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Trafficking of Endogenous Smooth Muscle Cell Cholesterol: A Role for Serum Amyloid A and Interleukin-1ß

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Abstract

Objective—Intracellular cholesterol distribution impacts cell function, however processes influencing endogenous cholesterol trafficking remain largely unknown. Atherosclerosis is associated with vascular inflammation and these studies address the role of inflammatory mediators on smooth muscle cell cholesterol trafficking.

Methods and Results—Interestingly, in the absence of an exogenous cholesterol source, serum amyloid A increased [^{14}C] oleic acid incorporation into cholesteryl ester in rat smooth muscle cells, suggesting endogenous cholesterol trafficking to the endoplasmic reticulum. [^{3}H] cholesteryl ester accumulated in cells prelabeled with [^{3}H] cholesterol, confirming that serum amyloid A mediated the movement of endogenous cholesterol. Cholesterol movement was dependent upon functional endolysosomes. The cholesterol oxidase sensitive pool of cholesterol decreased in serum amyloid A-treated cells. Furthermore, the mechanism whereby serum amyloid A induced cholesterol trafficking was determined to be via activation of expression of secretory phospholipase A_2, group IIA ($sPLA_2$) and $sPLA_2$-dependent activation of sphingomyelinase. Interestingly, although neither tumor necrosis factor α nor interferon γ induced cholesterol trafficking, interleukin-1ß induced [^{14}C] cholesteryl ester accumulation that was also dependent upon $sPLA_2$ and sphingomyelinase activities. Serum amyloid A activates smooth muscle cell interleukin-1ß expression and although the interleukin-1 receptor antagonist inhibited the interleukin-1ß-induced cholesterol trafficking, it had no effect on the movement of cholesterol mediated by serum amyloid A.

Conclusions—These data support a role for inflammation in endogenous smooth muscle cell cholesterol trafficking from the plasma membrane to the endoplasmic reticulum.

Keywords

Cholesterol trafficking; smooth muscle cells; serum amyloid A; interleukin-1ß

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INTRODUCTION

The distribution of cholesterol varies widely among different cellular compartments such that the highest percentage (estimated to be greater than 60%) of the total cell cholesterol is found in the plasma membrane of cultured cells and a relatively low percentage (estimated to be approximately 5%) is found in the endoplasmic reticulum (1, 2). Cholesterol moves between cellular compartments and both vesicular and non-vesicular-mediated trafficking of cholesterol between organelles have been described. In addition to cholesterol movement within intracellular compartments, uptake from exogenous sources and efflux to cholesterol acceptors contribute to cholesterol homeostasis (1). Importantly, the distribution of cell cholesterol in various compartments is known to have functional implications ranging from signaling initiated at plasma membrane “lipid rafts” and caveolae to regulation of gene transcription by cholesterol in the endoplasmic reticulum (3-5).

It is believed that the differential equilibrium of cholesterol in various membranes is maintained by differences in lipid composition (6). Thus, it has been noted that the sphingomyelin content of the membrane is critical to maintaining levels of cholesterol and treatment with exogenous sphingomyelinase results in the liberation of plasma membrane cholesterol that moves to the endoplasmic reticulum (7, 8).

Little is known about what influences endogenous trafficking of cholesterol and in particular, whether inflammatory processes associated with atherosclerosis affect intracellular cholesterol movement. Acute phase serum amyloid A (SAA) and interleukin-1ß (IL-1ß) are expressed in atherosclerotic lesions (9, 10). Recently, we showed that SAA activates expression of another acute phase protein, secretory phospholipase A_2, group IIA ($sPLA_2$) in aortic smooth muscle cells (11). Likewise, IL-1ß induces smooth muscle cell $sPLA_2$ gene expression (11-14). This report addresses the hypothesis that SAA- and IL-1ß-induced $sPLA_2$ mediates cholesterol trafficking to the endoplasmic reticulum. The studies demonstrate that SAA and IL-1ß induce the trafficking of endogenous cholesterol to the endoplasmic reticulum in aortic smooth muscle cells and that the trafficking of cholesterol is dependent upon activation of expression of $sPLA_2$. This work demonstrates for the first time that inflammatory processes associated with atherosclerosis are likely to have an impact on vascular smooth muscle cell function due to changes in the distribution of intracellular cholesterol pools.

METHODS

Isolation, culture and treatment of neonatal rat aortic smooth muscle cells

In accordance with practices approved by Boston University’s Institutional Animal Care and Use Committee (IACUC), neonatal aortic smooth muscle cells were isolated by digestion of the aortae of three-day-old Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), as previously described (15, 16). The cells were seeded in Dulbecco’s modified Eagle’s Medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM MEM non-essential amino acids and 1 mM MEM sodium pyruvate solution (DMEM; all from Mediatech, Inc., Manassas, VA) supplemented with 20% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells maintained in primary stage for approximately 1 week were then seeded into first passage at a density of 2×10^4 cells/cm^2 in DMEM containing 10% FBS. Experiments were performed on cells in passages 1 - 3, plated at a density of 2×10^4 cells/cm^2. Before the addition of experimental reagents, the media were removed, the cells washed twice and media containing 10% lipoprotein-deficient serum (LDS), prepared as described previously (15, 17), were added. ß-migrating very low density lipoprotein **(**ßVLDL) was isolated as previously described by density gradient

ultracentrifugation from serum of male New Zealand White rabbits fed a diet rich in cholesterol and peanut oil (15, 17). Recombinant SAA (PeproTech, Rocky Hill, NJ) was used at concentrations of 2 - 4 μM as activity of various preparations differs somewhat. The reagents [ßVLDL, SAA, IL-1ß (eBioscience, San Diego, CA), murine tumor necrosis factor α (TNFα; Peprotech), rat interferon γ (IFNγ; eBioscience)] were then added unless inhibitors [chloroquine (MP Biomedicals, Solon, OH), bafilomycin A1 (Sigma-Aldrich, St. Louis, MO), U18666A (Sigma-Aldrich), Ro 23-9358 (Sigma-Aldrich), arachidonyl trifluoromethylketone (AACOCF3; Enzo Life Sciences, Plymouth Meeting, PA), 3-O-methyl sphingomyelin (3-OMe sphingomyelin; Enzo Life Sciences), chicken egg yolk sphingomyelin (Sigma-Aldrich) and IL-1 receptor antagonist (IL-1Ra; R&D Systems, Minneapolis, MN)] were used, in which case they were added 1 hour prior to the addition of SAA or IL-1ß. To study the effect of lipid-associated SAA, high density lipoprotein (HDL; Calbiochem, La Jolla, CA)-associated SAA was prepared in DMEM-LDS by adding HDL (150 μg protein/ml) followed by SAA (2 μM) and incubating first at room temperature for 15 min while shaking, followed by 15 min at 37°C. Samples were then added to the cells and comparisons were made to similarly-treated samples containing SAA or HDL alone. Media (and reagents) were changed twice weekly and always 1 day prior to harvest.

