins from bovine spleen, horse

spleen, and canine liver (Fig. 1). In addition, when the plate was coated with apoB, bovine spleen ferritin bound strongly to the apoB-coated plate, as verified by detection with ALP-conjugated anti-bovine spleen antibody (Fig. 2). Both horse spleen ferritin and liver ferritin also bound strongly to the apoB-coated plate, as verified by detection with the corresponding anti-ferritin antibody conjugated with ALP (Orino et al. 2004, 2006) (data not shown). However, apoB did not bind significantly to bovine spleen apoferritin; little improvement in binding activity was observed after iron incorporation into the apoferritin (Fig. 2). Sandwich ELISA standard curves of bovine spleen ferritin and its apoferritin with anti-bovine spleen antibody and its ALP conjugate showed the same dose response (data not shown), suggesting that the antigenicity of apoferritin was the same as holoferritin irrespective of the presence of iron. These results indicated that the human apoB protein itself did not recognize ferritin. Horse spleen apoferritin has been reported to bind hemin

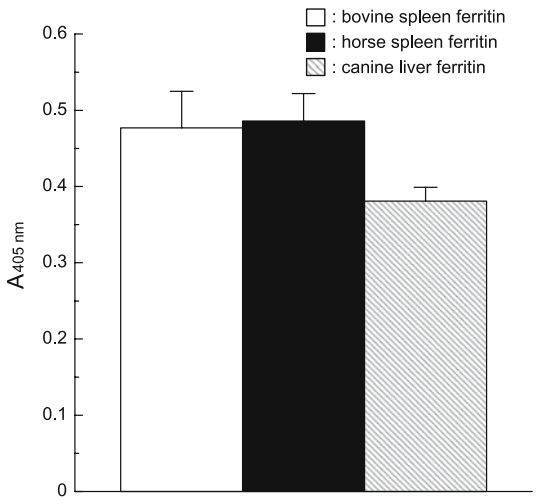

Fig. 1 Binding of human apoB to bovine spleen, horse spleen, and canine liver ferritins was conducted on ferritin-coated plate (each 100 ng per well) followed by detection with goat anti-human apoB–100 antibody and ALP-labeled rabbit anti-goat IgG(Fc) antibody. A 100 µl of human apoB (5 µg ml^{-1}) was added to microtiter plate wells coated ferritin from bovine spleen (*open bar*), horse spleen (*solid bar*), and canine liver (*hatched bar*). The apoB bound to the well was detected by goat anti-human apoB antibody followed by detection with ALP-labeled rabbit anti-goat IgG(Fc) antibody. Each *bar* represents the mean ± SD of four determinations

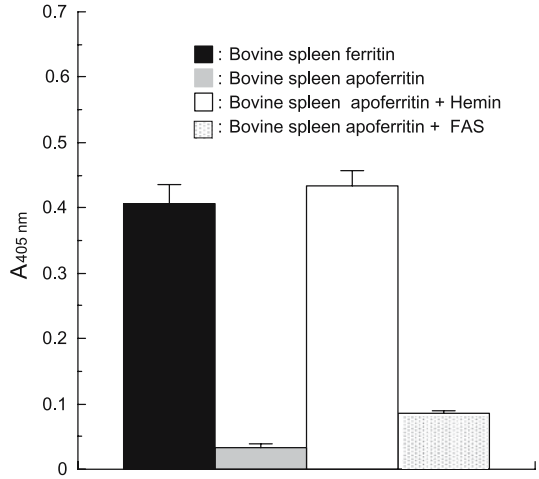

Fig. 2 Hemin-dependent binding of human apoB to bovine spleen apoferritin. A 0.5 ml-bovine spleen apoferritin (5 µg ml^{-1}) was incubated with FAS and hemin with molar ratios of 5,000:1 and 20:1 to bovine spleen apoferritin, respectively, at 37°C for 60 min. FAS and hemin were immediately removed by ultrafiltraion through centrifugation with Microcon YM–100 at 14,000 × *g*, and further ulrtrafilatration after addition of 0.5 ml PBS. Finally, concentrated ferritin samples were obtained, and adjusted to initial volume with PBS. Bovine spleen ferritin and its apoferritin were prepared by same treatment. A 100 µl of aliquots of these ferritin samples were added to each well of human apoB-coated plate (100 ng per well) followed by detection with 100 µl of ALP labeled anti-bovine spleen ferritin antibody (250 ng ml^{-1}). Each *bar* represents the mean ± SD of four determinations

