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Biliary Anionic Peptide Fraction/Calcium Binding Protein Inhibits Apolipoprotein A-I-Mediated Cholesterol Efflux from Cultured Cells

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The ABC transporter ABCA1 has been implicated to control cholesterol efflux in a variety of cell types including macrophages, fibroblasts, and intestinal epithelial cells. In this study we have investigated whether the 6-kD protein anionic peptide fraction/calcium binding protein (APF/CBP) which has homology to apolipoprotein AI may regulate efflux mediated by lipoproteins. APF/CBP was purified from T-tube bile by ultracentrifugation and preparative reversed phase HPLC. Cholesterol efflux to a variety of acceptors was determined using cultured fibroblasts from controls and patients with Tangiers disease. APF/CBP (0.1 to 2.4 μg/ml) inhibited ApoA-1 (2 μg/ml) mediated cholesterol efflux from normal fibroblasts in a dose dependent manner but had no effect on aspecific efflux to methyl-β-cyclodextrin or phosphatidylcholine liposomes. In ABCA1 deficient fibroblasts no effect of APF/CBP on efflux was seen. We conclude that APF/CBP specifically interferes with ApoA-I mediated cholesterol trafficking. We hypothesize that competitive binding to ABCA1 may explain the decreased ApoA-I mediated efflux from fibroblasts.

Recent studies have suggested that the ABC transporter ABCA1 plays a role in regulation of cholesterol efflux from peripheral cells (1–5). The protein has also been implicated in regulation of intestinal cholesterol uptake (6). Expression of the protein is strictly controlled by the nuclear receptor heterodimer LXR/RXR (6). Whether ABCA1 mediated cholesterol efflux can also be controlled at the lumenal side of the cell has not yet been studied. Activity of ABCA1 seems linked to the presence of apolipoprotein A-I, hence homologous proteins may be good candidates for such a regulatory function. Anionic Peptide Fraction/Calcium Binding Protein (APF/CBP) is a small anionic protein which has been shown to be present in serum and bile (7–10). The protein is present in cholesterol and pigment gallstones where it constitutes part of the protein matrix (11). The physiological relevance of the protein has not yet been elucidated. Interestingly APF/CBP shares epitopes with the N- and C-terminal parts of apolipoprotein A1 (9) and thus could interfere with ApoA-1 function. In this study we investigated whether APF/CBP indeed is able to influence cholesterol efflux from cultured cells. Fibroblasts with and without activity of ABCA1 were used as a model system.

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MATERIALS AND METHODS

Materials. ApoA-I and HDL were purchased from Calbiochem (La Jolla, CA). Fatty acid free BSA, Hepes, cholesterol, and phosphatidylcholine (egg yolk) were from Sigma (St. Louis, MO). [1,2-^{3}H]cholesterol (40–50 Ci/mmol) was purchased from Amersham (Little Chalfont, UK). Methyl-β-cyclodextrin was obtained from Cyclodextrin Techn. Dev. Inc. (Gainesville, FL).

Isolation of APF/CBP. APF/CBP was isolated from human hepatic bile and tested for purity as described previously modified as follows (12). In the last step of the procedure the protein was fractionated on a semipreparative HPLC column (Val-U-Pak, Regis, IL). The mobile phase consisted of (A) H_2O and (B) 100% acetonitril. The following gradient was used: 1% B till 11% B in 20 min. Thereafter in 1 min from 11% B to 21% B. The last step was 21% B till 35% B in 24 min. Subsequently, the column was washed during 5 min with 100% B and thereafter equilibrated with 1% B for 10 min prior to the next injection. The flow was 2 ml/min. The peak fractions were collected and lyophilized. After reconstitution activity of the protein was assessed in a calcium phosphate crystallization assay as described by Afdahl *et al.* (13). Purity of the protein was checked on SDS–polyacrylamide gelelectrophoresis as described previously (12).

Cell culture. Normal and ABCA1 deficient human skin fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM; BioWhittaker, Verviers, Belgium) supplemented with penicillin/streptomycin and 10% fetal calf serum (BioWhittaker) in a humidified 10% CO_2 incubator. For efflux experiments the cells were seeded into 24-multiwell plates in a 1:2 split ratio and grown till confluence during 7 days.

Cholesterol efflux assays. To load confluent cells with [^{3}H]cholesterol, fibroblasts were incubated for 24 h with DMEM supplemented with 30 μg/ml cholesterol, 0.5 μCi/ml [^{3}H]cholesterol, 10 mM Hepes pH 7.4 and 0.2% fatty acid free BSA.