[^{14}C] Oleic acid and [^{3}H] cholesterol incorporation into cholesteryl ester

[^{14}C] oleic acid incorporation into cholesteryl ester was determined essentially as described (17). Briefly, cells were incubated with reagents in media containing 10% LDS in the presence of [^{14}C] oleic acid (Perkin Elmer, Waltham, MA; NEC-317) complexed to albumin (0.43 μCi/ml). At the end of the indicated incubation, the lipids were extracted from the cell layer with hexane:isopropyl alcohol (3:2) and [^{14}C] cholesteryl oleate accumulation determined by thin layer chromatography (either by liquid scintillation counting or autoradiographic imaging of cholesteryl ester spots; if the latter was used, the data were normalized to account for the difference in efficiency between the techniques). In addition, cell layer protein was solubilized with 0.2 N NaOH and total protein determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific Inc., Rockford, IL) as per manufacturer's instructions. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD.

A double label approach was also used to determine if endogenous cholesterol trafficked to the endoplasmic reticulum. Cells were prelabeled with [^{3}H] cholesterol (Perkin Elmer, NET-139) by removing media, washing twice and incubating overnight with [^{3}H] cholesterol (2-3 μCi/ml) in DMEM containing LDS. Cells were washed 4-5 times, medium containing LDS was added and cells allowed to incubate overnight to equilibrate cellular cholesterol pools. Cells were treated with (or without) SAA and [^{14}C] oleic acid as described above. At the end of the indicated incubation, lipids were extracted with hexane/isopropyl alcohol (3:2) and [^{14}C] cholesteryl oleate accumulation and [^{3}H] cholesteryl ester determined by subjecting the extracts to thin layer chromatography. In addition, cell layer protein was solubilized with 0.2 N NaOH and total protein determined. The data are expressed as [^{14}C] cholesteryl oleate or [^{3}H] cholesteryl oleate (dpm)/μg protein ± SD.

Cell proliferation assay

Cell proliferation was determined using the MTT Cell Proliferation Assay (American Type Culture Collection, Manassas, VA) according to manufacturer's instructions.

Cholesterol oxidase-sensitive pool of cholesterol

Cells were labeled with [^{3}H] cholesterol as described above. After equilibration, cells were treated with SAA for 7 days, at which time the cholesterol oxidase-sensitive pool of cholesterol was determined essentially as previously described (18). Briefly, media were

removed, cells were washed with 1% fatty acid free bovine serum albumin in TBS (50 mM TRIS pH 7.5, 150 mM NaCl), then with phosphate buffered saline (PBS; 21-040, Mediatech, Inc.), fixed with 1% glutaradehyde in PBS, washed with PBS, and those cells to be treated received cholesterol oxidase (Sigma-Aldrich, C8649; 2 units/ml DMEM) for 30 min at 37°C. Cells were washed with PBS, lipids were extracted with hexane:isopropyl alcohol (3:2) and subjected to thin layer chromatography using a developing solution of hexane:ethyl ether:glacial acetic acid (130:30:2). Spots corresponding to cholesterol and cholestenone were measured by liquid scintillation counting. The data are expressed as [^{3}H] cholestenone as a percentage of the total [^{3}H] cholesterol ± SD calculated as follows: {[^{3}H] cholestenone (cpm)/{[^{3}H] free cholesterol (cpm) + [^{3}H] cholestenone (cpm)}} × 100.

Enzyme activity assays

The activity of $sPLA_2$ in culture media harvested from cells treated in the presence or absence of SAA was determined using a commercially available kit (Cayman Chemical, Ann Arbor, MI) as previously described (11). Enzyme activity is expressed as a function of the area of the cell culture surface (nmol product/min/cm^2) ± SD.

To determine neutral sphingomyelinase activity using the commercially available Amplex® Red Sphingomyelinase Assay Kit (Invitrogen, Grand Island, NY), cells treated in the presence or absence of SAA were washed twice with cold PBS on ice and then scraped in 1X reaction buffer supplied with the kit, frozen at −80°C overnight, then sonicated twice and centrifuged at 3000 × g for 10 min at 4°C. The supernatants were evaluated for enzyme activity according to the manufacturer's instructions with fluorescence readings taken at 1 min intervals for 1 hour, with an excitation wavelength of 530 nm and emission at 590 nm. Supernatants were also evaluated for total protein (BCA) and enzyme activity is expressed as the relative fluorescence emission units (mRFU) per min during the linear range of enzyme activity, normalized to the total cell protein (mRFU/min/μg protein ± SD).

Real time polymerase chain reaction (PCR)

As previously described (11), total RNA was extracted, reverse transcribed and the resultant cDNA used to determine mRNA expression levels of rat Pla2g2a by real time PCR analysis using SYBR Green primers synthesized by Eurofins MWG Operon (Huntsville, AL) (5′GTGACTCATGACTGTTGTTAC3′ and 5′CAAAACATTCAGCGGCAGC3′). An 18S rRNA TaqMan primer set (Applied Biosystems, Grand Island, NY) was used for normalization. Calculations were carried out using the ΔΔCt method of relative quantitation (if expression levels were low such that there was no Ct value after 40 cycles, data were calculated using a Ct of 40. The data are expressed as relative mRNA levels ± SD (with expression in SAA- or IL-1ß-treated cells set to a value of 1).

Statistical analyses

The data, expressed as mean ± SD of samples from representative experiments, were analyzed by analysis of variance. Statistically significant differences of relevant comparisons were determined by Bonferroni post-hoc analysis and reported when $P<0.05$.

RESULTS

An acute phase protein, $sPLA_2$ is a member of the phospholipase family that cleaves fatty acids in the sn2 position of the phospholipid backbone. Its expression is stimulated by inflammatory cytokines including IL-1β (19). Moreover, we showed that SAA activates smooth muscle cell expression of the Pla2g2a gene, resulting in an increase in $sPLA_2$ activity (11). It has been shown that arachidonic acid stimulates the activity of sphingomyelinase (20) and that sphingomyelinase-mediated degradation of plasma

membrane-associated sphingomyelin induces cholesterol movement to the endoplasmic reticulum (7). Thus, a hypothesis was formulated, that SAA-induced smooth muscle cell $sPLA_2$ expression increases endogenous sphingomyelinase activity, thereby liberating plasma membrane-associated cholesterol that mobilizes to the endoplasmic reticulum. To test the hypothesis, rat smooth muscle cells were treated with SAA and cholesterol trafficking to the endoplasmic reticulum was monitored. Free cholesterol is esterified in the endoplasmic reticulum by acyl coenzyme A:cholesterol acyltransferase and measurements of cholesterol esterification reflect the accumulation of cholesterol in this compartment (2). Thus, to test the potential role of acute phase SAA on cholesterol movement to the endoplasmic reticulum, the incorporation of [^{14}C] oleic acid into [^{14}C] cholesteryl ester was measured in cultured aortic smooth muscle cells. Figure 1 shows that as expected, supplying the cells with an exogenous source of cholesterol from hyperlipoproteinemic ßVLDL (containing 0.4 or 2.0 μg protein/ml) increased cholesterol esterification. Interestingly, the data show that SAA stimulated [^{14}C] cholesteryl ester accumulation, supporting the notion that SAA induced the movement of cholesterol to the endoplasmic reticulum. It is noteworthy that although the higher concentration of ßVLDL increased accumulation of [^{14}C] cholesteryl ester in comparison to the lower dose, the increase did not reflect the 5-fold increase in lipoprotein concentration, suggesting that cholesterol mobilization by ßVLDL in the endoplasmic reticulum was at or near maximum and that SAA was therefore quite efficient in inducing cholesterol movement.

