Serum albumin acts as a shuttle to enhance cholesterol efflux from cells

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Abstract An important mechanism contributing to cell cholesterol efflux is aqueous transfer in which cholesterol diffuses from cells into the aqueous phase and becomes incorporated into an acceptor particle. Some compounds can enhance diffusion by acting as shuttles transferring cholesterol to cholesterol acceptors, which act as cholesterol sinks. We have examined whether particles in serum can enhance cholesterol efflux by acting as shuttles. This task was accomplished by incubating radiolabeled J774 cells with increasing concentrations of lipoprotein-depleted sera (LPDS) or components present in serum as shuttles and a constant amount of LDL, small unilamellar vesicles, or red blood cells (RBC) as sinks. Synergistic efflux was measured as the difference in fractional efflux in excess of that predicted by the addition of the individual efflux values of sink and shuttle alone. Synergistic efflux was obtained when LPDS was incubated with cells and LDL. When different components of LPDS were used as shuttles, albumin produced synergistic efflux, while apoA-I did not. A synergistic effect was also obtained when RBC was used as the sink and albumin as shuttle. The previously observed negative association of albumin with coronary artery disease might be linked to reduced cholesterol shuttling that would occur when serum albumin levels are low. Serum albumin acts as a shuttle to enhance cholesterol efflux from cells. J. Lipid Res. 2013. 54: 671–676.

Supplementary key words aqueous diffusion • coronary artery disease • low density lipoprotein

There are a number of mechanisms that have been shown to contribute to the flux of cholesterol molecules between cells and extracellular particles. These include pathways mediated by scavenger receptor class B type I (SR-BI), ATP binding cassette transporter A1 (ABCA1), ATP binding cassette transporter G1 (ABCG1), and unmediated aqueous transfer. All of these mechanisms have been extensively studied, and a number of reviews discuss them in detail (1–3). The earliest cholesterol flux mechanism that was identified was aqueous transfer, in which there is a description of free cholesterol (FC) molecules from a donor, such as a cell membrane, into the aqueous phase followed by diffusion of the molecules and their subsequent collision with and incorporation into an acceptor particle (such as a lipoprotein) (4). This diffusional movement of FC can be enhanced by what has been described as a “shuttle and sink” mechanism in which some compounds present in the aqueous phase can serve as FC shuttles, enhancing the flux of cholesterol between donor and acceptor particles (5, 6). Perhaps the best examples of shuttles are cyclodextrins, which act as catalysts that bind FC, greatly increasing the bidirectional flux of FC through the aqueous phase (6). Although the studies using cyclodextrins clearly demonstrated the feasibility of shuttles and sinks enhancing FC flux, there was no evidence that synergistic efflux could be the result of the interaction of physiologically relevant particles and that such a process was taking place in serum.

Little research has been done on the shuttle/sink mechanism since the initial reports. However, a recent publication by Hoang et al. (7) studying the flux of cholesterol in human sera after infusion of reconstituted HDL (rHDL) yielded data that were consistent with a shuttle/sink mechanism. The results of this study indicated that although rHDL are good cholesterol acceptors, they have only a limited capacity to store cholesterol. This property of rHDL would result in rHDL performing the role of a cholesterol shuttle. In contrast, the data from this infusion study...
indicated that the plasma reservoirs with a large capacity to hold cholesterol are the apoB-containing LDL and VLDL, thus playing the role of cholesterol sinks. In the present study, we have examined the possibility that a synergistic enhancement of cholesterol flux occurs through the interaction of components that are present in serum. For these studies, we have employed J774 mouse macrophages labeled with [3H]cholesterol as cholesterol donors, plasma components as shuttles, and LDL, small unilamellar vesicles (SUV), or red blood cells (RBC) as cholesterol sinks. To investigate synergistic effects, we quantitated the FC efflux from cells incubated with a constant amount of LDL, or other potential sinks, and increasing concentrations of lipoprotein-depleted sera (LPDS), or components present in LPDS. Synergy was measured as the difference in fractional efflux in excess of that predicted by the addition of the individual efflux values of sink and shuttle alone.