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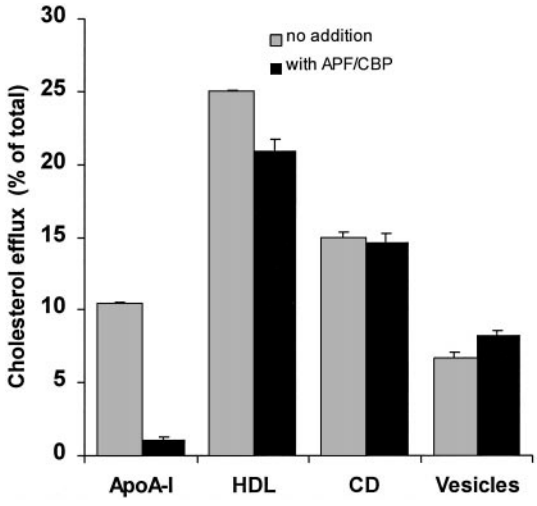

FIG. 1. Effect of APF/CBP on cholesterol efflux from normal human skin fibroblasts to four different cholesterol acceptors. Fibroblasts were loaded and labeled with 30 μg/ml [^{3}H]cholesterol as described under Materials and Methods. Efflux was started by adding culture medium containing 2 μg/ml ApoA-I, or 50 μg/ml HDL, or 0.5 mM methyl-β-cyclodextrin (CD) or 0.5 mg/ml PC vesicles with or without 0.6 μg/ml APF/CBP. After 5 h (PC vesicles) or 20 h (ApoA-I, HDL, CD) incubation the amount of [^{3}H]cholesterol in medium and cells was determined. Cholesterol efflux is calculated as the % total [^{3}H]cholesterol (cells plus medium) released into the medium. Results are the means ± SD of duplicate wells from two to three experiments.

Cells were washed four times with PBS/BSA before addition of efflux medium. Efflux was started by adding efflux medium (culture medium without FCS, supplemented with 10 mM HEPES, pH 7.4) containing the cholesterol acceptors ApoA-I, HDL, phosphatidylcholine vesicles or methyl-β-cyclodextrin. CBP/APF (0 to 2.4 μg/ml) was added as indicated together with the cholesterol acceptors before starting the efflux procedure. After a 5- (for PC vesicles) or 20-h (for ApoA-I, HDL or CD) incubation in a humidified 10% CO_2 incubator, the medium was collected and centrifuged (10,000*g;* 5 min). The remaining cellular lipids were determined after a 2-h extraction with 2-propanol. The amount of [^{3}H]cholesterol in the medium and cellular extract was quantified by liquid scintillation counting. The percentage efflux was calculated by dividing the radioactive counts in the efflux medium by the sum of the counts in the medium plus the cell extract.

Protein determination. The amount of CBP/APF was determined using a fluorimetric (fluorescamine) assay as described by Harvey *et al.* (14).

RESULTS

The effect of APF/CBP (0.6 μg/ml) on cholesterol efflux to four different acceptors is shown in Fig. 1. APF/CBP inhibited efflux to ApoA-1 almost completely, efflux to HDL was inhibited by 20% at this concentration of APF/CBP whereas no significant effect was seen with the aspecific acceptors methyl-β-cyclodextrin or phosphatidylcholine liposomes. Also higher amounts of APF/CBP, up to 2.4 μg/ml failed to exert effect on the latter two acceptors. In the absence of acceptors addition of APF/CBP even at very high concentration (5 μg/ml) did not influence efflux indicating that the protein itself was not able to accept appreciable amounts of cholesterol.

Figure 2 shows a dose response effect of APF/CBP on ApoA-I mediated cholesterol efflux from normal human skin fibroblasts. Two concentrations of Apo A-I were used. There was no difference between 2 and 10 μg/ml ApoA-I induced efflux in 20 h. Under both conditions efflux was about 12% indicating saturation of the process. Addition of 0.3 to 2.4 μg/ml APF/CBP to 2 μg/ml ApoA-I resulted in a significant inhibition (30 to 95%) of cholesterol efflux. At 10 μg/ml of ApoA-I the inhibiting effect of APF/CBP was less pronounced. At the highest CBP/APF concentration cholesterol efflux was almost fully blocked at both concentrations of ApoA-1. To investigate whether the inhibitory effect of APF/CBP on the cholesterol efflux was specific for the ABCA1 dependent pathway the effect on the HDL mediated efflux from normal and fibroblasts derived from a patient with Tangiers disease was determined. Efflux to apoA-I in these cells is close to zero and could therefore not be used. Efflux to HDL contains both a ABCAI specific and aspecific component. As shown in Fig. 3, APF/CBP decreased the HDL dependent cholesterol efflux from normal fibroblasts in a concentration dependent manner. At the highest CBP/APF concentration (2.4 μg/ml) a 40 percent inhibition was seen. In