To further verify the role of endogenous cholesterol movement by SAA, a double label approach was taken. To study the movement of cell-associated cholesterol, smooth muscle cells were prelabeled with [^{3}H] cholesterol. SAA was then added in the presence of [^{14}C] oleic acid and at 3, 6 and 24 hours, the cells were harvested and the accumulation of both [^{14}C] cholesteryl ester and [^{3}H] cholesteryl ester were studied. The data in Figures 2A and 2B show that there was a time-dependent accumulation of cholesteryl ester derived either from exogenous [^{14}C] oleic acid or cell-associated [^{3}H] cholesterol, respectively. These data confirm that SAA mediated the movement of endogenous cholesterol to the endoplasmic reticulum. It has been shown that both vesicular and non-vesicular pathways of cholesterol trafficking exist (21). To determine whether cholesterol movement by SAA is mediated by the endolysosomal system, its trafficking was measured in the presence of inhibitors of lysosomal function. As it is known that exogenous cholesterol delivered to the endoplasmic reticulum via ßVLDL requires lysosomes(22), it was first determined whether cholesterol movement to the endoplasmic reticulum mediated by ßVLDL could be inhibited in these cultures using known inhibitors. The data (not shown) demonstrated that pretreatment with either chloroquine (10 μM), bafilomycin A1 (10 nM) or the hydrophobic amine U18666A (5 μM) (23) inhibited ßVLDL-induced cholesterol esterification, so these conditions were used to determine if lysosomal function is necessary for SAA-mediated cholesterol transport. Moreover, using the MTT assay, it was shown that inhibiting lysosomal function for 24 hours did not cause cell toxicity (data not shown). The data in Figure 3 show that the SAA-induced increase in cholesteryl ester accumulation was inhibited by treatment with chloroquine, bafilomycin A1 or U18666A.

To determine if chronic SAA-mediated cholesterol trafficking resulted in a decrease in plasma membrane cholesterol, cells prelabeled with [^{3}H] cholesterol were treated with (or without) SAA for 7 days, at which time the cholesterol oxidase-sensitive pool of cholesterol was determined. SAA-treated cells had less plasma membrane cholesterol as evidenced by a significant decrease of cholesterol oxidase-sensitive cholesterol when compared to control-treated cells (Figure 4).

The role of $sPLA_2$ in SAA-induced trafficking of cholesterol to the endoplasmic reticulum was explored using an inhibitor of $sPLA_2$ activity, Ro 23-9358. Previously, we showed that

Ro 23-9358 inhibits the activity of SAA-induced $sPLA_2$ in media harvested from smooth muscle cells that were treated with SAA (11). To determine its effectiveness when incubated directly with the cells, it was added to the cultures and 24 hours later, media were collected and tested for $sPLA_2$ activity. The data in Figure 5A indicate that Ro 23-9358 decreased the SAA-induced $sPLA_2$ activity that accumulated during the time of incubation with SAA. Cholesterol esterification was measured in cells pretreated with Ro 23-9358 prior to the addition of SAA and Ro 23-9358 decreased the SAA-induced cholesterol trafficking, suggesting a role for $sPLA_2$ in this process (Figure 5B). Likewise, the inhibition of SAA-mediated expression of $sPLA_2$ in the presence of HDL as we previously reported (11) (Figure 5C) also inhibited cholesterol trafficking to the endoplasmic reticulum (Figure 5D).

To determine if cytosolic PLA_2, group IV ($cPLA_2$) plays a role in SAA-induced cholesterol trafficking, [^{14}C] cholesteryl ester accumulation was measured in cells treated with SAA in the presence of AACOCF3, an inhibitor of $cPLA_2$ activity (24). Interestingly, AACOCF3 completely blocked SAA-induced cholesterol trafficking (Figure 6A); moreover, inhibition of $cPLA_2$ prevented the SAA-induced expression of $sPLA_2$ (Figure 6B).

Jayadev *et al.*, (20) showed that free arachidonic acid, a product of PLA_2 activity, stimulates the activity of sphingomyelinase. Previous studies have shown that sphingomyelinase stimulates energy-independent transport of cholesterol to the endoplasmic reticulum (25). A role for sphingomyelinase in SAA-mediated cholesterol trafficking was explored; neutral sphingomyelinase activity was measured and SAA induced a chronic increase in activity (Figure 7A). To determine if the increase in sphingomyelinase activity contributed to the SAA-mediated cholesterol trafficking, cells were treated with 3-OMe sphingomyelin to inhibit sphingomyelinase activity (26) and it completely inhibited SAA-induced cholesterol trafficking (Figure 7B). Moreover, replenishing the cells with sphingomyelin prevented SAA-induced cholesterol trafficking (Figure 7C).

Other inflammatory mediators were evaluated for their ability to traffic cholesterol. As we and others showed previously (11-14), IL-1ß activated the expression of $sPLA_2$ mRNA but neither TNFα nor IFNγ activated $sPLA_2$ mRNA expression (Figure 8A). Interestingly, consistent with our hypothesis on the role of $sPLA_2$ in cholesterol trafficking, neither TNFα nor IFNγ induced cholesterol esterification but cholesteryl ester accumulation increased in cells treated with IL-1ß (Figure 8B). Moreover, inhibiting $sPLA_2$ activity with Ro 23-9358 blocked the IL-1ß-induced cholesterol esterification (Figure 8C). Likewise, inhibiting sphingomyelinase activity with 3-OMe sphingomyelin completely reversed the IL-1ß-mediated movement of cholesterol (Figure 8D).

We previously showed that SAA activates smooth muscle cell IL-1ß gene expression (11), so the possibility that the effect of SAA on cholesterol trafficking was mediated by IL-1ß signaling was explored. IL-1Ra inhibited the IL-1β-induced cholesterol esterification but SAA-induced trafficking was unaffected by IL-1Ra (Figure 9).

