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The Lipidation Status Of Acute-Phase Protein Serum Amyloid A Determines Cholesterol Mobilization Via Scavenger Receptor Class B, Type I

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ABSTRACT

During the acute-phase reaction, serum amyloid A (SAA) replaces apoA-I as the major high-density lipoprotein (HDL)-associated apolipoprotein. A remarkable portion of SAA exists in a lipid-free/lipid-poor form and promotes ATP-binding cassette transporter A1 (ABCA1)-dependent cellular cholesterol efflux. In contrast to lipid-free apoA-I and apoE, lipid-free SAA was recently reported to mobilize scavenger receptor class B, type I (SR-BI)-dependent cellular cholesterol efflux (Van der Westhuyzen, D. R., Cai, L., de Beer, M. C., and de Beer, F. C. (2005) J. Biol. Chem. 280, 35890-35895). This unique property could strongly affect cellular cholesterol mobilization during inflammation. However, here we show that overexpression of SR-BI in HEK cells (devoid of ABCA1) failed to mobilize cholesterol to lipid-free or lipid-poor SAA. Only reconstituted vesicles containing phospholipids and SAA promoted SR-BI-mediated cholesterol efflux. Cholesterol efflux from HEK and HEK[SR-BI] cells to lipid-free and lipid-poor SAA was minimal while efficient efflux was observed from fibroblasts and CHO cells (both expressing functional ABCA1). Overexpression of SR-BI in CHO cells strongly attenuated cholesterol efflux to lipid-free SAA even in the presence of an SR-BI blocking IgG. This implies that SR-BI attenuates ABCA1-mediated cholesterol efflux in a way that is not dependent on SR-BI-mediated re-uptake of cholesterol. The present in vitro experiments demonstrate that the lipidation status of SAA is a critical factor governing cholesterol acceptor properties of this amphipathic apolipoprotein. In addition, we demonstrate that SAA mediates cellular cholesterol efflux via the ABCA1 and/or SR-BI pathway in a similar way as apoA-I.

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INTRODUCTION

The serum amyloid A (SAA) family of proteins is encoded by multiple genes, which display allelic variation and a high degree of homology in mammals (1). In humans, the highly homologous *SAA1* and *SAA2* genes code for the corresponding non-glycosylated acute-phase SAA1 and SAA2 proteins, both commonly referred to as SAA. Triggered by inflammation through stimulation of hepatocytes by lymphokine-mediated processes, the concentrations of SAA may increase during the acute-phase reaction to levels 500-2000-fold greater than that found in the non-inflammatory state (20-50 μg/ml). Besides its role as a major acute-phase reactant (2), SAA acts as the precursor protein during secondary reactive amyloidosis and as an apolipoprotein, respectively (3,4). SAA associates with lipoproteins of the high-density range (HDL), in particular with lipid-rich α-migrating HDL-particles subclass 3 (HDL_3). Coupled with an inflammation-related decrease of apoA-I (the major HDL-associated apolipoprotein under non inflammatory conditions), an increased content of SAA (up to 87 % of total HDL-protein content (5)) modulates the metabolic properties of its physiological carrier during inflammation. A major function of HDL comprises its role during reverse cholesterol transport. However, HDL-mediated cholesterol efflux capacity from peripheral tissue as well as HDL-mediated cholesterol/cholesteryl ester (CE) delivery to the liver and/or steroidogenic tissues is drastically altered during the acute-phase response. Both, a decreased capacity of acute-phase (SAA-enriched) HDL to acquire cellular cholesterol from macrophages (6-8) and a decreased capacity to deliver CEs to hepatic cells (9) have been reported. Besides changes in the apolipoprotein composition, changes in the lipid composition may lead to an increased particle diameter of acute-phase HDL (10-12) that may be considered another determinant modulating the turnover of HDL-particles and its constituents.

The only receptor able to mediate effective bidirectional lipid/cholesterol flux is scavenger receptor class B, type I (SR-BI). SR-BI, a multiligand scavenger receptor, can bind a variety of ligands including native and modified lipoprotein particles (13). Evidence from genetically engineered mouse models highlights its physiological role as HDL-receptor. SR-BI binds discoidal reconstituted HDL containing apoA-I and other apolipoproteins, as well as spherical HDL-particles. A structural motif recognized by SR-BI is an amphipathic helix present in all HDL-associated apolipoproteins (14).

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Another important sterol transporter contributing to cholesterol efflux and HDL-assembly/remodeling is ATP-binding cassette transporter A1 (ABCA1) (15). ABCA1 mediates the transfer of cellular phospholipids and cholesterol to extracellular lipid-free apoA-I or preβ-migrating HDL particles. Through a series of intermediate steps, lipidated apoA-I then proceeds to discoidal HDL-particles that are converted to spherical lipoprotein particles by lecithin:cholesterol acyltransferase, generating a CE-rich hydrophobic core within the lipoprotein particle. The resulting β-migrating HDL_3-particles can interact with SR-BI or undergo conversion to large HDL_2-like particles and preβ-HDL (16). Recent observations suggested that lipid-free SAA, following internalization by macrophages, can inhibit intracellular acylCoA:cholesterol acyltransferase but may activate neutral cholesterol hydrolase (17). This imbalance could promote a shift in the cellular cholesterol pool towards free cholesterol that in turn can be mobilized via the ABCA1 pathway (17). Lipid-free SAA may bind cholesterol and modulate cholesterol flux (18) apparently by acting as a cholesterol acceptor via the ABCA1 pathway (19,20).

Recent findings provide evidence that SR-BI acts as a candidate receptor for SAA and/or SAA containing lipoproteins (21,22). Lipid-free SAA has further been reported to promote SR-BI-dependent cholesterol efflux (23). This observation is of importance as a remarkable portion (up to 15 % (24)) of total plasma SAA concentrations (ranging up to 100 mg/dl during the acute-phase reaction (2)) exists in a lipid-free (non lipid-associated) or lipid-poor form and may contribute to cholesterol homeostasis as reported for other apolipoproteins. However, lipid-free apoA-I (although acting as a ligand for SR-BI (25)) and apoE (another HDL-associated apolipoprotein) failed to promote SR-BI-dependent cellular cholesterol efflux. Only lipidation of these apolipoproteins promotes cellular cholesterol efflux via SR-BI (25,26).

Therefore the goal of the present study was to reveal whether the extent of lipidation of SAA governs SR-BI-/ABCA1-dependent cholesterol efflux to this apolipoprotein.

MATERIALS AND METHODS

Materials

Radiochemicals were purchased from DuPont/NEN. Ham's-F12K medium was from Gibco (Life Technologies) and fetal bovine serum (FBS) was from Boehringer Ingelheim

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Bioproducts. Plastic-ware used for tissue culture was obtained from Costar. BLT-1 (2-hexyl-1-cyclopentanone thiosemicarbazone) was from ChemBridge and Probucol from Sigma. All other chemicals were obtained from Merck, except where indicated.

Methods

a.) Preparation of HDL, apoA-I and SAA

ApoE-free HDL_3 (d = 1.125 to 1.21 g/ml) was prepared by discontinuous density ultracentrifugation of plasma obtained from normolipidemic blood donors (27).

For preparation of human apoA-I, HDL_3 was delipidated, apolipoproteins were redissolved in 50 mM glycine/0.5 M NaCl/6 M urea (pH 8.8) and separated by size-exclusion chromatography on a Sephacryl S-200 column (28). The fractions containing the major peak were pooled, dialyzed against 50 mM NH_4HCO_3 and freeze-dried. The product was homogeneous as assessed by SDS-PAGE, reverse-phase HPLC and amino acid analysis (28).