MATERIALS AND METHODS

Materials

Tissue culture plasticware was purchased from Thermo Fisher (Rochester, NY) and Corning (Corning, NY). Minimum essential medium Eagle (MEM), RPMI 1640, and Dulbecco’s phosphate-buffered saline (DPBS) were purchased from Mediatech Cellgro (Rochester, NY) and Corning (Corning, NY). MEM buffered with 10 mM HEPES (pH 7.4) was prepared in the laboratory using HEPES purchased from Bio-Whittaker (Walkersville, MD). Fetal bovine serum (FBS), gentamicin, trypsin EDTA, acyl-CoA cholesterol acyltransferase (ACAT) inhibitor (Sandoz 58-035), Cpt-cAMP, ovalbumin, and fatty acid-free BSA were purchased from Sigma-Aldrich (St. Louis, MO). As a local standard for gas liquid chromatography (GLC) analysis. A fraction of the extracted lipid was used to measure total cholesterol radioactivity incorporated into cellular lipids using scintillation counting. The remaining lipid was prepared for GLC to measure cholesterol mass as previously described (11). The extent of loss of cell FC mass was calculated based on the cholesterol content of the cells at t0, prior to the efflux phase. Cell protein was measured by the method described by Markwell et al. (12). Cell proteins were measured before the incubation period (w) and after the incubation period (samples); there were no statistically significant changes in cell growth between the end of equilibration period and at the end of efflux period.

Preparation of lipoproteins and lipoprotein-depleted serum

Human LPDS, HDL, LDL, and VLDL were isolated by sequential ultracentrifugation from serum obtained with approved consent from healthy, normolipemic individuals as previously described (HDL, d = 1.06–1.21; LDL, d = 1.01–1.06; and VLDL, d = 0.95–1.01) (8). To obtain acetylated low-density lipoprotein (acLDL), LDL was modified using acetic anhydride, as previously described (9). ApoA1 was purified from delipidated HDL using ethanol/diethyl ether followed by anion-exchange chromatography on a Q-Sepharose column. The apoA1 fractions were pooled, dialyzed against 5 mM NH4HCO3, lyophilized, and stored at −20°C. The apoA1 was resolubilized in 6 M guanidine hydrochloride and dialyzed extensively against saline (0.15 M NaCl, pH 7.4) prior to incubating with the cells. Prior to use, LPDS, HDL, LDL, and VLDL were all extensively dialyzed against 0.15 M NaCl (pH 7.4) and sterilized by filtration using a 0.45 µm Millipore filter. Egg PC SUVs were made as previously described (10).

Cell culture

J774 mouse macrophage stock cells were maintained in RPMI growth medium supplemented with 10% FBS and gentamicin.

For experiments, J774 cells were plated in RPMI growth medium in 24-well plates at a density of 150,000 cells/well for 24 h. Cholesterol-normal cells were labeled for 24 h with 1 µg/ml [3H]cholesterol in medium supplemented with 5% FBS and 2 µg/ml ACAT inhibitor. In experiments using cholesterol-enriched cells, J774 cells were incubated with the same labeling medium plus 25 µg/ml acLDL. After labeling plus or minus cholesterol enrichment, the cells were washed 2× with MEM HEPES and equilibrated with RPMI medium containing 0.2% BSA and cAMP (0.3 mmol/l) for 18 h. After this equilibration period, the cells were washed 2× with MEM-HEPES buffer and incubated with MEM-HEPES media containing exogenous acceptors alone or in combination (shuttle and sink) for 4 h, or as indicated in the figures, to measure the labeled FC efflux. Aliquots of media were collected to measure the release of radiolabeled cholesterol. J774 cell monolayers at time zero (t0) were washed 2× with DPBS, and cholesterol was extracted using isopropanol. The percentage efflux is calculated based on the [3H]cholesterol in the cells at t0. The ACAT inhibitor was used in all phases of experiments (from labeling to efflux phase) to ensure pools of radiolabeled cholesterol ester would not be present as this could complicate the determination of the fractional release of labeled cholesterol from the cells.