DISCUSSION

It is well known that cholesterol entering the cell from extracellular sources *i.e.* from plasma-derived lipoprotein, traffics to the endoplasmic reticulum, thereby contributing to stringent regulation of cellular lipid metabolism (5). However, the influences of endogenous cholesterol trafficking remain largely unexplored. Moreover, although it is known that the distribution of cholesterol influences cell function, the role of inflammation on cholesterol repositioning has not been addressed. Smooth muscle cells are critical to proper vascular function; however, functional changes induce a phenotype that contributes to lesion formation in atherosclerosis (27). Therefore, the mechanisms inducing cholesterol

movement in this cell type are of considerable interest. Recently, we reported that SAA activates smooth muscle cell expression of the $sPLA_2$ gene (11) and it has been shown that IL-1ß activates smooth muscle cell $sPLA_2$ gene expression (11-13). This report examines the hypothesis that SAA induces the trafficking of endogenous plasma membrane cholesterol to the endoplasmic reticulum in aortic smooth muscle cells and that the trafficking is dependent upon $sPLA_2$ and sphingomyelinase activities. Moreover, the hypothesis that $sPLA_2$ induced by IL-1ß also mobilizes cholesterol to the endoplasmic reticulum was studied. The data show that smooth muscle cell cholesterol esterification was stimulated by SAA as well as by IL-1ß and that the accumulation of cholesterol in the endoplasmic reticulum was $cPLA_2$-, $sPLA_2$- and sphingomyelinase-dependent. The data support the hypothesis that the activation of expression of $sPLA_2$ results in the liberation of free fatty acids that activate endogenous sphingomyelinase which degrades plasma membrane sphingomyelin, resulting in the release of plasma membrane cholesterol and its trafficking to the endoplasmic reticulum.

Evidence that supports a role for $sPLA_2$ in SAA-induced cholesterol trafficking to the endoplasmic reticulum includes the finding that the pharmacologic inhibitor of $sPLA_2$ activity, Ro 23-9358, decreased the SAA-induced cholesterol trafficking. Ro 23-9358 didn't fully inhibit the SAA-mediated trafficking of cholesterol but it was noted that the inhibition of SAA-induced $sPLA_2$ activity was not complete under these experimental conditions. In our previous report (11), the inhibitor was added directly to media after it was harvested from SAA-treated cultures and even in lower doses than reported here, it was more effective in reducing enzyme activity than what is shown in Figure 5A. This discrepancy between the efficacy of Ro 23-9358 added to the cell cultures prior to the incubation of SAA *vs.* its efficacy when added to the enzyme-containing media samples just before assaying activity is presumably due to a loss of activity of the inhibitor during the 24 hour incubation. It is likely that the lack of an even more robust reduction in SAA-induced cholesterol trafficking by Ro 23-9358 was due to this loss of activity with time in culture *i.e.* the role of $sPLA_2$ in this process is likely more profound than the pharmacologic inhibitor studies indicated.

Slotte and Bierman (7) first demonstrated that neutral sphingomyelinase treatment of skin fibroblasts results in the movement of cholesterol to the acyl coenzyme A:cholesterol acyltransferase-sensitive pool as measured by cholesterol esterification. Moreover, arachadonic acid, a product of PLA_2 enzymes was shown to stimulate the activity of sphingomyelinase (20). These studies show that SAA activated neutral sphingomyelinase activity, and that inhibition of neutral sphingomyelinase or exogenous addition of sphingomyelin prevented the SAA-mediated trafficking of cholesterol. Chatterjee (28) showed that TNFα activates neutral sphingomyelinase and induces cholesteryl ester accumulation in human skin fibroblasts and it is interesting to speculate that this too was an $sPLA_2$-mediated effect, particularly in light of the finding that arachadonic acid mediates TNFα-induced sphingomyelin hydrolysis in HL-60 cells (20). PLA_2 isolated from Naja Naja caused, if anything, a small decrease in cholesterol ester accumulation in isolated renal tubules (29) and this could represent the difference in the source of PLA_2 such that the endogenous enzyme might be effective due to parameters including proper localization of the reaction products, making them available to activate the endogenous sphingomyelinase. It has been shown by others that TNFα and IFNγ activate the expression of $sPLA_2$. TNFα-mediated expression of $sPLA_2$ was shown in endothelial cells (30), HepG2 cells (31), rat mesangial cells (32), astrocytes (33, 34) and fetal rat calvarial bone-forming cells (35). Lindbom et al. showed that IFNγ induced $sPLA_2$ expression in human airway epithelial cells, whereas TNFα did not (36). Of particular interest is the study using human smooth muscle cells in which IFNγ induced $sPLA_2$ expression whereas TNFα only caused a transient increase in expression and in fact, it antagonized the IFNγ-mediated response (37). In another study, TNF increased $sPLA_2$ expression in rat smooth muscle cells (38). In the

current report however, neither IFNγ nor TNFα induced cholesterol trafficking tothe endoplasmic reticulum (Figure 8B), consistent with the lack of induction of $sPLA_2$ expression (Figure 8A).

It has been suggested that sphingomyelin and cholesterol form complexes of higher affinity than those formed between phosphatidyl choline and cholesterol. Recently however, the nature of the physical interaction between sphingomyelin and cholesterol has been questioned and an alternative explanation for sphingomyelinase-induced cholesterol trafficking has been suggested, which is that ceramide generated by sphingomyelinase might displace cholesterol from the membrane (1). In any case, degradation of sphingomyelin in the plasma membrane leads to a liberation of cholesterol from that pool and in the studies reported here, the SAA- or IL-1ß-induced increase in the activity of sphingomyelinase mediated the release of cholesterol, enabling its trafficking.

Resynthesis of sphingomyelin restored cholesterol to the plasma membrane within hours after treatment of BHK-21 cells with sphingomyelinase (39). In this study, cells were treated with SAA for 24 hours or more and accumulation of cholesteryl ester measured during that time. Moreover, sphingomyelinase activity increased and the cholesterol oxidase-sensitive pool of cholesterol *i.e.* the plasma membrane cholesterol, decreased in cells chronically treated with SAA for up to 7 days. These data suggest that the effects of chronic exposure during atherogenesis can potentially overcome physiologic mechanisms that maintain cholesterol pools when not challenged. There is evidence that the so-called acute phase isoforms of SAA are expressed constitutively in low levels (40, 41) in normal tissues, suggesting that SAA may affect normal tissue function and/or host defense. It is interesting to consider the possibility that the ability of SAA to mobilize endogenous cholesterol stores might play a role in this regard.

Previous work has suggested crosstalk between $sPLA_2$ and $cPLA_2$ enzymes in that $cPLA_2$ activity impacts on IL-1ß/TNFα-induced $sPLA_2$ expression in rat fibroblastic 3Y1 cells (42, 43) and IL-1β/TNFα-induced expression of $cPLA_2$ increases by introducing $sPLA_2$ either by exogenous addition or by transfection in the $sPLA_2$-deficient mouse MC3T3-E1 cell line (44). Likewise, $sPLA_2$ activates rat glomerular mesangial cell $cPLA_2$ (45). Moreover, studies in P388D1 macrophages and overexpression of $sPLA_2$ and $cPLA_2$ in HEK293 cells indicate functional crosstalk such that the activity of $sPLA_2$ is dependent upon the action of $cPLA_2$ (43, 46, 47). The data shown here support a role for $cPLA_2$ in the $sPLA_2$-mediated cholesterol trafficking induced by SAA due to a $cPLA_2$-stimulated increase in expression of $sPLA_2$.