SAA1, the most abundant SAA isoform during inflammation (accounting up to 90 % of total SAA (29)), was isolated from human plasma obtained from patients undergoing plasmapheresis for therapeutic purposes. Briefly, SAA was isolated by hydrophobic interaction-fast protein liquid chromatography on octylsepharose followed by fast protein liquid-gel permeation chromatography on a Superdex TM^{75} column exactly as described (30). Further purification of SAA from coeluting apoC's and apoA-II was achieved by preparative isoelectric focusing technique by a Rotofor™ IEF cell between pH 5-7 (30). Purity of SAA1 was checked by immunochemical techniques and amino acid analysis. Cholesterol and phospholipid content in SAA1 and apoA-I preparations (estimated as described (7)) were below the detection limits.

b.) Generation of reconstituted particles

Complexes comprising phosphatidylcholine (PC, Sigma) with apolipoproteins were prepared using the sodium cholate dialysis method as described (31). PC was suspended in 10 mM Tris-HCl (pH 8, 150 mM NaCl, 0.01 % EDTA) by vortexing. The mixture was held on ice for 1 h followed by adding sodium cholate (the final cholate:PC molar ratio was 1). SAA or apoA-I was added and the mixture was incubated for further 20 h at 4°C. Subsequently sodium cholate was removed by extensive dialysis against PBS at 4°C. The PC:apolipoprotein complexes were prepared using a weight ratio of 2.71:1.

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c.) Labeling procedures

SAA labeling with ^{125}INa-iodination was performed as described (7) using N-bromosuccinimide as the coupling agent. Routinely, 100 µCi of ^{125}INa was used to label 200 µg of protein. This procedure resulted in specific activities between 500 and 800 cpm/ng of protein.

HDL_3 was labeled with [cholesteryl-1,2,6,7-^{3}H] palmitate by CE-transfer protein-catalyzed transfer from donor liposomes (27). Briefly, 200 µCi of the corresponding label and 100 µg of egg yolk lecithin were dried under argon, followed by the addition of 1 ml of 10 mM PBS. The mixture was shaken for 2 min at 37°C and sonicated. Lipoproteins (1 ml, containing 3-6 mg of protein), 1 ml of lipoprotein-deficient serum (as a source of CE-transfer protein), and 1 ml of PBS were added. The mixture was incubated under argon at 37°C in a shaking water bath overnight. Subsequently, the labeled HDL_3 fractions were reisolated in a TLX120 bench top ultracentrifuge in a TLA100.4 rotor (Beckman). The HDL_3 band (d = 1.125-1.21 g/ml) was aspirated and dialyzed against 10 mM PBS, pH 7.4. This labeling procedure resulted in specific activities of 5-9 cpm/ng of protein.

d.) Cell culture

Chinese hamster ovary (CHO) cells : ldlA cells (clone 7, an LDL-receptor-deficient CHO cell line) were cultured in Ham's-F12K medium containing 5 % (v/v) FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (32). Stable transfectants expressing murine SR-BI (ldlA[mSR-BI]) were maintained in medium containing 0.5 mg/ml G-418.

Fibroblasts : Human skin fibroblasts were from explants of skin biopsies obtained from healthy volunteers (33) and maintained in DMEM containing 10 % FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

Human embryonic kidney (HEK) cells : HEK-293 cells were maintained in DMEM containing 10 % FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

e.) Construction of recombinant human SR-BI (hSR-BI) adenovirus and generation of HEK cells transiently expressing hSR-BI

The adenoviral plasmid shuttle vector (pAvCvSv) and pJM17 vector were kindly supplied by Dr. L. Chan (Baylor College of Medicine, Houston, Texas). The hSR-BI cDNA (kindly supplied by Dr. H. Hauser, ETH, Zürich, Switzerland), which was originally inserted into pcEXV-3 vector, was partially restricted with EcoR I and the 2.5 kb band was eluted from gel. This band was subcloned into pBluescript using EcoR I site, amplified, restricted

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with Kpn I and this fragment was finally partially restricted with BamH I. Plasmid shuttle vector was opened using Kpn I and Bgl II and Kpn I/BamH I restricted hSR-BI cDNA was inserted. These modifications were necessary to enable insertion of hSR-BI cDNA under the control of the CMV promoter in the plasmid shuttle vector. To obtain transiently infected cells, adenovirus infection of HEK-293 cells with hSR-BI (termed HEK[hSR-BI]) or ß-galactosidase (β-gal, termed HEK[β-gal]) as control was performed as described (34,35). No cell toxicity was observed under these experimental conditions.

HEK cells were cultivated in 12-well culture dishes. At a density of 5 x 10^4 cells/cm^2 they were rinsed once with PBS and infected with recombinant adenovirus (multiplicity of infection (moi) = 1 to 10 plaque forming units) in 250 µl of infection media (DMEM medium containing 50 U/ml penicillin and 50 µg/ml streptomycin) for 2 h.

f.) Efflux experiments

Efflux of ^{3}H-cholesterol from cells was measured by appearance of ^{3}H-cholesterol in the cellular supernatant. Cells were incubated in the presence of ^{3}H-cholesterol (0.5 µCi/ml) for 24 h as described (36). After cholesterol loading, cells were rinsed twice with TBS (containing 5 % [w/v] BSA) and twice with TBS and incubated for further 2 h in DMEM containing 0,1 % BSA. In some experiments, Probucol was added at a final concentration of 20 µM. Subsequently, the cells were rinsed again with TBS and efflux experiments were initiated by the addition of SAA, apoA-I or HDL_3. In some experiments, BLT-1 was added at 10 µM final concentrations. In other experiments using ldlA7 and ldlA[mSR-BI] cells, cholesterol efflux was studied in the presence of a polyclonal rabbit anti-mSR-BI IgG, shown to block both CE-association and cellular cholesterol efflux (36,37). After 2 - 4 h, the culture medium was removed and the cells were lysed to estimate cell-associated radioactivity and radioactivity present in the medium. Efflux of ^{3}H-cholesterol to culture medium was calculated as % of total by subtracting cell-associated radioactivity + radioactivity present in culture medium from radioactivity present in culture medium x 100.

g.) Association studies

Association studies of ^{3}H-CE-labeled-HDL_3 to HEK cells were performed at 37°C for 2 h with 20 µg/ml of ^{3}H-CE-labeled-HDL_3 in the absence (total association) or presence of 1 mg of protein/ml (unspecific association) of unlabeled autologous lipoprotein (27). Subsequently the medium was aspirated, and the cells were rinsed twice with TBS (containing 5 % (w/v) bovine serum albumin) followed by two washes with TBS. Cells were then lysed

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with 0.3 N NaOH. The radioactivity and protein content of the cell lysate was measured in the same aliquot. Specific cell-association was calculated as the difference between the total and nonspecific cell-association.

h.) SDS-PAGE and immunoblotting

The samples were separated by SDS-PAGE on 7 % linear gels and blotted onto nitrocellulose membranes (7). Subsequently, blots were incubated with a sequence-specific rabbit anti-SR-BI peptide (496-509, dilution 1:1500) antibody (Abcam), a rabbit polyclonal anti-ABCA1 antibody (Genosphere Biotechnologies, dilution 1:1000), or a rabbit polyclonal anti-ABCG1 antibody (Novus, dilution 1:1500). After incubation with peroxidase-conjugated secondary antibodies (1:1000), visualization of immunoreactive bands (84 kDa for SR-BI; 250 kDa for ABCA1; 63 kDa for ABCG1) was achieved using the ECL technique.