(Crichton et al. 1997), suggesting that mammalian ferritins contain a heme-binding site at physiological conditions. We successfully induced binding of apoB to apoferritin by incubating apoferritin with hemin. Such incubation resulted in apoB binding to apoferritin at the same level as the binding between apoB and holoferritin.

Binding of bovine apoferritin with biotinylated hemin

Biotinylated hemin is applied to detect the specific interaction of hemoprotein with heme as described previously (Ishida et al. 2003). To examine whether apoferritin binds hemin directly, we prepared biotinylated hemin and its concentration was determined by conversion using hemin's molar extinction coefficient. Bovine spleen apoferritin was incubated with biotinylated

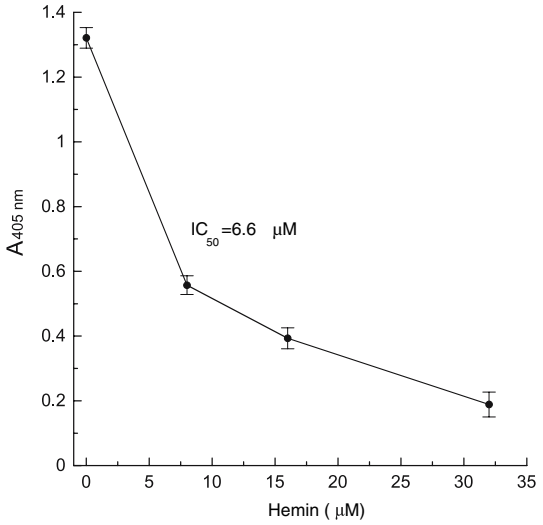

Fig. 3 Inhibitory effect of hemin on binding of bovine spleen apoferritin to biotinylated hemin. A 1.0 ml-bovine spleen apoferritin (5 μg ml^{-1}) was incubated with biotinylated hemin with molar ratios of 20:1 to bovine spleen apoferritin at 37°C for 60 min. A 100 μl of aliquots of the apoferritin sample were added to each well of avidin-coated plate (1 μg per well) with various concentrations of hemin adjusted to be 160 μM of final concentration of NaOH. The detection of ferritin was performed in as described in Fig. 2. Each *bar* represents the mean ± SD of four determinations

hemin at molar ratios of 20:1 to the apoferritin. Figure 3 shows the direct binding of the apoferritin with biotinylated hemin using avidin-coated plate and ALP labeled anti-bovine spleen ferritin antibody. Hemin dose-dependently inhibits the binding between two molecules with an inhibitory concentration 50 (IC_{50}) of 6.6 μM.

Inhibitory effects of hemin and protoporphyrin IX on binding of apoB and LDL to ferritin

Figure 4A shows that hemin inhibited binding of human apoB to bovine spleen ferritin in a dose-dependent manner with IC_{50} of 60 nM. Hemin also similarly inhibited the binding of apoB to horse spleen and canine liver ferritins (data not shown). These results support the hypothesis that apoB reacted with heme located on the surface of ferritin, rather than the amino acids of ferritin itself. In order to examine direct binding of apoB with ferritin in the circulation, human LDL