The functions of the SAA family, the members of which are the products of 4 genes, remain uncertain (9, 41). In inflammatory conditions, circulating plasma levels of the acute phase isoforms can be quite high, exceeding 1 mg/ml (48). Elevated plasma SAA has been shown to be an indicator of an increased risk for heart disease (49-51). SAA is expressed in human and mouse atherosclerotic lesions (52-55), suggesting that it may play a role in atherosclerosis, although whether SAA is pro-atherogenic or anti-atherogenic is controversial (9). Our previous work demonstrated that IL-1α induces synthesis of SAA in cultured aortic smooth muscle cells (56) and that SAA down-regulates endogenous lipid biosynthesis (57). It is intriguing to speculate that due to an inflammatory process in the developing plaque, available IL-1α induces local synthesis of SAA by smooth muscle cells and that the SAA then acts in an autocrine fashion to induce intracellular movement of smooth muscle cell plasma membrane cholesterol to the endoplasmic reticulum. Likewise, IL-1ß, which accumulates in atherosclerotic lesions and is known to activate smooth muscle cell $sPLA_2$ expression, may contribute to functional changes due to the redistribution of intracellular cholesterol pools.

In summary, this study demonstrates that SAA and IL-1ß induce aortic smooth muscle cell trafficking of endogenous cholesterol via $cPLA_2$-dependent induction of expression of $sPLA_2$, the products of which in turn activate sphingomyelinase, which then cleaves plasma membrane sphingomyelin leading to the liberation of cholesterol that then traffics to the endoplasmic reticulum (Figure 10). To our knowledge, this is the first demonstration of regulation of intracellular trafficking of endogenous smooth muscle cell cholesterol by the inflammatory mediators SAA and IL-1ß. This represents a novel mechanism for regulation of smooth muscle cell cholesterol trafficking with potential for a role in inflammation-induced changes in cell function during atherosclerosis.

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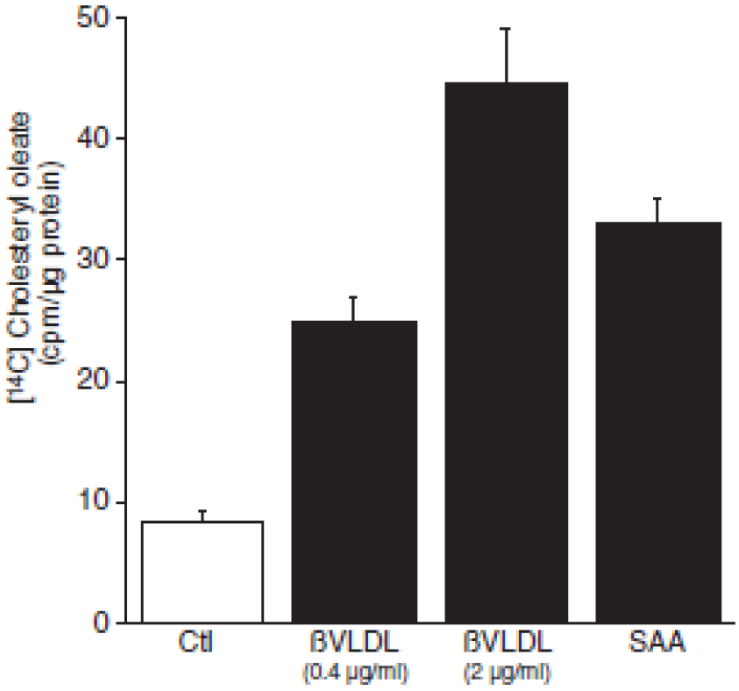

Figure 1.

Serum amyloid A (SAA) induces cholesterol trafficking to the endoplasmic reticulum. Smooth muscle cells were treated in the absence (control [Ctl]) or presence of β-mignating very-low-density lipoprotein (βVLDL: at the indicated dose) or SAA (4 μmol/L) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate(cpm)/μg protein ± SD(n=3). [^{14}C] cholesteryl oleate accumulation in SAA-treated cells (P<0.001) and βVLDL-treated cells at both doses (P<0.001) was significantly different from that in Ctl-treated cells.

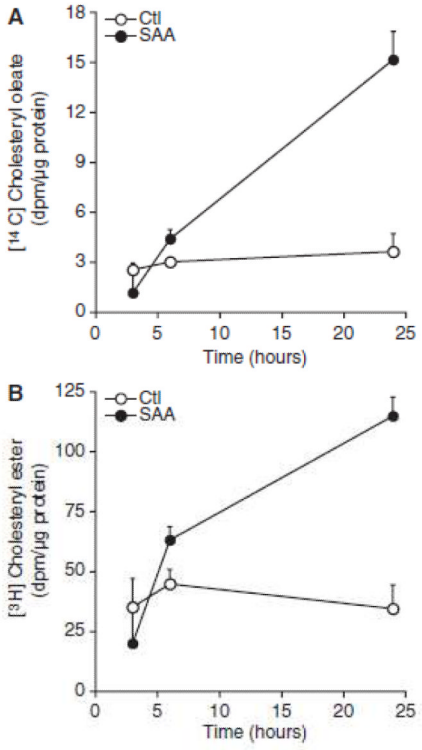

Figure 2.

Serum amyloid A (SAA) induces endogenous cholesterol trafficking to the endoplasmic reticulum. Smooth muscle cells were prelabeled with [^{3}H] cholesterol as described in the Materials and Methods section. After an overnight equilibration, cells were treated in the absence (control [Ctl]) or presence of SAA (2 μmol/L with [^{14}C] oleic acid. At the indicated time points, (**A**) [^{14}C] cholesteryl oleate and (**B**) [^{3}H] cholesteryl ester accumulation were measured as described in the Materials and Methods section The data are expressed as (**A**) [^{14}C] cholesteryl oleate (dpm)/μg protein ± SD (n=3) and (**B**) [^{3}H] cholesteryl ester (dpm)/μg protein ± SD (n=3). **A**, [^{14}C] cholesteryl oleate accumulation in SAA-treated cells at 6 hours and 24 hours (P<0.05) was significantly different from that in Ctl-treated cells. **B**, [^{3}H] cholesteryl Oleate accumulation in SAA-treated cells at 24 hours (P<0.05) was significantly different from that in Ctl-treated cells.

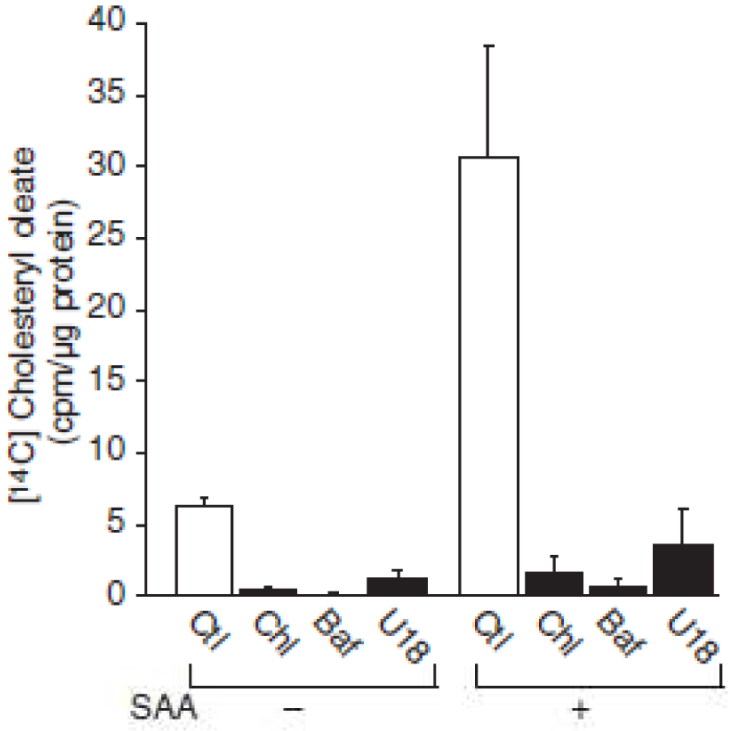

Figure 3.