RESULTS

1.) Effect of SR-BI expression on cholesterol efflux to lipid-free SAA

We first tested whether SR-BI affects the ability of lipid-free SAA to promote cholesterol efflux. To perform these experiments, HEK cells overexpressing human SR-BI were used. Expression and functionality of SR-BI was confirmed by Western blot experiments and by measuring CE-uptake. As expected, an increased expression of SR-BI protein is paralleled by an increased cell-association of ^{3}H-CE labeled HDL_3 to HEK[hSR-BI] cells (Figure 1A). Next, the ability of lipid-free apolipoproteins to promote cholesterol efflux from ^{3}H-cholesterol loaded HEK and HEK[hSR-BI] cells was tested. Figure 1B shows that increasing SR-BI expression was without effect on cholesterol efflux to lipid-free SAA. As anticipated, lipid-free apoA-I also failed to promote cholesterol efflux from HEK[hSR-BI] cells. As HEK cells in general do not express detectable levels of ABCA1, ABCA1-mediated lipidation of lipid-free SAA or apoA-I during the incubation period of cholesterol efflux experiments are negligible. Only HDL_3-induced cholesterol efflux correlated with SR-BI expression levels. Therefore, lipid-free SAA, in a similar manner as lipid-free apoA-I, is unable to induce SR-BI-mediated cholesterol efflux.

2.) Effect of SR-BI expression on cholesterol efflux to lipid-poor SAA

Next, we tested whether fibroblasts could lipidate SAA and apoA-I in an ABCA1-dependent manner. For these experiments, ^{3}H-cholesterol loaded fibroblasts were incubated

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with lipid-free SAA or apoA-I and cholesterol efflux was measured. As shown in Figure 2A, both lipid-free apolipoproteins promoted cellular cholesterol efflux from 1.6 % (SAA) to 1.8 % (apoA-I) of total radioactivity (measured after 4 h). This apolipoprotein-mediated cholesterol efflux was mainly mediated by ABCA1 as approx. 70 % inhibition of cholesterol efflux was achieved by the specific ABCA1 inhibitor Probucol (38). As fibroblasts do not express detectable SR-BI protein levels (39), a contribution of this receptor during cholesterol efflux can be excluded.

The next set of experiments aimed to study whether SR-BI could mediate cellular cholesterol efflux to lipid-poor SAA or apoA-I. To generate lipid-poor (minimally-lipidated) apolipoproteins, fibroblasts were incubated with lipid-free SAA or apoA-I for 4 h. After this preincubation period, the cellular supernatant (containing lipid-poor apolipoproteins generated via ABCA1-dependent mechanisms of fibroblasts) was transferred to ^{3}H-cholesterol loaded HEK[hSR-BI] cells. Figure 2B shows that cholesterol efflux to lipid-free apolipoproteins is lower (0.1 % for apoA-I and 0.3 % for SAA) as observed with lipid-poor apolipoproteins (about 0.6 % of total cell-associated radioactivity for SAA or apoA-I). No difference became apparent between HEK and HEK[hSR-BI] cells. Based on these findings (Figure 2), we conclude that SR-BI does not contribute to lipid-free nor lipid-poor SAA- or apoA-I-induced cholesterol efflux.

3.) Inhibition of ABCA1-mediated cholesterol efflux by SR-BI

To confirm findings obtained above (Figure 2B) and to test whether the presence of ABCA1 besides SR-BI modulates the ability of SAA to mobilize cholesterol, experiments were performed with ldlA7 and ldlA[mSR-BI] cells (32). Both cell lines express ABCA1 at similar levels (Figure 3A). High expression of SR-BI is only present in ldlA[mSR-BI] cells while endogenous SR-BI expression is very low on ldlA7 cells. Both cell lines were loaded with ^{3}H-cholesterol and efflux experiments were performed with lipid-free SAA or apoA-I as acceptors. Of note, ldlA[mSR-BI] cells (compared with ldlA7 cells) showed a decreased cholesterol efflux capacity to SAA (Figure 3B). Similar data were obtained using apoA-I as cholesterol acceptor. This observation is in line with a previous report demonstrating an inhibitory effect of SR-BI on ABCA1-mediated cholesterol efflux (40). These authors (40) suggested that SR-BI could re-uptake cholesterol effluxed by ABCA1. To test this assumption, experiments were performed in the presence of a specific anti-SR-BI IgG (Figure 3C). Using ldlA[mSR-BI] cells, cholesterol efflux to SAA was not affected by anti-SR-BI

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IgG, while cholesterol efflux to apoA-I was slightly increased (0.45±0.06% vs. 0.79±0.15%). As anticipated, the anti-SR-BI IgG blocked SR-BI-dependent cholesterol efflux from ldlA[mSR-BI] cells to HDL_3. When the same experiments were performed with control ldlA7 cells, no difference on cellular cholesterol efflux to lipid-free SAA or apoA-I and HDL were observed after preincubating the cells with either non-immune IgG or anti-SR-BI IgG. From these experiments we conclude that the inhibitory activity of SR-BI on ABCA1-dependent cholesterol flux to SAA is not mediated by re-uptake of cholesterol via SR-BI.

4.) Reconstituted phospholipid:apolipoprotein vesicles induce SR-BI-mediated cholesterol efflux

Since lipid-free and lipid-poor SAA failed to promote SR-BI-mediated cholesterol efflux (Figure 1 to 3), we tested whether more extensive lipidation of SAA favors SR-BI-dependent cholesterol efflux. Recent findings demonstrated that reconstituted vesicles containing lipids and apoA-I or apoE effectively promoted SR-BI-mediated cholesterol efflux (26,41,42). HEK[hSR-BI] cells were used for these experiments. To avoid bidirectional cholesterol transfer, the reconstituted phospholipid vesicles used as cholesterol acceptors contained only PC and the corresponding apolipoprotein. As shown in Figure 4, vesicles containing SAA or apoA-I effectively promoted SR-BI-dependent cholesterol efflux from HEK[hSR-BI] cells when compared to HEK and HEK[β-gal] cells. From these results (Figure 1 to 4) we conclude that sufficient lipidation of SAA, similar as observed with apoA-I (Figure 3, reference (25)) and apoE (26), is a prerequisite to mobilize SR-BI-dependent cholesterol efflux.

5.) The role of BLT-1 in cholesterol efflux

Recent data (23) demonstrated an inhibition of cholesterol efflux from hepatoma cells to lipid-free SAA by BLT-1, an inhibitor of SR-BI-mediated cholesterol transport (43). As we could show that cellular cholesterol efflux to lipid-free or lipid-poor SAA (Figure 1 to 3) occurs independently of SR-BI, we were interested to assess the specificity of BLT-1 by using three different cell models. The first set of experiments was performed with HEK (controls) and HEK[hSR-BI] cells that do not express ABCA1. Figure 5A shows that BLT-1 specifically inhibited SR-BI-mediated cholesterol efflux to HDL_3. No inhibitory effect of BLT-1 was found in controls and HEK[β-gal] infected cells. Probucol had no effect on SR-BI-mediated cellular cholesterol efflux from HEK, HEK[β-gal], and HEK[hSR-BI] cells, respectively.