Protein and cholesterol mass determination

At the end of the efflux experiment, the cell monolayers were washed with DPBS, and cell lipid was extracted using 0.5 ml of 2-propanol containing 5 µg/ml of cholesteryl methyl ether (CME; Sigma, St. Louis, MO) as an internal standard for gas liquid chromatography (GLC) analysis. A fraction of the extracted lipid was used to measure total cholesterol radioactivity incorporated into cellular lipids using scintillation counting. The remaining lipid was prepared for GLC to measure cholesterol mass as previously described (11). The extent of loss of cell FC mass was calculated based on the cholesterol content of the cells at t0, prior to the efflux phase. Cell protein was measured by the method described by Markwell et al. (12). Cell proteins were measured before the incubation period (w) and after the incubation period (samples); there were no statistically significant changes in cell growth between the end of equilibration period and at the end of efflux period.

Statistical analysis

All statistical analyses were performed using Prism (4.0) software, GraphPad Inc. (San Diego, CA). Data were presented as mean ± SD. Statistical significance was determined by unpaired t test unless otherwise indicated. Significance was assessed at P < 0.05.

RESULTS AND DISCUSSION

Shuttle and sink mechanism

The earliest mechanism demonstrated to play a role in the movement of FC molecules between cells and lipoproteins was termed aqueous diffusion. In this proposed mechanism, molecules of FC desorb from the cell membrane and are then either incorporated into a phospholipid-containing acceptor, such as a lipoprotein, or reincorporated into the plasma membrane (4, 10, 13). This physicochemical mechanism is ubiquitous and contributes to the exchange of FC between cells and lipoproteins as well as the exchange of FC between lipoproteins (14). Because of the limited solubility of FC in an aqueous environment, the FC flux
between cells and acceptors by aqueous transfer is relatively inefficient, although a number of recent studies have demonstrated that aqueous transfer does contribute significantly to total cell FC efflux (2, 15). The fractional contribution of aqueous transfer to total efflux is determined by the level of other competing flux pathways present in the cell. The inefficiency associated with aqueous transfer could be enhanced if a cholesterol carrier were present that had the ability to loosely associate with FC and speed the flux between cholesterol donor and acceptor. A number of years ago this concept led us to propose a model termed “shuttle and sink” in which a cell would act as a FC donor, and a lipoprotein or phospholipid vesicle would serve as a FC acceptor (i.e., “sink”) (6, 16). Since these prior studies used nonphysiological compounds as shuttles and sinks (cyclodextrins and phospholipid vesicles), we initiated the present study to determine whether there are compounds in serum that act as shuttles, increasing the movement of FC between cells and lipoproteins that serve as sinks.

Shuttling by LPDS

The experimental design that allowed us to identify and quantify the contribution of shuttles to efflux employed human LDL as a sink and human LPDS as a source of potential shuttles. The fractional efflux of FC from [3H]cholesterol-labeled J774 cells produced by each of these two components, alone and in combination, was determined. Any efflux obtained from the combination of LDL and LPDS that was greater than the additive values of the LDL and LPDS alone resulted from synergy, attributable to the presence of a shuttle in the LPDS (see Fig. 1). This synergy was dependent on the LPDS dose (Fig. 2) and can be expressed either as the actual fractional efflux of the shuttle component (Fig. 2A) or as the percentage of the total efflux (Fig. 2B). Even though the absolute synergistic efflux values are relatively small (1–3.5% depending on LPDS concentration), the contribution of this efflux to the total efflux is much larger (1–25% depending on LPDS concentration, Fig.2B). In related experiments, the LDL was replaced with human VLDL which also served as a cholesterol sink, although the level of synergy was less than that obtained with LDL.

A series of experiments was conducted in an effort to identify the compound in serum that contributed to the synergy illustrated in Figs. 1 and 2. A likely candidate was HDL, and when added as a potential shuttle at concentrations ranging from 2.5 to 50 µg/ml, low levels of shuttling was produced at concentrations between 2.5 and 15 µg/ml, but no significant shuttling was evident at higher levels of HDL (Fig. 3). The presence of shuttling at low HDL concentrations and the loss of this effect as HDL was increased may be attributed to the high cholesterol efflux efficiency of HDL. Thus, as HDL concentration is increased and total efflux is elevated, the relative contribution of shuttling to total efflux becomes progressively less.

