Modification of LDL with human secretory phospholipase A_2 or sphingomyelinase promotes its arachidonic acid-releasing propensity

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Abstract **Oxidation and lipolytic remodeling of LDL are believed to stimulate LDL entrapment in the arterial wall, expanding the inflammatory response and promoting atherosclerosis. However, the cellular responses and molecular mechanisms underlying the atherogenic effects of lipolytically modified LDL are incompletely understood. Human THP-1 monocytes were prelabeled with [^{3}H]arachidonic acid (AA) before incubation with LDL or LDL lipolytically modified by secretory PLA_2 ($sPLA_2$) or bacterial sphingomyelinase (SMase). LDL elicited rapid and dose-dependent extracellular release of AA in monocytes. Interestingly, LDL modified by $sPLA_2$ or SMase displayed a marked increase in AA mobilization relative to native LDL, and this increase correlated with enhanced activity of cytosolic PLA_2 ($cPLA_2$) assayed in vitro as well as increased monocyte tumor necrosis factor-α secretion. The AA liberation was attenuated by inhibitors toward $cPLA_2$ and $sPLA_2$, indicating that both PLA_2 enzymes participate in LDL-induced AA release. In conclusion, these results demonstrate that LDL lipolytically modified by $sPLA_2$ or SMase potentiates cellular AA release and $cPLA_2$ activation in human monocytes. From our results, we suggest novel atherogenic properties for LDL modified by $sPLA_2$ and SMase in AA release and signaling, which could contribute to the inflammatory gene expression observed in atherosclerosis.**—Oestvang, J., D. Bonnefont-Rousselot, E. Ninio, J. K. Hakala, B. Johansen, and M. W. Anthonsen. **Modification of LDL with human secretory phospholipase A_2 or sphingomyelinase promotes its arachidonic acid-releasing propensity. *J. Lipid Res.* 2004.** 45: **831–838.**

Supplementary key words low density lipoprotein remodeling • inflammation • atherosclerosis • monocytes • oleic acid

Atherosclerosis, the underlying cause of myocardial infarction, stroke, and vascular occlusive disease, may be characterized as an inflammatory disease that is initiated by the entrapment of LDL in the arterial wall (1). In this process, the oxidation and lipolytic remodeling of LDL is recognized to be important for the subendothelial retention of LDL, causing LDL aggregation, foam cell formation, endothelial dysfunction, and macrophage chemotaxis (2). Secretory nonpancreatic phospholipase A_2 ($sPLA_2$) and sphingomyelinase (SMase) have been implicated in the lipolytic modification of LDL in vitro, thereby generating LDL that has increased binding strength to human aortic proteoglycans and stimulates foam cell formation (3–6). Both $sPLA_2$ and SMase are found in the arterial intima (7, 8), and several reports link these enzymes to atherogenesis: atherosclerotic lesions and levels of biologically active oxidized phospholipids are increased in $sPLA_2$ transgenic mice (9), and $sPLA_2$ has been identified as an independent risk factor for coronary artery disease (10). Also, mice that are knockouts for the acid SMase gene show resistance to atherosclerotic lesion development (11). Finally, the activities of $sPLA_2$ and secretory SMase are upregulated during acute systemic inflammation, which could specifically link these enzymes to the inflammatory events in atherosclerosis (12).

PLA_2 hydrolyzes the *sn*-2 fatty acid ester bond of glycerophospholipids (13), whereas SMase cleaves sphingo-

Abbreviations: AA, arachidonic acid; BEL, bromoenol lactone; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; LDL_{nat}, native LDL; MAFP, methyl arachidonyl fluorophosphonate; MDA, malondialdehyde equivalents; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OA, oleic acid; PLA_2, phospholipase A_2; SMase, sphingomyelinase; $sPLA_2$, secretory phospholipase A_2; TNF-α, tumor necrosis factor-α.

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Manuscript received 14 July 2003 and in revised form 19 December 2003.

Published, JLR Papers in Press, February 1, 2004.
DOI 10.1194/jlr.M300310-JLR200

myelin that is present in cell membranes and lipoproteins (14). The phospholipid hydrolysis of LDL by PLA_2 and SMase generates FFA and lipid mediators (lysophospholipids and ceramide) that could possibly initiate diverse atherogenic processes in various cell types at sites of lipoprotein deposition in the arterial wall. Also, the physiochemical properties, including the charge and conformation of LDL, are changed upon phospholipid hydrolysis by $sPLA_2$ and SMase, which could account for the observed enhanced interaction between lipolytically modified LDL and cells in the arterial wall. Despite the findings that lipolytic modification of LDL enhances the retention of aortic proteoglycans and accelerates foam cell formation, the molecular mechanisms underlying the atherogenic properties of lipolytically modified LDL are incompletely understood. Therefore, the present study was undertaken to examine other cellular effects of lipolytically modified LDL. Stimulus-induced mobilization of arachidonic acid (AA), mediated by the group IV Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) and the 14 kDa Ca^{2+}-dependent $sPLA_2$s, has been assigned an important role in lipid-mediated cellular signaling associated with inflammatory responses (15). In this study, we investigated whether LDL and lipolytically modified LDL may contribute to monocyte AA signaling. Our findings show that *1*) LDL elicits rapid AA release mediated by PLA_2 enzymes in human THP-1 monocytes, and *2*) LDL lipolytically modified by SMase or $sPLA_2$ exerts strongly enhanced AA-inducing capacity compared with native LDL (LDL_{nat}). These results suggest novel atherogenic properties for LDL, $sPLA_2$-modified LDL, and SMase-modified LDL through the induction of monocyte AA release that may contribute to inflammatory signaling and gene expression promoting atherogenesis.