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Next we tested whether BLT-1 interferes with ABCA1-dependent cholesterol efflux. Therefore, cholesterol efflux studies were performed with fibroblasts and ldlA7 cells that express high levels of functional ABCA1; fibroblasts do not express SR-BI (39) while low expression of SR-BI is observed on ldlA7 cells (Figure 3). When experiments were performed with fibroblasts (Figure 5B), BLT-1 inhibited apolipoprotein-mediated cellular cholesterol efflux from 20 % (SAA) to 26 % (apoA-I). Probucol was more effective (80% inhibition) (Figure 5B). No additive effects were observed when BLT-1 and Probucol were used in combination. Cholesterol efflux to HDL_3 was not affected by BLT-1, data in line with the fact that fibroblasts do not express detectable levels of SR-BI (39). However, when the same experiments were performed with ldlA7 cells, cholesterol efflux to lipid-free SAA or apoA-I was inhibited by BLT-1 up to 90 %. A similar inhibitory activity was observed when Probucol was used (Figure 5B). Again, BLT-1 and Probucol in combination did not further alter cellular cholesterol efflux. From this set of experiments, we conclude that BLT-1 inhibits almost completely ABCA1-dependent cholesterol efflux in ldlA7 cells. However, BLT-1 showed only minimal inhibitory activity on HDL_3-promoted cholesterol efflux (Figure 5B), data in line with low levels of endogenous SR-BI expression in ldlA7 cells (Figure 3A).

Our findings suggest that BLT-1 apparently interferes with ABCA1-mediated cholesterol efflux to lipid-free apolipoproteins. This observation could explain observations by others, that BLT-1 may inhibit cholesterol efflux to lipid-free or lipid-poor SAA (23).

DISCUSSION

ApoA-I levels decrease during acute and chronic inflammatory conditions while SAA, a positive acute-phase reactant, becomes the predominant apolipoprotein by modulating properties of HDL as well as enzymes activities involved in HDL-remodeling (3). A decreased activity of lecithin:cholesterol acyltransferase (8) together with an increased activity of phospholipid transfer protein (11,12,44) during the acute-phase are determinant factors for decreased plasma concentrations of HDL but increased concentrations of lipid-free/-poor apoA-I. The latter particles act as acceptors for cellular cholesterol via ABCA1-dependent mechanisms under physiological (non inflammatory) conditions. Accumulating evidence suggests that lipid-free/-poor SAA (when abundantly available during inflammatory conditions) also contributes to cholesterol efflux via ABCA1-dependent mechanisms ((19); Figure 2A and 5). This pathway could contribute to prevention of local cholesterol accumulation at sites of tissue destruction (45). Alternatively, SAA – when present on HDL –

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could contribute to delivery of acute-phase HDL-associated cholesterol to cells involved in tissue repair at sites of inflammation (46).

The fact that lipid-associated SAA acts as a specific ligand for SR-BI is apparently related to the secondary structure of the protein. SAA contains two α-helical regions, and the N-terminal portion contains a short amphipathic α-helix that is likely involved in lipid binding (47,48). Both apoA-I and synthetic amphipathic peptides containing a structural α-helical motif, effectively competed for SAA binding to hSR-BI-transfected HeLa and THP-1 cells (21); this indicates that one or more α-helices of SAA are recognized by SR-BI.

The fact that neither lipid-free nor lipid-poor SAA acquires cellular cholesterol via SR-BI-dependent mechanisms (Figure 1B, 2B) is a major finding of the present study; an adequate lipidation status of SAA (as shown here with PC:SAA containing vesicles) facilitates cholesterol efflux of this apolipoprotein via the SR-BI pathway. From these data we conclude that under *in vivo* conditions, SAA (as commonly known for other apolipoproteins e.g. apoA-I) has to be lipidated through a series of intermediate steps to form spherical SAA-enriched HDL-particles that in turn may mobilize cholesterol via SR-BI. Our results, using purified human SAA1, are in contrast to previous findings (23). Van der Westhuyzen and colleagues (23) showed supporting evidence for SR-BI-mediated cholesterol efflux to lipid-free or lipid-poor SAA; in that study BLT-1, an inhibitor of SR-BI (43), was found to inhibit cholesterol efflux from hepatoma cells (23). However, we here provide evidence that BLT-1 may interfere with ABCA1-mediated cholesterol efflux (Figure 5). In line with a recent study (49), we found that BLT-1 inhibited cholesterol efflux from human fibroblasts (cells that do not express SR-BI (39)) to lipid-free apoA-I by approx. 30 %. Of note, cholesterol efflux from ldlA7 cells to lipid-free SAA or apoA-I was inhibited by BLT-1 or Probucol to a similar extent (about 90 %). Thus, BLT-1 apparently inhibits ABCA1-dependent efflux from ldlA7 cells almost completely.

Another important observation of the present study is that SR-BI inhibits ABCA1-mediated cholesterol efflux to lipid-free SAA. From our experiments we conclude that the inhibitory activity of SR-BI on ABCA1-mediated cholesterol efflux cannot be sufficiently explained by re-uptake of ABCA1 effluxed cholesterol via SR-BI as suggested (40) as anti-SR-BI IgG failed to increase cholesterol flux from ldlA[mSR-BI] cells to SAA. Accordingly, other - not yet identified - mechanism(s) may contribute (19). Direct binding of cholesterol to

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SR-BI may lead to a decreased ABCA1-accessible cholesterol pool (50). Alternatively, SR-BI could alter membrane dynamics and properties (as already reported (37,51,52)) and these alterations may inactivate ABCA1.

In line with a recent study (20) we found that lipid-free SAA or apoA-I only minimally releases cholesterol from ABCA1-deficient HEK cells. Compared to results obtained with lipid-free SAA, minimal lipidation of SAA (resulting in lipid-poor SAA) moderately enhanced the ability to induce cholesterol efflux (Figure 2B, 0.3 % vs. 0.7 % of total radioactivity). A possible role of ABCG1 mediating sequential lipidation of ABCA1-lipidated SAA - as shown for apoA-I (53) - could be excluded as we were unable to detect ABCG1 on HEK-cells by Western blot experiments (data not shown); data in line with others (54).

Summarizing, we show that ABCA1- and SR-BI-dependent pathways are operative for cholesterol removal by SAA. ABCA1 is the major player mediating cholesterol flux to lipid-free SAA. This is of importance during inflammatory conditions where expression of ABCA1 on macrophages is significantly altered (8). Most importantly, SR-BI-mediated cholesterol efflux is only operative following extensive lipidation of SAA, a phenomenon similar to that observed with other amphipathic apolipoproteins (25,26).

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FIGURE LEGENDS

Figure 1.

Cholesterol mobilization to lipid-free SAA or apoA-I occurs independently of SR-BI expression levels.