Serum amyloid A (SAA)–mediated cholesterol trafficking is lysosome–dependent. Smooth muscle cells were treated in the absence or presence of SAA (4 μmol/L) and either no inhibitor (control [Ctl]), chloroquine (Chl; 10 μmol/L), bafilomycin A1 (Baf; 10 nmol/L), or U18666A (U18; 5 μmol/L) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section, The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation In Chl-treated (P<0.05). Baf-treated (P<0.05), and SAA-treated cells (P< 0.001) was significantly different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate accumulation in SAA plus Chl-treated (P<0.001), SAA plus Baf-treated (P<0.001), and SAA plus U18-treated cells (P<0.001) was significantly different from that in SAA-treated cells.

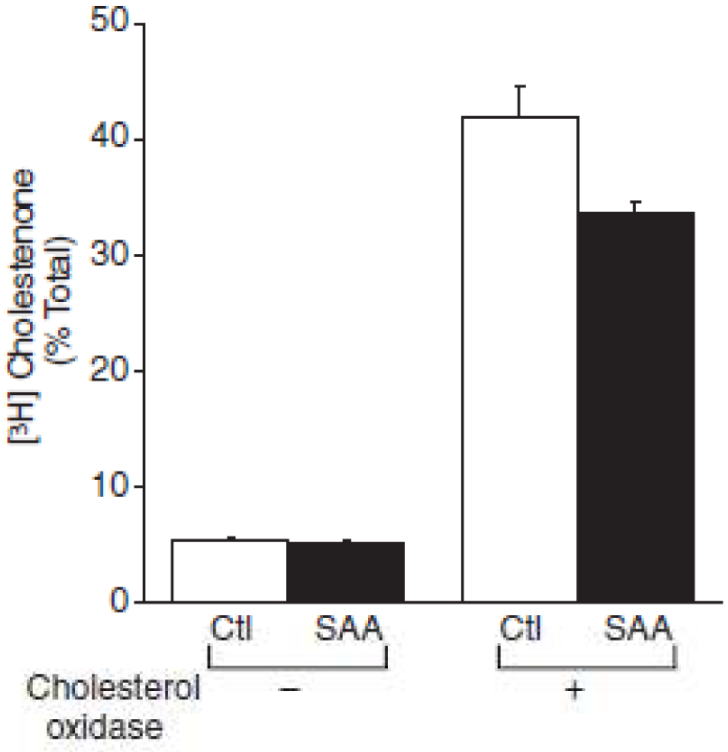

Figure 4.

Serum amyloid A (SAA) decreases plasma membrane cholesterol. Smooth muscle cells were prelabeled with [^{3}H] cholesterol and then treated In the absence (control [Ctl]) or presence of SAA (2 μmol/L) for 7 days, at which time the cholesterol oxidase–sensltlve pool of cholesterol was determined as described in the Materials and Methods section. The data are expressed as [^{3}H] cholestenone as a percentage of the total [^{3}H] cholesterol ± SD (n=3) calculated as follows: ([^{3}H] cholestenone [cpm]/{[^{3}H] free cholesterol [cpm]+[^{3}H] cholestenone [cpm]})×100 ± SD (n=3). The percentage of [^{3}H] cholestenone in SAA-treated cells (P< 0.001) was significantly different from that in control (Ctl)-treated cells In the samples treated with cholesterol oxidase.

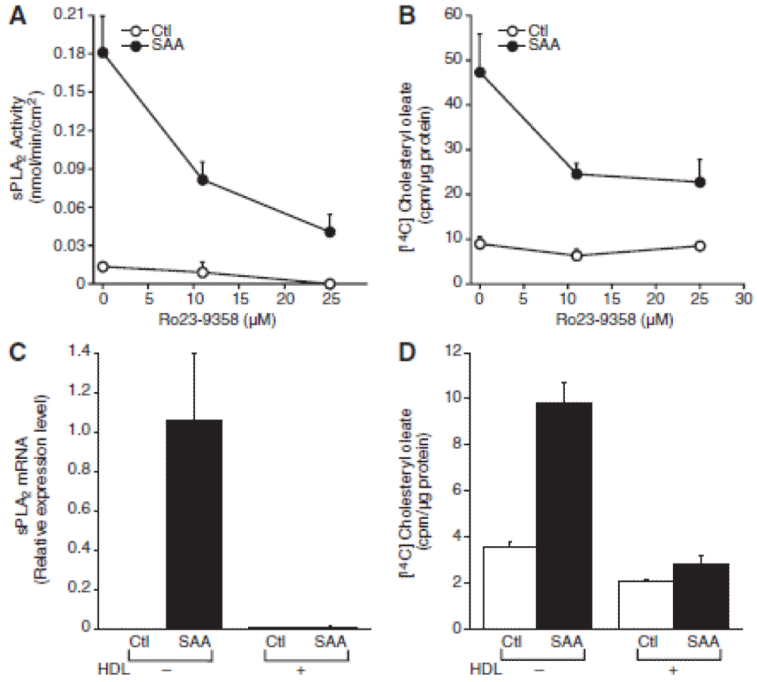

Figure 5.

Serum amyloid A (SAA)-mediated cholesterol trafficking is secretory phospholipase A_2, group IIA [$sPLA_2$-dependent. **A**, Smooth muscle cells were treated in the absence or presence of Ro23–9358 (at the indicated dose) and either in the absence (control [Ctl] or presence of SAA (4 μmol/L) for 24 hours, at which time $sPLA_2$ enzyme activity was measured as described in the Materials and Methods section. The data are expressed as nmol product/min/cm^2 ± SD (n=3). Enzyme activity in SAA-treated cells (P<0.0001) was significantly different from that in Ctl-treated cells. Enzyme activity in SAA plus Ro23-9358-treated cells at both doses (P<0.0001) was significantly different from that in SAA-treated cells. **B**, Smooth muscle cells were treated in the absence or presence of Ro23-9358 (at the indicated dose) and either in the absence (Ctl) or presence of SAA (4 μmol/L) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in SAA-treated cells (P<0.001) was significantly different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate accumulation in SAA plus Ro23-9358–treated cells at both doses (P<0.001) was significantly different from that in SAA-treated cells. **C**, Smooth muscle cells were treated in the absence or presence of high-density lipoprotein (HDL; 150 μg protein/mL) and either in the absence (Ctl) or presence of SAA (2 μmol/L) for 24 hours, at which time total RNA was extracted and analyzed by real-time polymerase chain reaction for $sPLA_2$ mRNA as described in the Materials and Methods section. The data are expressed as relative $sPLA_2$ mRNA levels ± SD [n=3; with SAA-treated cells set to a value of 1). The level of $sPLA_2$ mRNA in SAA-treated cells (P<0.05) was significantly different from that in Ctl-treated cells. The level of $sPLA_2$ mRNA in SAA plus HDL-treated cells (P<.05) was significantly different from that in SAA-treated cells. **D**, Smooth muscle cells were treated in the absence or presence of HDL [150 μg protein/mL) either in the absence (Ctl) or presence of SAA (2 μmol/L) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in SAA-treated cells (P<0.001) was significantly

different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate accumulation in SAA plus HDL-treated cells (P<0.001) was significantly different from that in SAA-treated cells.