Since apolipoproteins could potentially serve as shuttles, we replaced the LPDS with increasing concentrations of human apoA-I ranging from 2 to 10 µg/ml. Interestingly, rather than a synergistic response, we observed a consistent reduction in total efflux so that the efflux obtained in a system with apoA-I and LDL was less than the additive values of apoA-I and LDL alone (average 36% reduction, range 26–42%, data not shown). The reason for this less than additive value is not clear, but it may be related to a possible interaction of apoA-I with either the LDL sink or the donor cells since these J774 cells expressed ABCA1. A somewhat similar phenomenon was reported by Zhao and Marcel (17) in which cholesterol efflux from fibroblasts to media containing both serum albumin and Lp2A-I HDL was less than the sum of the efflux induced by each component alone. Since it has been demonstrated that the activity of LCAT can influence the flux of FC between cells and lipoproteins by shifting the FC/phospholipid ratio of acceptor lipoproteins and thus shifting the FC equilibrium.

Fig. 1. Cholesterol efflux from J774 cells when LPDS is used as a shuttle and LDL as a sink. Radiolabeled J774 cells were incubated with increasing concentrations of LPDS alone or in combination with 25 µg/ml LDL. Cholesterol efflux was measured after 4 h. Synergy, expressed as percentage efflux per 4 h was calculated by subtracting the individual efflux values obtained by incubating the cells with LPDS or LDL alone from that of the total efflux obtained from the combination of LPDS and LDL. The synergy obtained was LPDS concentration dependent. % LDL efflux, open bar; % LPDS efflux, hatched bar; % synergy efflux, solid bar. Data are presented as mean ± SD (n = 9).

Fig. 2. Synergy obtained from J774 cells when LPDS is used as a shuttle and LDL as a sink. Radiolabeled J774 cells were incubated with increasing concentrations of LPDS alone or in combination with 25 µg/ml LDL, and cholesterol efflux was measured after 4 h. Synergy was calculated as the actual fractional efflux (difference between efflux values from the combination of LPDS+LDL and the additive value of LPDS and LDL added alone). Data are presented as the percentage efflux that is attributed to synergy (A, filled squares) or the percentage contribution of synergy to total efflux (B, filled circles). Both methods for expressing synergy were LPDS concentration dependent. Data are presented as mean ± SD (n = 9). The error bars are contained within the symbols.
between cells and lipoproteins (18–22), we treated the LPDS with n-ethylmaleimide (NEM) at a concentration that inhibited 95% of the LCAT activity present in LPDS. When added to the cell system at increasing concentration, the LPDS shuttling capacity was not changed by LCAT inhibition (data not shown).

Shuttling by albumin

Another component of LPDS that could be responsible for FC shuttling is albumin. A number of studies have demonstrated that some FC can associate with albumin (17, 23) and that albumin can serve as a cholesterol acceptor, although the fractional efflux produced by the incorporation of albumin in the media is less than that obtained with HDL or LPDS (17, 24, 25). It has been demonstrated that albumin mediates the bidirectional flux of cellular FC and functions as a low affinity, high capacity cholesterol transporter (17). These properties of albumin are similar to those of cyclodextrins, which have been shown to be efficient shuttles moving FC between donors and acceptors.

Data obtained with albumin demonstrate synergy when expressed as percentage synergy (Fig. 4A) or percentage of total efflux (Fig. 4B), similar to that obtained with LPDS (compare Fig. 2 with Fig. 4). There was no difference in synergy when BSA was compared with HSA (data not shown); however, replacement of BSA with ovalbumin demonstrated the specificity of serum albumin since no synergy was produced by ovalbumin (data not shown). This result could be anticipated, as Zhao and Marcel (17) previously demonstrated that ovalbumin could not stimulate the efflux of FC from fibroblasts when compared with HSA.

Even though there are no specific binding sites for FC on albumin (26) and the association of FC with purified albumin is limited, removal of albumin from plasma by immunoaffinity chromatography reduces total efflux by as much as 40% (23). Thus purified albumin added to the culture medium alone is a relatively poor promoter of efflux, whereas the presence of lipoproteins with albumin, as is the case with whole plasma, enhances efflux. In addition to studies in which cell cholesterol efflux to albumin was directly measured, we conducted an experiment to determine the change in fractional efflux upon removal of albumin from LPDS. The removal of albumin resulted in a 22% reduction in total efflux and a 91% reduction in the efflux attributed to synergy (see supplementary data for details).