HEK cells were infected with hSR-BI at indicated multiplicity of infection (moi) as described in the methods section. (**A**) SR-BI-expression was verified by Western blot analysis using sequence-specific rabbit anti-SR-BI peptide antibody. Association of ^{3}H-CE-labeled HDL_3 by HEK cells and hSR-BI-infected HEK cells was measured following incubation of cells for 2 h with 20 μg/ml ^{3}H-CE-labeled HDL_3. Cell-associated radioactivity was determined as described in the methods section. (**B**) HEK and HEK[hSR-BI] cells were examined for their ability to efflux ^{3}H-cholesterol to indicated acceptors. The cells were labeled with ^{3}H-cholesterol as described in the methods section and incubated with lipid-free SAA (10 μg/ml), lipid-free apoA-I (10 μg/ml) or HDL_3 (50 μg/ml) at 37°C for 4 h. Media and cells were separately collected, and the radioactivity was measured by liquid scintillation counting. Efflux was determined as a fraction of the cpm in media over the total (cpm in media plus cpm in cells). Specific cholesterol efflux was calculated by subtracting efflux in the absence of acceptors from efflux in presence of acceptors. Results represent mean values averages of triplicate determinations from one representative experiment out of three and error bars represent ± SD.

Figure 2.

Cholesterol mobilization to lipid-poor SAA or apoA-I occurs independently of SR-BI expression levels.

(**A**) [^{3}H]-cholesterol loaded fibroblast cells were incubated with lipid-free SAA or apoA-I (10 μg/ml) at 37°C for 2 h. Where indicated, cells were preincubated with Probucol (20 μM) as described in the Methods section. (**B**) Lipid-poor SAA or apoA-I was obtained by preincubating SAA or apoA-I (10 μg/ml) in DMEM with fibroblasts for 4 h. The lipid content of lipid-poor SAA or apoA-I ranged from 4.6 and 6.8 ng cholesterol/μg protein and 9.3 to 12.6 ng PC/μg protein and was measured as described (7). After the incubation period, the medium containing SAA or apoA-I was collected and added to ^{3}H-cholesterol-labeled HEK or HEK[hSR-BI] cells and incubated for further 4 h at 37°C. In parallel, lipid-free SAA or apoA-I (10 μg/ml) was added to cells. Subsequently, radioactivity in media and cells was measured and cholesterol efflux determined. Results represent mean values of duplicate

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determinations from one representative experiment out of two, and error bars represent the ranges of the measurements.

Figure 3.

SAA- and apoA-I-induced cholesterol efflux from ldlA[mSR-BI] and ldlA7 cells.

(**A**) SR-BI and ABCA1 expression by ldlA[mSR-BI] and ldlA7 cells (controls) was analyzed by Western blot analysis using sequence-specific rabbit anti-SR-BI peptide antibody or polyclonal anti-ABCA1 antibody. (**B**) ^{3}H-cholesterol-labeled ldlA7 and ldlA[mSR-BI] cells were incubated in the absence (control) or presence of lipid-free SAA or apoA-I (10 µg/ml, 4 h, 37°C) at or (**C**) in the presence of lipid-free SAA or apoA-I (10 µg/ml) or HDL_3 (50 µg/ml) at 37°C for 4 h in the presence of anti-SR-BI IgG or non-immune IgG. Subsequently, radioactivity in media and cells was measured and cholesterol efflux determined. Results represent mean values of triplicate determinations from one representative experiment out of four, and error bars represent ± SD.

Figure 4.

Effect of SR-BI-expression on cholesterol efflux to SAA:phospholipid vesicles.

HEK (control), HEK[β-gal] (moi = 10) (virus control) and HEK[hSR-BI] cells (moi = 10) were examined for their ability to efflux ^{3}H-cholesterol to phosphatidylcholine (PC) vesicles (generated as described in the methods section) containing SAA or apoA-I (25 µg protein/ml). The PC:apolipoprotein complexes were prepared using a weight ratio of 2.71:1. Cells were incubated for 4 h at 37°C in the presence of indicated acceptors and cholesterol efflux was determined as described. Results represent mean values of duplicate determinations from one representative experiment out of two, and error bars represent the ranges of the measurements.

Figure 5.

Effect of BLT-1 and Probucol on lipid-free SAA- or apoA-I-induced cholesterol efflux.

(A) ^{3}H-cholesterol-labeled HEK (control), HEK[β-gal] (moi = 10) (virus control) and HEK[hSR-BI] cells (moi = 10) HEK cells, **(B)** fibroblasts or ldlA7 cells were incubated with lipid-free SAA or apoA-I (10 µg/ml) (B) or HDL_3 (20 µg/ml) (**A**, **B**) for 2 h in the absence or presence of BLT-1 (10 µM). Probucol (20 µM) was added to the cells 2 h prior to cell experiments as described in the methods section. Cholesterol efflux was determined as described. Inhibition of cholesterol efflux (expressed in percentage) was calculated as the difference of efflux from cells without inhibitors compared to cells in presence of respective

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inhibitors. Results represent mean values of duplicate determinations from one representative experiment out of three, and error bars represent the ranges of the measurements.