METHODS

Materials

Fatty acid-free BSA, *Bacillus cereus* SMase, chondroitinase ABC from *Proteus vulgaris*, heparinase III from *Flavobacterium heparinum*, and chloroquine were from Sigma. Methyl arachidonyl fluorophosphonate (MAFP) and bromoenol lactone (BEL) were obtained from Cayman Chemicals. [5,6,8,9,11,12,14,15-^{3}H]AA (specific activity, 180–240 Ci/mmol) and [1-^{14}C]oleic acid (OA; specific activity, 40–60 mCi/mmol) were purchased from New England Nuclear. The $sPLA_2$ inhibitor SB203347 was kindly provided by Dr. Lisa Marshall (SmithKline Beecham Pharmaceuticals). Purified human recombinant group IIa $sPLA_2$ and the mouse monoclonal antibody BA11, generated against human recombinant group IIa $sPLA_2$, were generously provided by Dr. Jeff Browning (Biogen, Inc.). Prof. John McGregor [Institut National de la Santé et de la Recherche Médicale (INSERM) U331, Lyon, France] kindly provided the 10/5 mouse monoclonal anti-CD36 blocking antibody.

Cell culture

Human monocyte-like THP-1 cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 0.3 mg/ml glutamine, and 0.1 mg/ml gentamicin in a humidified air/5% CO_2 atmosphere at 37°C.

Lipoprotein purification

Human LDL (d 1.019–1.050 g/ml) was isolated from the plasma of normolipidemic donors by sequential ultracentrifugation (16) in the presence of EDTA (1.08 mM) and thereafter extensively dialyzed at 4°C against RPMI 1640 with EDTA (10 mM) and subjected to a short (1–2 h) dialysis in RPMI 1640. LDL lipid composition was determined to check the purity of each preparation, and protein concentration was determined by a pyrogallol red technique (Elitech Diagnostics, Sees, France) (17), which gave results similar to those of the bicinchoninic acid kit. Determination of the content of thiobarbituric acid-reactive substances, expressed as malondialdehyde equivalents (MDA), provided an estimation of the degree of lipid peroxidation (18). Typically LDL_{nat} preparations contained $<0.9 \pm 0.1$ nmol MDA/mg LDL protein. Also, conjugated dienes were determined by monitoring the changes in absorbance at 234 nm (19).

Treatment of LDL with $sPLA_2$ and SMase

LDL (100 μg/ml) was incubated with purified human recombinant group IIa $sPLA_2$ or *B. cereus* SMase in RPMI 1640 medium with 5 mM $MgCl_2$ and 2 mM $CaCl_2$ at 37°C for the periods of time indicated in the figures. Lipolysis was stopped by the addition of EDTA to a final concentration of 10 mM EDTA. SMase- and $sPLA_2$-treated LDL are denoted SMase/LDL and $sPLA_2$/LDL, respectively. The oxidative status of LDL (measured as thiobarbituric acid-reactive substances or conjugated dienes) was not changed after incubation with $sPLA_2$ or SMase or after incubation of LDL species with THP-1 cells for up to 2 h.

Measurement of AA and OA release

THP-1 cells were labeled with [^{3}H]AA (0.4 μCi/ml) and [^{14}C]OA (0.07 μCi/ml) as previously described (20). Extracellular release of total [^{3}H]AA and [^{14}C]OA radioactivity was assessed by liquid scintillation counting of supernatants. The results are given as released [^{3}H]AA or [^{14}C]OA in the supernatant relative to [^{3}H]AA or [^{14}C]OA incorporated into the cells (dissolved in 1 M NaOH) and are normalized to show fold induction of treated cells relative to untreated cells.

Assay of $cPLA_2$ activity

$cPLA_2$ activity measurements were performed as previously described (20) using 100 μg of supernatant protein and sonicated vesicles of 1-palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphorylcholine (100 μM) containing 100,000 cpm of 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol-3-phosphorylcholine.

Tumor necrosis factor-α assay

Tumor necrosis factor-α (TNF-α) activity in supernatants from treated cells was determined in the WEHI 164 clone 13 bioassay as previously described (21).