LDL served as the cholesterol sink in the experiment presented in Figs. 1–4. Because LDL contains FC, the shuttling capacity of albumin contributes to both the influx of cholesterol into cells and the efflux of FC, thereby altering FC exchange. Under these conditions, movement of isotopic cholesterol out of the donor cells would be generally matched by the influx of unlabeled cholesterol into the cells (27–29). This bidirectional flux results in the appearance of [3H]cholesterol in the media, but no change in cell cholesterol mass. This was the case in a number of attempts to detect changes in the cholesterol content of donor J774 cells incubated with albumin and LDL that were unsuccessful. To measure the ability of albumin acting as a shuttle to influence cell cholesterol mass, it was necessary to provide a cholesterol sink that lacked cholesterol, thus eliminating the influx arm of the bidirectional flux system. In the present study, LDL was replaced with SUV, which served as a cholesterol-free sink. In such a system, reductions in cell FC mass could be measured after a short incubation time (6 h), as shown in Table 1. Using both FC normal and enriched cells, there was a significant loss of cell cholesterol mass with the combination of albumin as a shuttle and SUV as a sink, illustrating that the shuttling capacity of albumin can contribute to movements of cholesterol mass, if sufficient FC gradients are established. Under the experimental conditions used in this experiment, significant reductions in donor cell cholesterol mass were not observed with SUV or albumin alone (Table 1).

The data collected using either LDL or SUV as a sink are consistent with the role of albumin acting as a shuttle...
TABLE 1. Effect of albumin on the reduction of cholesterol mass from J774 cells when incubated with SUV

<table>
<thead>
<tr>
<th>Media Supplement</th>
<th>Normal Cells</th>
<th>Cells Enriched with AcLDL (25 µg/ml)</th>
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<tr>
<td></td>
<td>µg cholesterol/mg protein</td>
<td>µg cholesterol/mg protein</td>
</tr>
<tr>
<td>SUV (200 µg/ml)</td>
<td>14.7 ± 0.6</td>
<td>55.9 ± 0.2</td>
</tr>
<tr>
<td>BSA (1 mg/ml)</td>
<td>15.1 ± 0.4</td>
<td>50.6 ± 3.7</td>
</tr>
<tr>
<td>BSA+SUV</td>
<td>12.8 ± 0.3*</td>
<td>40.8 ± 3.8*</td>
</tr>
</tbody>
</table>

J774 cells were incubated for 6 h with media supplemented with 200 µg/ml of SUV, 1.0 mg/ml BSA, or a combination of BSA plus SUV. Total cholesterol from cell monolayers was measured using GLC and is expressed as µg cholesterol/mg protein. *Significantly different (P < 0.05, n = 3) compared with their corresponding t₀ value (t₀ for normal cells = 15.6 ± 0.6 µg cholesterol/mg protein; t₀ for cells enriched with 25 µg/ml of AcLDL = 54.1 ± 2.6 µg cholesterol/mg protein).

and enhancing the aqueous diffusion of FC, and not influencing flux via a receptor-mediated process. To assess the capacity of albumin to function as a shuttle, we conducted an experiment in which radiolabeled J774 cells served as FC donors and RBC as acceptor. Since aqueous diffusion of FC is ubiquitous and can play an important role in cell cholesterol flux, albumin acting as a shuttle exerts its effect by enhancing aqueous diffusion, as illustrated by the synergy observed when SUV or RBC served as FC acceptor. Since aqueous diffusion of FC is ubiquitous and can play an important role in cell cholesterol flux (15), the enhancement of aqueous diffusion by albumin could have a significant impact on cholesterol turnover in vivo. This might not be a major factor at normal serum albumin levels (4.0–5.6 g/dl) where the synergistic potential of albumin might be saturated and variations within this normal range will not influence the contribution of albumin to synergistic efflux. However, in hypoalbuminemic individuals with albumin levels less than 3.5 g/dl, changes in serum albumin levels may reduce synergistic cholesterol efflux. There have been numerous reports suggesting that lower serum albumin concentrations in humans may be associated with increased risk of coronary artery disease (CAD) (31, 92), and a number of hypotheses have been proposed to explain this association, including arterial inflammation, antioxidant effects, control of blood pressure, and association with HDL concentrations (33). It is possible that the association of albumin with CAD is a reflection of the ability of albumin to shuttle FC between a variety of acceptors. Such shuttling would increase the movement of FC down its concentration gradient between the numerous cholesterol pools present in both plasma and tissues, thereby facilitating the restoration of steady-state levels as cholesterol is metabolized. A particularly important cholesterol pool that could be influenced by the shuttling capability of albumin is RBC cholesterol. The cholesterol concentration of RBC is roughly equivalent to that found in plasma lipoproteins (34), and it has been recently shown that RBC plays a role in reverse cholesterol transport (34). FC in the membrane of RBC rapidly exchanges with other pools of cholesterol via an aqueous diffusion mechanism (35–37). Thus, both the flux of cholesterol between plasma lipoproteins and RBC, as well as the delivery of RBC cholesterol to hepatocytes (34), is enhanced by the ability of albumin to serve as a cholesterol shuttle.