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Figure 1A

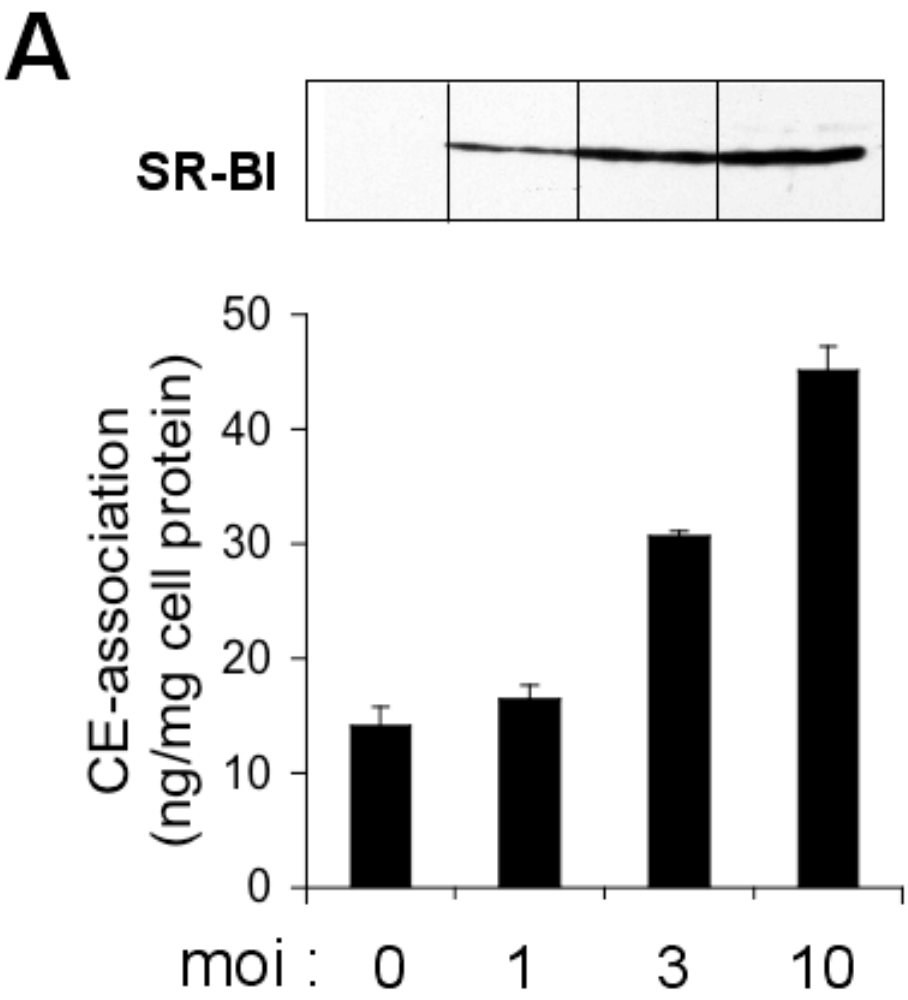

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Figure 1B

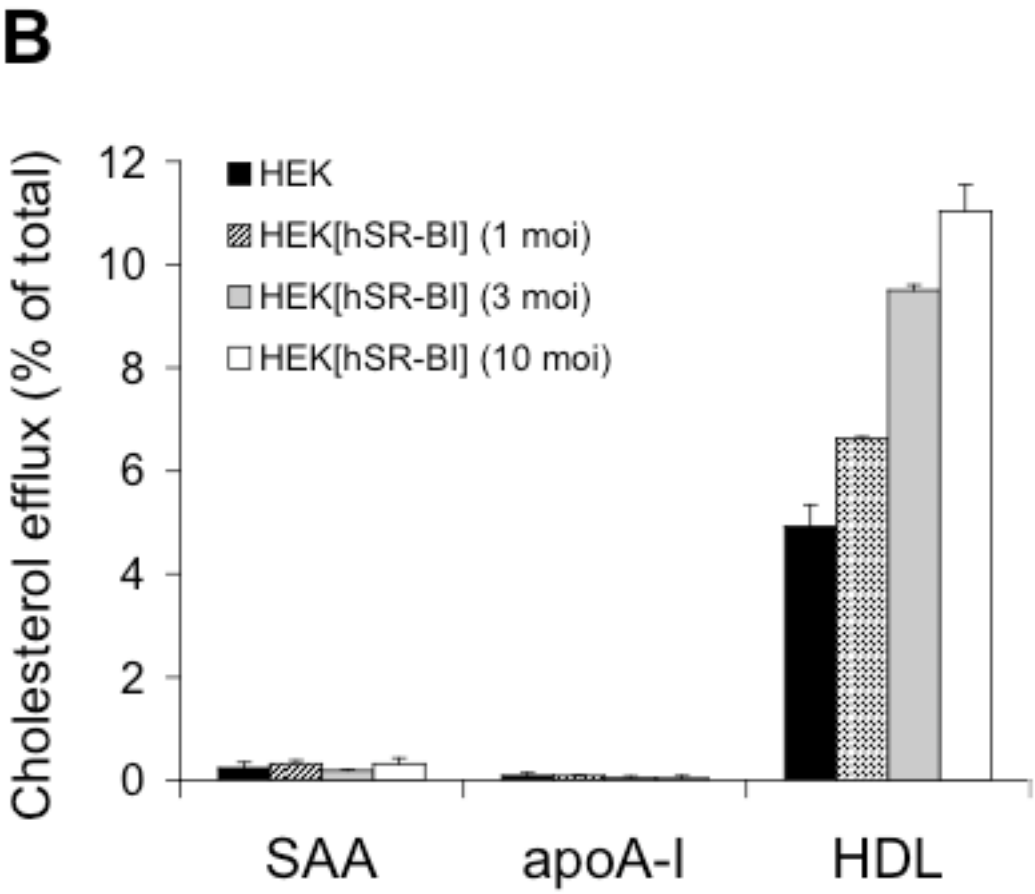

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Figure 2A

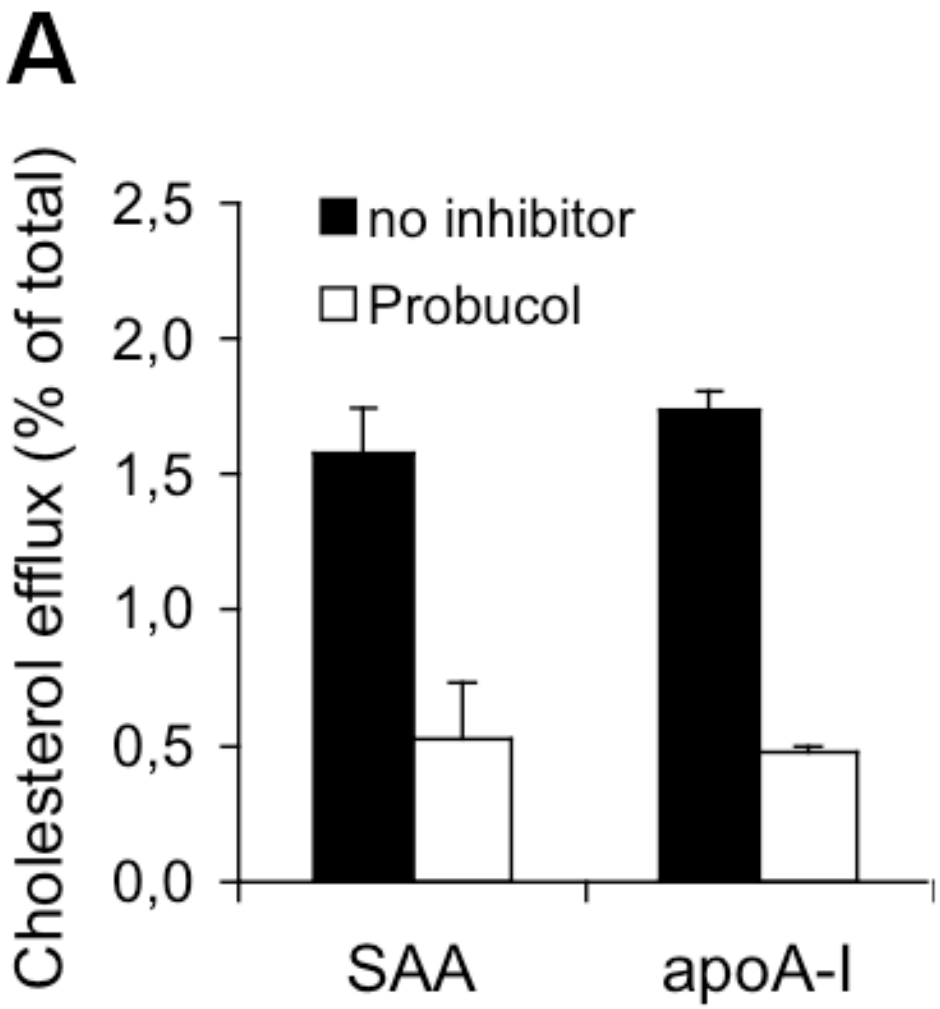

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Figure 2B