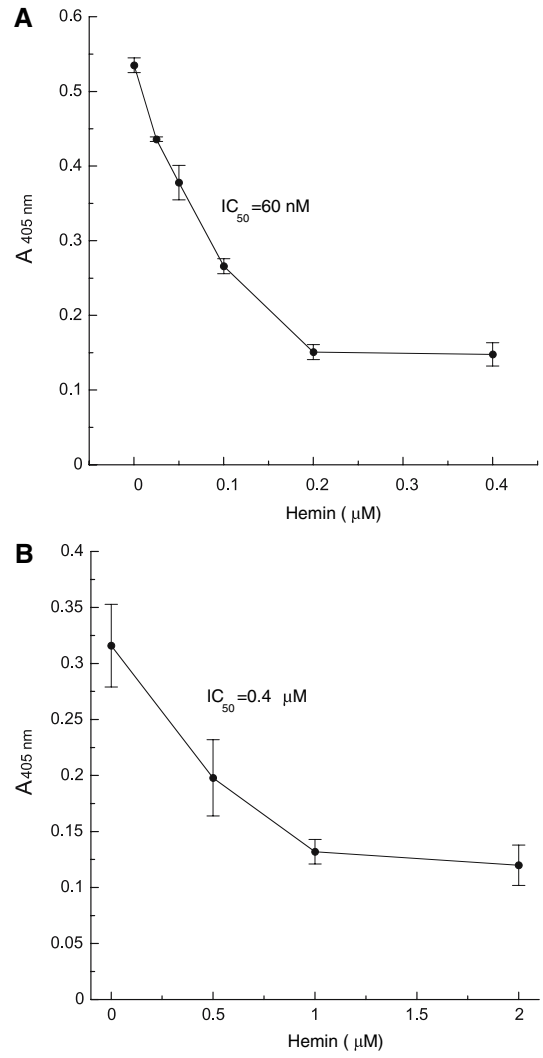

Fig. 4 Inhibitory effect of hemin on binding of human apoB (**A**) and LDL (**B**) to bovine spleen ferritin. A 100 μl aliquots of bovine spleen ferritin (5 μg ml^{-1}) were added to each well of apoB-coated plate (100 ng per well) and LDL-coated plate (200 ng per well) with various concentrations of hemin adjusted to be 2.1 and 11 μM of final concentration of NaOH, respectively. The detection of ferritin was performed in as described in Fig. 2. Each *bar* represents the mean ± SD of four determinations

containing apoB was used. LDL binds bovine spleen ferritin and binding between two molecules was inhibited by hemin with IC_{50} of 0.4 μM (Fig. 4B). In addition, as shown in Fig. 5, human apoB bound biotinylated hemin, while the presence of hemin showed a dose-dependent inhibitory effect on binding between apoB and biotinylated hemin. To achieve 50% inhibition

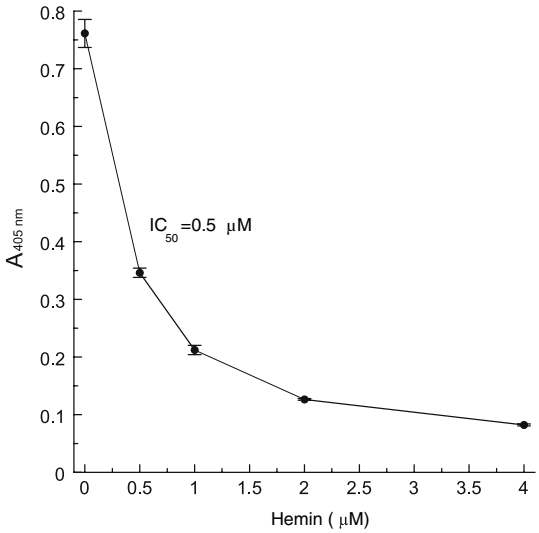

Fig. 5 Inhibitory effect of hemin on binding of human apoB to biotinylated hemin. A 100 μl aliquots of biotinylated hemin (47 nM) were added to each well of apoB-coated plate (100 ng per well) with various concentrations of hemin adjusted to be 21 μM of final concentration of NaOH. After incubation, biotinylated hemin bound to the well was detected by ALP-conjugated NeutrAvidin (2 μg ml^{-1}). Each *bar* represents the mean ± SD of four determinations