Physiological significance

Although albumin does not have specific binding sites for cholesterol (26), it can solubilize small amounts of cholesterol; however, because of the high concentrations of albumin in plasma, the contribution of this protein to efflux from cells is considerable. This was demonstrated in an early study by Fielding and Moser (23) who showed that the removal of albumin from plasma by immunoaffinity resulted in a reduction of approximately 40% in cholesterol efflux from fibroblasts. A detailed study by Zhao and Marcel (17) indicated that albumin could participate in the bidirectional flux of cholesterol between fibroblasts and lipoproteins, although this ability to stimulate efflux is less than that obtained with HDL or reconstituted HDL (rHDL) particles. The ability of albumin to solubilize FC would allow it to function as a shuttle, similar to that shown to operate when cyclodextrins increase the flux of cholesterol between donor cells and acceptors, such as lipoproteins particles (30). Although there are a number of pathways that can participate in cell FC flux, albumin acting as a shuttle exerts its effect by enhancing aqueous diffusion, as illustrated by the synergy observed when SUV or RBC served as FC acceptor. Since aqueous diffusion of FC is ubiquitous and can play an important role in cell cholesterol flux (15), the enhancement of aqueous diffusion by albumin could have a significant impact on cholesterol turnover in vivo. This might not be a major factor at normal serum albumin levels (4.0–5.6 g/dl) where the synergistic potential of albumin might be saturated and variations within this normal range will not influence the contribution of albumin to synergistic efflux. However, in hypoalbuminemic individuals with albumin levels less than 3.5 g/dl, changes in serum albumin levels may reduce synergistic cholesterol efflux. There have been numerous reports suggesting that lower serum albumin concentrations in humans may be associated with increased risk of coronary artery disease (CAD) (31, 92), and a number of hypotheses have been proposed to explain this association, including arterial inflammation, antioxidant effects, control of blood pressure, and association with HDL concentrations (33). It is possible that the association of albumin with CAD is a reflection of the ability of albumin to shuttle FC between a variety of acceptors. Such shuttling would increase the movement of FC down its concentration gradient between the numerous cholesterol pools present in both plasma and tissues, thereby facilitating the restoration of steady-state levels as cholesterol is metabolized. A particularly important cholesterol pool that could be influenced by the shuttling capability of albumin is RBC cholesterol. The cholesterol concentration of RBC is roughly equivalent to that found in plasma lipoproteins (34), and it has been recently shown that RBC plays a role in reverse cholesterol transport (34). FC in the membrane of RBC rapidly exchanges with other pools of cholesterol via an aqueous diffusion mechanism (35–37). Thus, both the flux of cholesterol between plasma lipoproteins and RBC, as well as the delivery of RBC cholesterol to hepatocytes (34), is enhanced by the ability of albumin to serve as a cholesterol shuttle.

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TABLE 2. Effect of albumin on exchange of cholesterol between J774 cells and RBC

<table>
<thead>
<tr>
<th>Media Supplements</th>
<th>% [³H]cholesterol efflux / 2 h</th>
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<tr>
<td>RBC alone</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>BSA alone</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>BSA+RBC⁺⁺</td>
<td>2.5 ± 0.4</td>
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</table>

Radiolabeled J774 cells were incubated for 2 h with media supplemented with 5 × 10¹⁰ RBC, 1.5 mg/ml BSA, or a combination of BSA plus RBC. % efflux is based on the [³H]cholesterol in the J774 cells at time 0 (t₀).

⁺⁺Statistically different from BSA or RBC alone (P < 0.05, n = 6).
REFERENCES