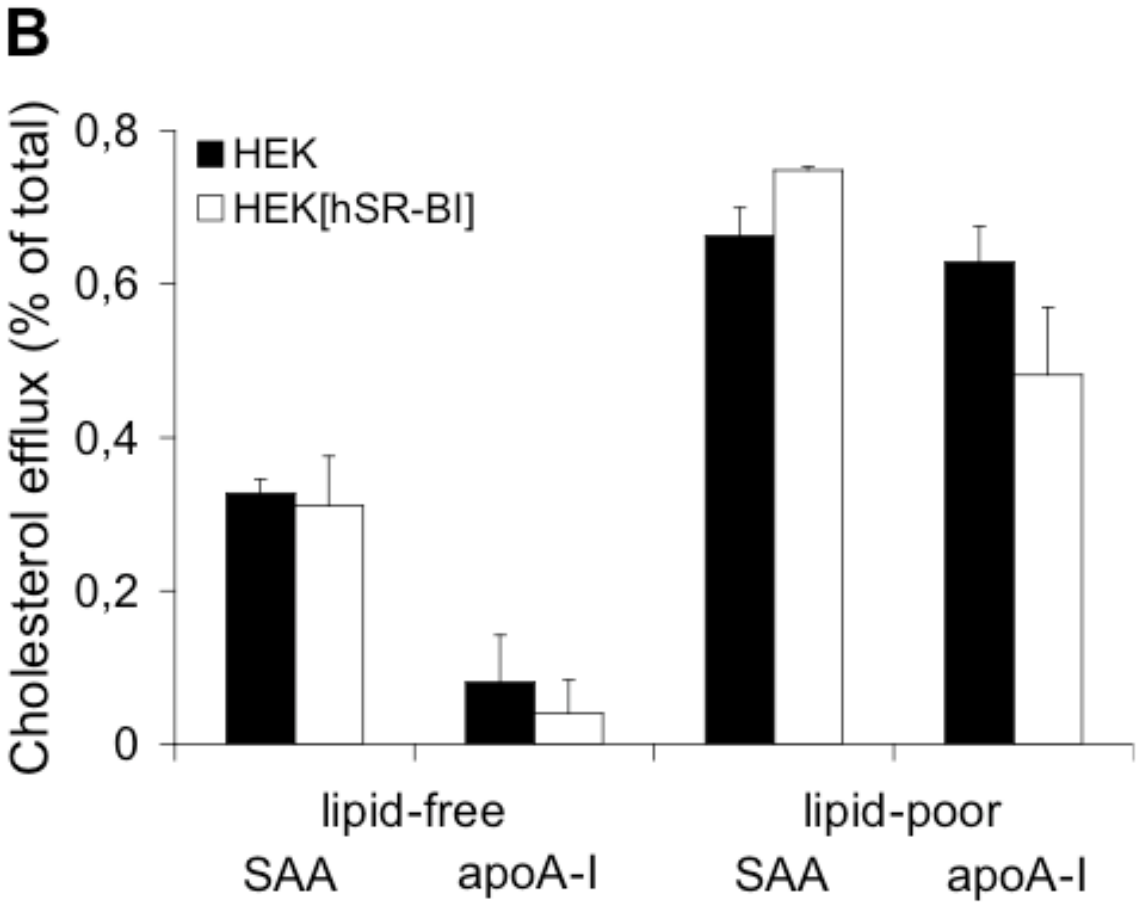

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Figure 3A

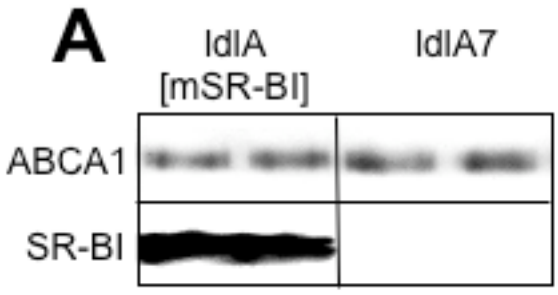

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Figure 3B

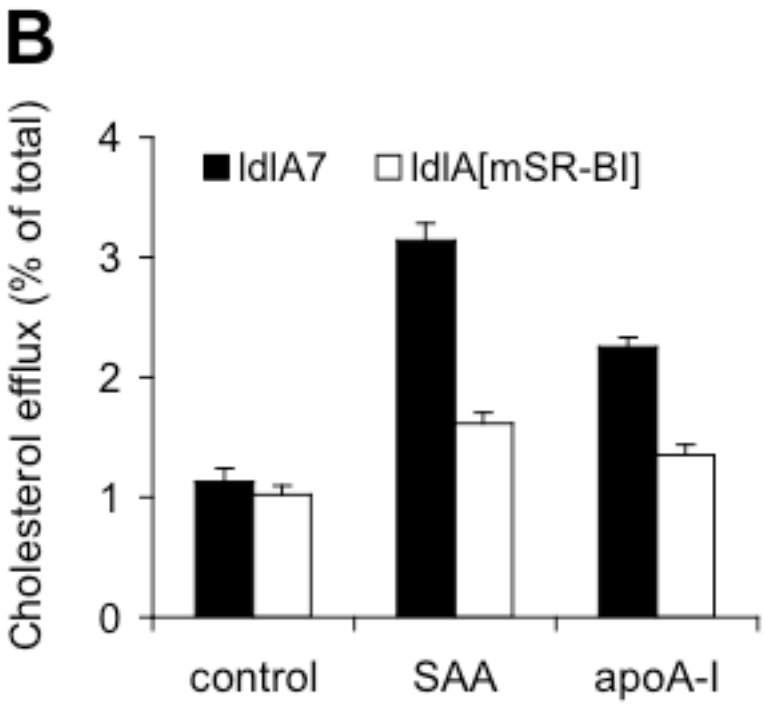

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Figure 3C

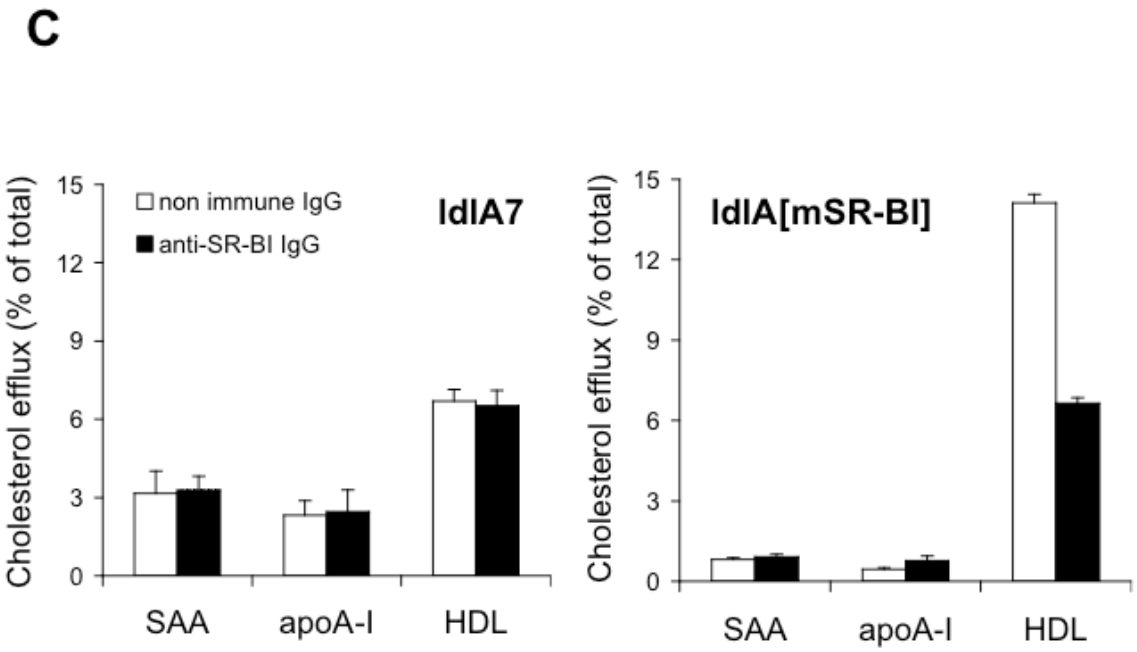