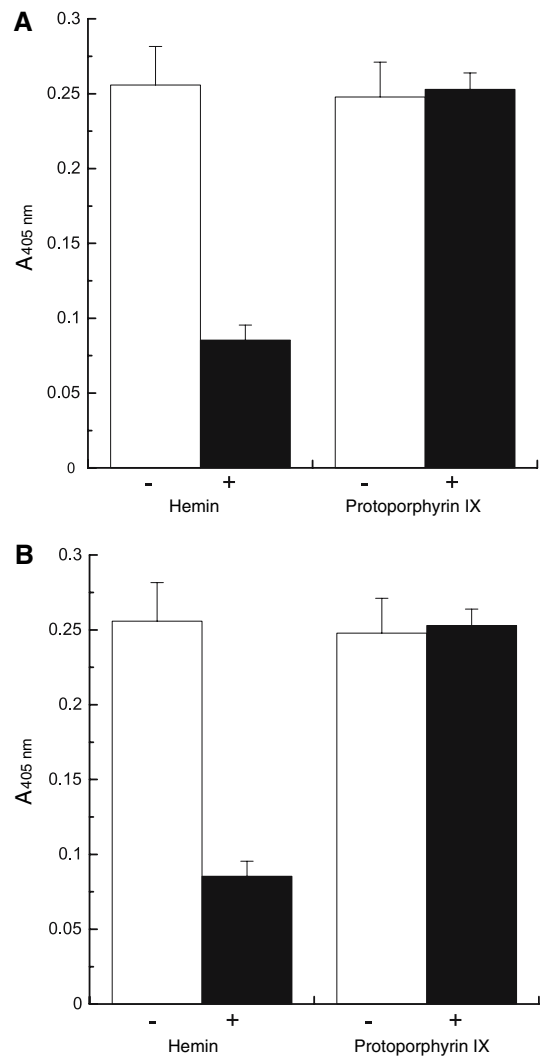

Fig. 6 Inhibitory effect of hemin and protoporphyrin IX of human apoB (**A**) and LDL (**B**) to biotinylated hemin. A 100 μl aliquots of biotinylated hemin (47 nM) were added to each well of apoB- or LDL-coated plate (apoB: 100 ng per well: LDL: 200 ng per well) with hemin (1 μg ml^{-1}) or protoporphyrin IX (1 μg ml^{-1}) adjusted to be 8.6 μM and 5.7 mM of final concentration of NaOH and HCl, respectively. After incubation, biotinylated hemin bound to the well was detected by ALP-conjugated NeutrAvidin (2 μg ml^{-1}). Each *bar* represents the mean ± SD of four determinations

against binding of biotinylated hemin (4.7 pmol per well) to human apoB (0.2 pmol per well), a molar ratio of 23.5:1, i.e., IC_{50} of 0.5 μM of hemin, was required. This inhibitory concentration of hemin to the binding of biotinylated hemin to human apoB was eight times higher in concentration than the corresponding inhibitory concentration of hemin to the binding of ferritin to apoB.

Although protoporphyrin IX is iron-free hemin, protoporphyrin IX did not inhibit the binding of apoB and LDL to biotinylated hemin (Fig. 6A, B).

Discussion

Rat ferritin has been identified as an apoB-binding protein (Rashid et al. 2002), and ferritin suppresses secretion of apoB (Hevi and Chuck 2003). Although our study here shows that human apoB binds to mammalian ferritins, apoB seems to bind directly to hemin but not to ferritin. ApoB did not bind significantly to bovine spleen apoferritin, and even after incorporation of iron into the apoferritin, little binding activity was observed. However, hemin-treated bovine spleen apoferritin showed binding activity at the same level as that of holoferritin after the removal of hemin by ultrafiltration because the unbound hemin caused

inhibition of hemin-mediated binding. Furthermore, binding of apoB to mammalian ferritins was inhibited by hemin in a dose-dependent manner. Therefore, we propose that apoB binds ferritin mediated through binding to hemin rather than through direct interaction between apoB and the amino acid structure of ferritin. Interestingly, it was reported in one study that horse spleen apoferritin binds hemin in order to demetallate the hemin and metallate protoporphyrin IX using the iron released from hemin (Crichton et al. 1997). The apoferritin binds with a stochiometry of 1 hemin molecule/2 subunits (Crichton et al. 1997). This study showed the direct binding between bovine spleen apoferritin and biotinylated hemin at 20 molar ratio of 20:1 to the apoferritin (1 hemin molecule/1 subunit), and binding between the two molecules was inhibited by hemin in a dose-dependent fashion. However, in this study, the binding between ferritin and biotinylated hemin can be detected even though ferritin binds one biotiniylated hemin. Taken together, these results suggest that purified ferritins retain heme-binding activity during purification steps, including heat and acid treatments (Kakuta et al. 1997), and that the reduction treatment of ferritin under acid conditions also causes the removal of heme as well as iron. In conclusion, naturally occurring mammalian ferritins bind heme at physiological conditions although biological and physiological significance of heme-binding ferritin remains to be clarified.