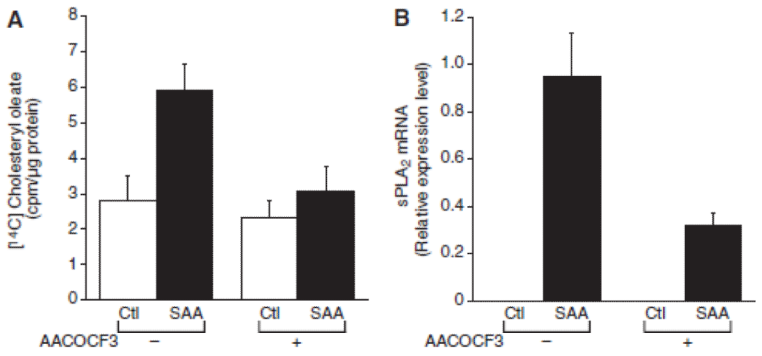

Figure 6.

Serum amyloid A(SAA)-mediated cholesterol trafficking is cyclic phospholipase (cPL) A_2–dependent. **A**, Smooth muscle cells were treated in the absence or presence of arachidonyl trifluoromethyl-ketone (AACOCF3; 10 μmol/L) and either in the absence (control [Ctl]) or presence of SAA (4 μmol/L) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in SAA-treated cells [P<0.001) was significantly different from that in Ctl-treated cells. [14C] cholesteryl oleate accumulation in SAA plus AACOCF3-treated cells (P<0.01) was significantly different from that in SAA-treated cells. **B**. Smooth muscle cells were treated in the absence or presence of AACOCF3 (10 μmol/L) and either in the absence (Ctl) or presence of SAA (2 μmol/L) for 24 hours, at which time total RNA was extracted and analyzed by real-time polymerase chain reaction for secretory phospholipase A_2, group IIA ($sPLA_2$) mRNA as described in the Materials and Methods section. The data are expressed as relative $sPLA_2$ mRNA levels ± SD (n=3; with SAA-treated cells set to a value of 1). The level of $sPLA_2$ mRNA in SAA-treated cells was significantly different from that in Ctl-treated cells (P<0.001). The level of $sPLA_2$ mRNA in SAA plus AACOCF3-treated cells (P<0.0001) was significantly different from that in SAA-treated cells.

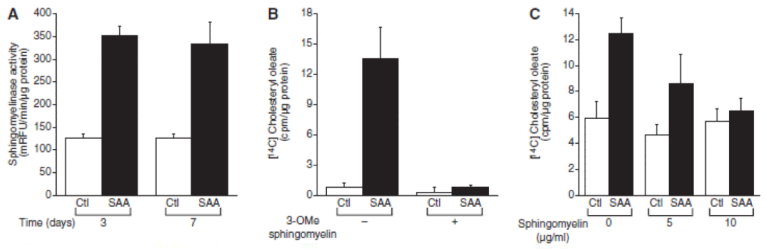

Figure 7.

Serum amyloid A (SAA)-mediated cholesterol trafficking is sphingomyelinase-dependent. **A**, Smooth muscle cells is were treated in the absence (control [Ctl]) or presence of SAA (2 μmol/L) for 3 days and 7 days, at which time sphingomyelinase activity was measured as described in the Materials and Methods section. The data are expressed as relative fluorescence emission units (mRFU)/min/μg protein ± SD (n=3). Enzyme activity in SAA-treated cells was significantly different from that in Ctl-treated cells at both time points (P<0.001). **B**, Smooth muscle cells were treated in the absence or presence of 3-O-methyl sphingomyelin (3-OMe sphingomyelin 75 μmol/L) and either in the absence (Ctl) or presence of SAA (4 μmol/L) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oieate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in SAA-treated cells (P< 0.0001) was significantly different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate accumulation in SAA plus 3-OMe sphingomyeKn–treated cells (P< 0.0001) was significantly different from that in SAA-treated cells. **C**, Smooth muscle cells were treated in the absence or presence of sphingomyelin [at the indicated dose) and either in the absence (Ctl) or presence of SAA (4 μmol/L) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in SAA-treated cells (P< 0.0001) was significantly different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate accumulation in SAA plus sphingomyelin-treated cells (5 μg/mL; P<0.05) was significantly different from that in sphmgomyelin-treated cells (5 μg/mL). [^{14}C] cholesteryl oleate accumulation in SAA plus sphingomyelin-treated cells at both doses (5 μg/mL, P<0.05: 10 μg/mL, P<0.001) was significantly different from that in SAA-treated cells.

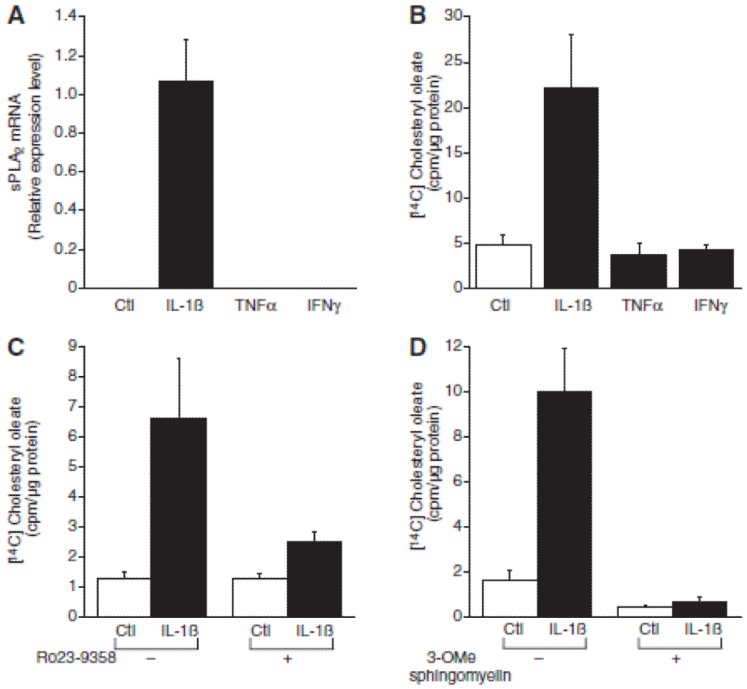

Figure 8.