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Figure 4

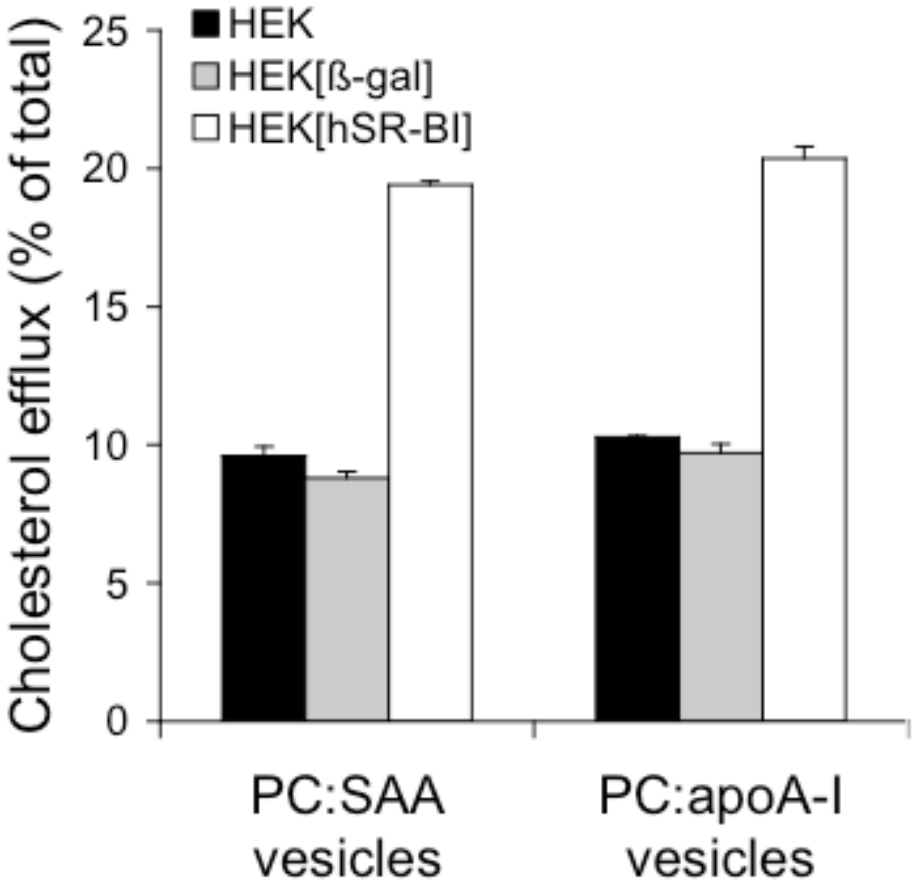

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Figure 5A

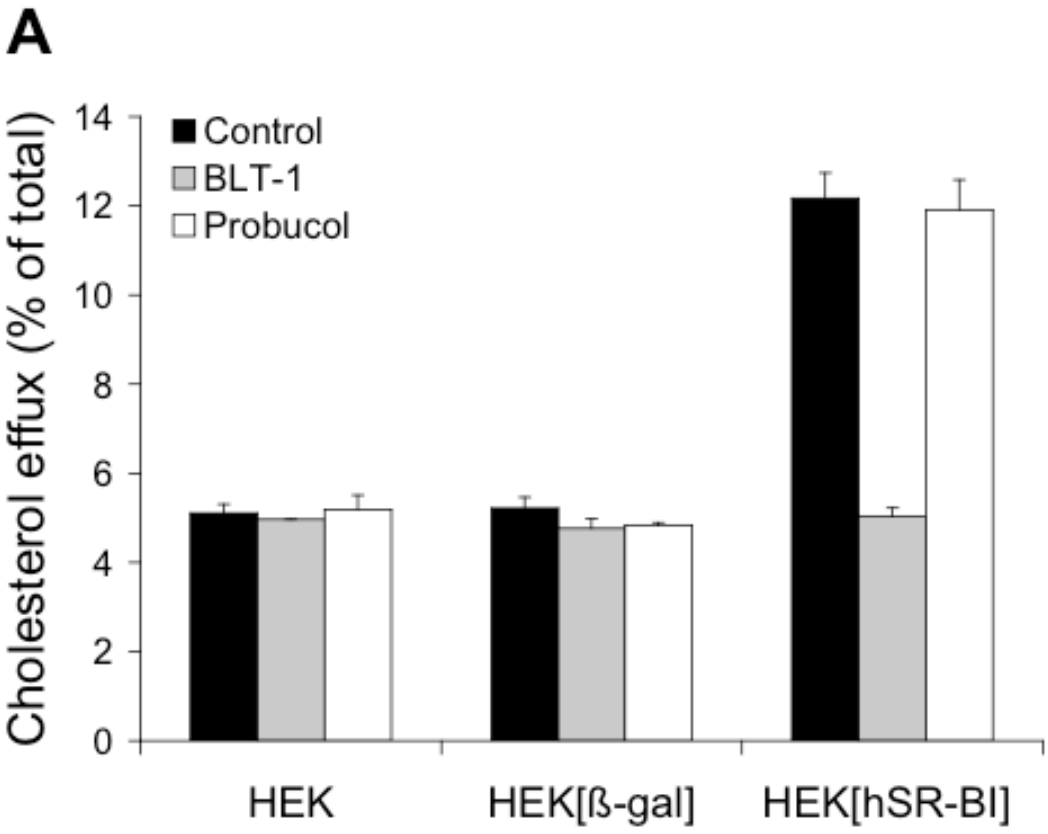

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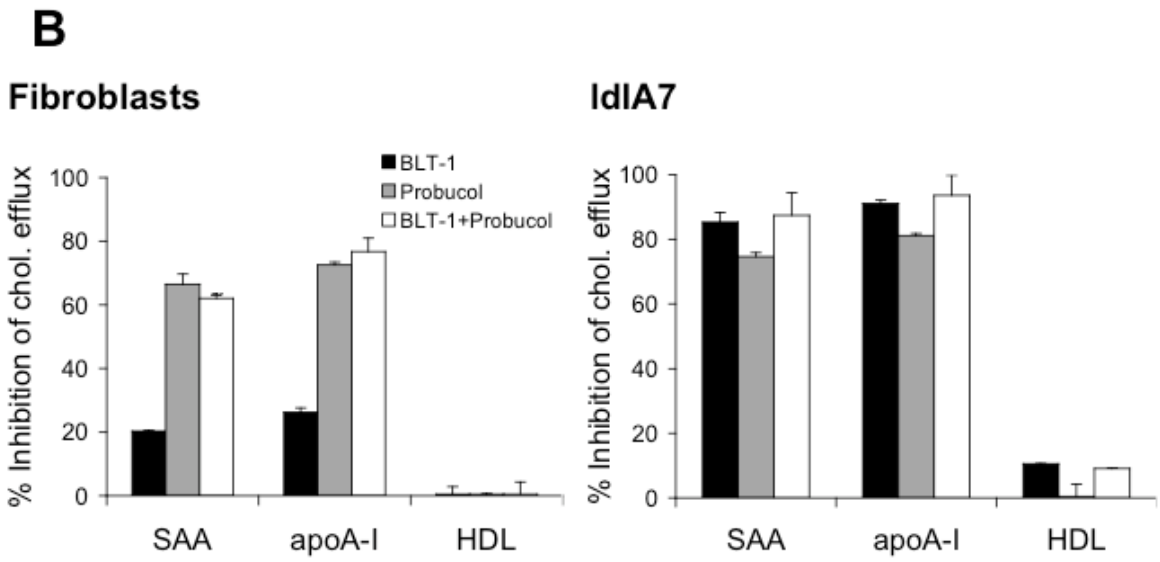
Figure 5B



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