Ferritin-binding proteins circulate in the form of ferritin complexes in mammals and a few have been identified: H-kininogen (Torti and Torti 1998), alpha–2-macroglobulin (Santambrogio and Massover 1989; Massover 1994), anti-ferritin autoantibodies (Orino et al. 2004, 2006), and fibrinogen (Orino et al. 1993). Although the ferritin-binding sites of H-kininogen (Parthasarathy et al. 2002) as well as canine anti-ferritin autoantibodies (IgM) (Orino et al. 2006) have been demonstrated, the presence of ferritin-binding sites or heme-binding sites in other ferritin-binding proteins has not been clarified yet. On the other hand, there are many reports in the literature on heme-binding proteins, including novel p22 HBP (Taketani et al. 1998), HBP23 (Iwahara et al. 1995), and HBP.93 (Tsutsui and Mueller 1982) as well as well-known globins (Rossmann and Argos 1975), cytochromes (Rossmann and Argos 1975), glutathione *S*-transferase (Harvey and Beutler 1982), and hemopexin (Delanghe and Langlois 2001) in mammalian circulation and tissues. However, to our knowledge, there is no report on a gross heme-binding protein like apoB. ApoB circulates in several forms (apoB–100, apoB–74, apoB–48, and apoB–26)(Kane et al. 1980). Although we assume that apoB has multiple binding sites based on the binding of biotinylated hemin to apoB at a molar ratio of 23.5:1, further study is needed to clarify the binding site of heme for apoB. In addition, apoB and LDL bound hemin, but not protoporphyrin IX (iron-free hemin), and it was proposed that that apoB recognize a subtle structural difference between hemin and protoporphyrin IX, but being inconsistent with previous report (Camejo et al. 1998). This difference in conditions of buffer in binding of apoB to protoporphyrin IX may be due to why apoB recognizes iron-free hemin (protoporphyrin IX).

The binding activity between ferritin and biotinylated hemin was highest because the concentration of hemin required for 50% binding inhibition was markedly high as compared with the binding activities between biotinylated hemin and apoB or ferritin, suggesting that ferritin binds hemin strongly. The binding activity between ferritin and apoB was remarkably lower than that between biotinylated hemin and apoB, based on the concentration of hemin required for 50% binding inhibition. Ferritin's heme-binding site may be on the surface of the ferritin molecule, but apoB itself apparently also can recognize the part of hemin that binds to the ferritin molecule. Ferrous iron released from ferritin can cause oxidative stress by generation of hydroxyl radicals thorough a Fenton reaction, which leads to DNA strand breaks, enzyme inactivation, and initiation of lipid peroxidation (McCord 1996). LDL also bound ferritin mediated through hemin-binding, and some LDL containing apoB may circulate in the form of a complex with either ferritin or heme (Camejo et al. 1998). The oxidation of LDL by iron or hemin is taken up by the receptors of macrophage scavengers observed in early atherosclerotic lesions (Camejo et al. 1998; Mertens and

Holvoet 2001). Although ferritin circulates in relatively low concentration, this ferritin is rapidly cleared from circulation, probably by the reticuloendothelial system (Worwood et al. 1982; Orino et al. 2004, 2006). Ferritin gene expression is enhanced in atherosclerotic areas (Pang et al. 1996), suggesting that iron or heme is incorporated after complex formation between LDL with ferritin or heme itself and induces ferritin gene expression (Klausner and Harford 1989; Iwasaki et al. 2006). Iron metabolism may be involved in transport of TG and cholesterol by apoB in normal and abnormal lipid metabolism.

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