Interleukin (IL)-1β induces cholesterol trafficking to the endoplasmic reticulum. **A**, Smooth muscle cells were treated in the absence (control [Ctl]) or presence of IL-1β (100ng/mL), tumor necrosis factor-α (TNFα; 100ng/mL) or interferon (IFNγ; 50U/mL) for 24 hours, at which time total RNA was extracted and analyzed by real-time polymerase chain reaction for secretory phospholipase A_2, group IIA ($sPLA_2$) mRNA as described in the Materials and Methods section. The data are expressed as relative $sPLA_2$, mRNA levels ± SD (n=3; with IL-1β–treated cells set to a value of 1). The levels of $sPLA_2$ mRNA in IL-1β–treated cells (P<0.01) were significantly different from that in Ctl-treated cells. **B**, Smooth muscle cells were treated in the absence (Ctl) or presence of IL-1β (100ng/mL), TNFα (10 ng/mL) or IFNγ (50 U/mL) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C]cholesteryl oleate accumulation in IL-lβ–treated cells (P<0.0001) was significantly different from that in Ctl-treated cells. **C**, Smooth muscle cells were treated in the absence or presence of Ro23-9358 (25 μmol/L) and either in the absence (Ctl) or presence of IL-1β (100ng/mL) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in IL-1β–treated cells (P<0.0001) was significantly different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate accumulation in IL-1β plus Ro23-9358-treated cells (P<0.0001) was significantly different from that in IL-1β–treated oells. **D**. Smooth muscle cells were treated in the absence or presence of 3-OMe sphingomyelin (3-O-methyl sphingomyelin; 75 μmol/L) and either in the absence (Ctl) or presence of IL-1β (100ng/mL) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in IL-1β–treated cells (P<0.0001) was significantly different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate

accumulation in IL-1β plus 3-OMe sphingomyelin–treated cells (P<0.0001) was significantly different from that in IL-1β–treated cells.

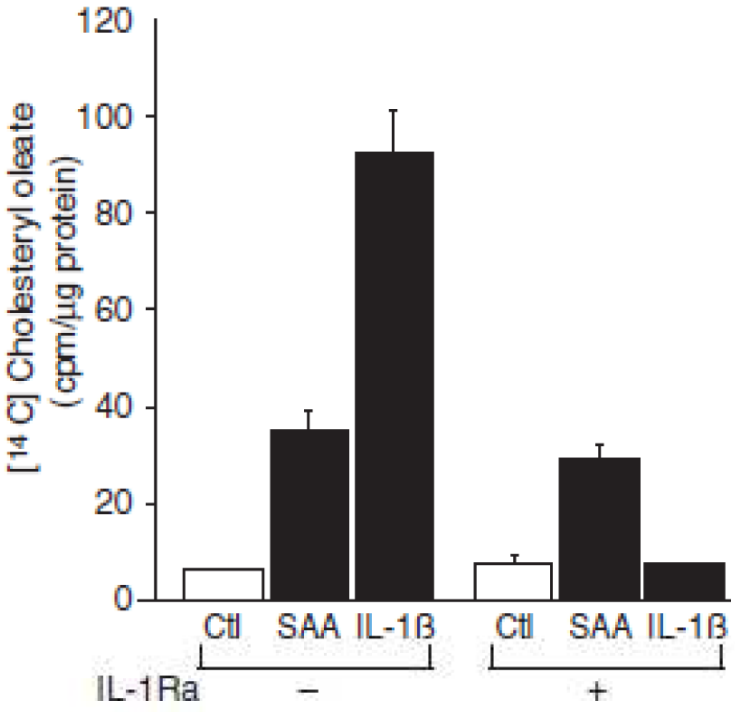

Figure 9.

Serum amyloid A (SAA)-induced cholesterol trafficking is interleukin (IL)-1 receptor independent. Smooth muscle cells were treated in the absence or presence of IL-1 receptor antagonist (IL-1Ra; 1 μg/mL) and either in the absence (Ctl) or presence of SAA(4 μmol/L) or IL-1β (100 ng/mL) with [^{14}C] oleic acid for 24 hours, at which time [^{14}C] cholesteryl oleate accumulation was measured as described in the Materials and Methods section. The data are expressed as [^{14}C] cholesteryl oleate (cpm)/μg protein ± SD (n=3). [^{14}C] cholesteryl oleate accumulation in SAA-treated (P<0.0001) and IL-1β–treated cells (P<0.0001) was significantly different from that in Ctl-treated cells. [^{14}C] cholesteryl oleate accumulation in IL-1β plus IL-lβ–treated cells (P<0.0001) was significantly different from that in IL-1β–treated cells.

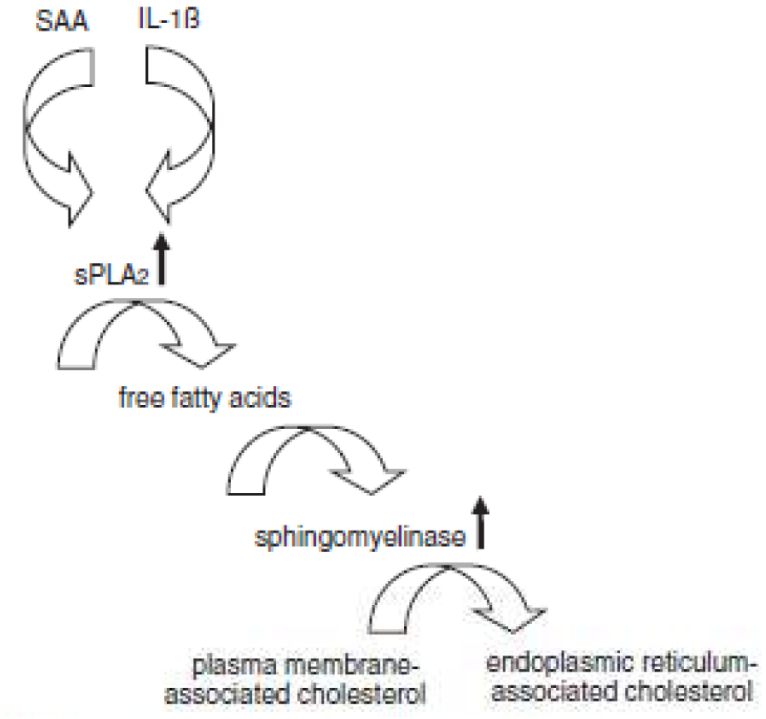

Figure 10.

Schema depicting serum amyloid A (SAA)– and interleukin (IL)-lβ–mediated smooth muscle cell cholesterol trafficking to the endoplasmic reticulum. SAA and IL-1β induce expression of secretory phospholipase A_2, group IIA ($sPLA_2$). the activity of which liberates free fatty acids from phospholipids. The fatty acids increase the activity of sphingomyelinase, which liberates cholesterol associated with sphingomyelin in the plasma membrane and the cholesterol traffics to the endoplasmic reticulum. Note that **small vertical arrows** indicate an increased enzyme activity and **larger arrows** point to a critical product of the pathway described but they are not meant to imply a specific enzyme reaction.