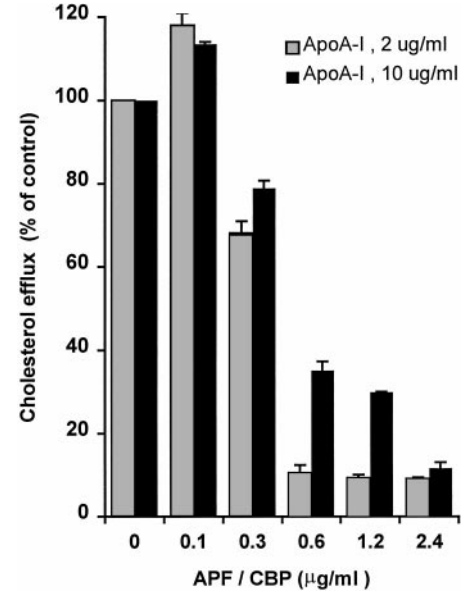

FIG. 2. Effect of APF/CBP on the ApoA-I mediated cholesterol efflux from normal human skin fibroblasts. Fibroblasts were loaded and labeled with 30 μg/ml [^{3}H]cholesterol as described in Methods. Efflux was started by adding culture medium containing 2 or 10 μg/ml ApoA-I, together with increasing amounts of APF/CBP as indicated. After 20 h incubation the amount of [^{3}H]cholesterol in medium and cells was determined. Cholesterol efflux is calculated as the % of total [^{3}H]cholesterol (cells plus medium) released into the medium. Efflux without APF/CBP (control) was set to 100%. Results are the means ± SD of duplicate wells from two experiments.

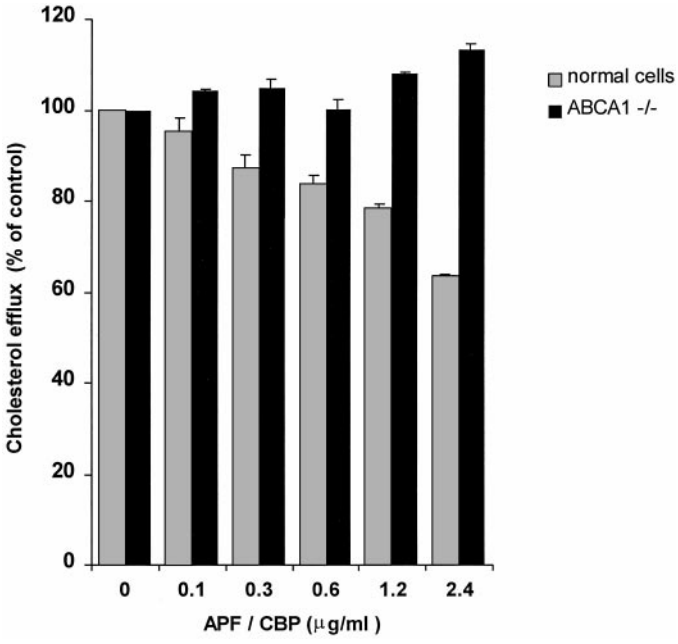

FIG. 3. Effect of APF/CBP on the HDL mediated free cholesterol efflux from normal and ABCAI deficient human skin fibroblasts. Fibroblasts were treated as described in the legend to Fig. 1. Efflux was started by adding culture medium containing 50 μg/ml HDL, together with increasing amounts of APF/CBP as indicated. After 20 h incubation the amount of [^{3}H]cholesterol in medium and cells was determined. Cholesterol efflux was calculated as described in the legend to Fig. 2. Results are the means ± SD of duplicate wells from two experiments.

contrast even at the highest concentration APF/CBP had no effect on the HDL dependent cholesterol efflux from ABCA1 deficient cells.

DISCUSSION

APF/CBP is a protease resistant protein which is secreted by the liver to both blood and bile (15). A physiological role for the protein has not yet been found. Here we show that the protein specifically interferes with apolipoprotein mediated efflux from cultured fibroblasts. APF/CBP dose dependently inhibited cholesterol efflux to ApoA-I. Aspecific efflux towards methyl-β-cyclodextrin or phosphatidylcholine vesicles was not inhibited and efflux towards HDL was partly affected. Our data suggest that ABCAI plays a crucial role in the effect. Efflux to HDL in fibroblasts derived from patients with Tangiers disease was not inhibited by APF/CBP. Since the protein has been shown to be present in HDL3 (15), it may play a regulating role in cholesterol uptake from peripheral tissue. In addition to its function in peripheral cholesterol efflux ABCA1 (2, 3, 16, 17) has been suggested to be involved in regulation of cholesterol uptake in the intestine. Massive upregulation of ABCA1 expression by RXR/LXR agonists significantly decreased cholesterol absorption in mice (6). Although other ABC transporters may also play a role in this stimulation of cholesterol efflux from the epithelial cells (18) there are no apolipoproteins present so an alternative acceptor for the cholesterol must be present in the intestinal lumen. The concentration of APF/CBP in bile is in the range of 1–2 mg/ml (19). Hence even after dilution in the intestinal lumen there is a relative high content of APF/CBP in the intestine. The protein itself has a low capacity for cholesterol but is has been shown to be able to mediate efflux towards bile acid micelles (7, 20). *In vivo* experiments in an animal model will have to be carried out to validate this functional role for APF/CBP.

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