Cytotoxicity assay

Cytotoxicity in the presence of different LDL species, PLA_2 chemical inhibitors, or inhibitor vehicles was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye uptake assay as previously described (22, 23).

Statistical analysis

Data are expressed as means ± SD of triplicate determinations within separate experiments. Similar results were obtained in three to five experiments. Differences between means were determined by Student's *t*-test for paired samples, and those at $P < 0.05$ were considered significant. In the figures, asterisks indicate that values are statistically different from the given reference values.

RESULTS

LDL induces AA release in human monocytes

Stimulus-induced mobilization of AA has been assigned important roles in cellular signaling that regulates inflammatory activation (15, 24). To investigate whether LDL also stimulates the extracellular release of AA and OA in monocytes, THP-1 cells were prelabeled with [^{3}H]AA and [^{14}C]OA before the addition of LDL. LDL rapidly and reproducibly increased cellular AA and OA liberation by 1.4- to 2.5-fold (**Fig. 1**), showing dose-dependent effects without exerting cytotoxic effects at the time and concentrations applied (as assessed by the MTT assay; data not shown).

LDL-elicited AA release is mediated by PLA_2 activity

The activation of PLA_2 enzymes provides a direct pathway for AA release (13). To examine whether PLA_2 enzymes mediate LDL-induced AA mobilization, we first investigated whether the observed AA release correlated with an increased activity of the AA-selective $cPLA_2$ enzyme. The activity of $cPLA_2$ was determined in vitro using sonicated vesicles of arachidonoylphosphatidylcholine as a substrate. Indeed, LDL enhanced the activity of $cPLA_2$ (**Fig. 2**), which was blocked by the $cPLA_2/Ca^{2+}$-independent PLA_2 ($iPLA_2$) inhibitor MAFP (25) (data not shown). These results indicate that $cPLA_2$, at least in part, mediates LDL-induced AA liberation. To further examine the role of PLA_2 enzymes in LDL-elicited AA release in THP-1 cells, we next investigated the influence of specific inhibitors targeted against distinct PLA_2 enzymes on LDL-induced fatty acid release. Pretreatment of THP-1 cells with the dual $cPLA_2/iPLA_2$ inhibitor MAFP (25, 26) potently reduced LDL induced AA release, resulting in complete inhibition at 10 μM (**Fig. 3**). Likewise, the active site-directed, $sPLA_2$-specific inhibitor SB203347, displaying 40-fold selectivity for $sPLA_2$ over $cPLA_2$ (27), dose-dependently attenuated LDL-stimulated AA liberation, displaying ~80% inhibition at 10 μM (Fig. 3). In contrast, the $iPLA_2$ inhibitor BEL or vehicles had no effect on AA release, and inhibitors were not cytotoxic as determined by MTT assay (data not shown). Therefore, these results suggest that both $cPLA_2$ and $sPLA_2$ contribute to the LDL-elicited AA release observed in THP-1 monocytes.

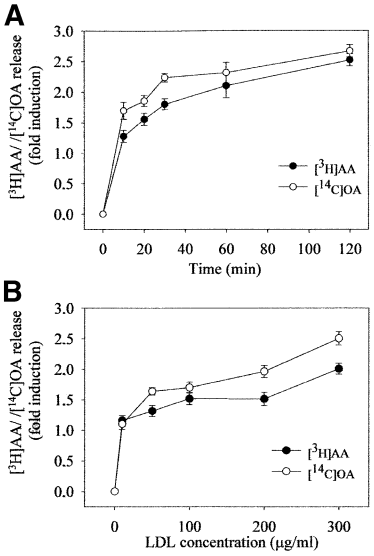

Fig. 1. LDL stimulates [^{3}H]arachidonic acid (AA) and [^{14}C]oleic acid (OA) mobilization in THP-1 cells. [^{3}H]AA- and [^{14}C]OA-labeled THP-1 cells were incubated with 100 μg/ml native LDL (LDL_{nat}) for the indicated time periods (A) or with increasing concentrations of LDL_{nat} for 30 min (B) before determination of [^{3}H]AA and [^{14}C]OA in the supernatants as described in Methods. Data are expressed as means ± SD of triplicate determinations within separate experiments.

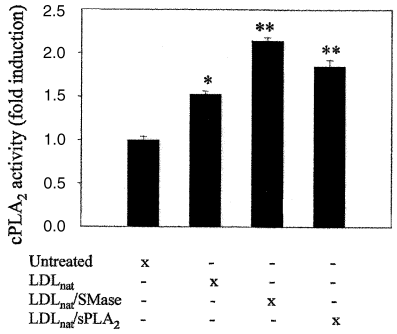

Fig. 2. LDL_{nat} and LDL modified by secretory phospholipase A_2 ($sPLA_2$) or sphingomyelinase (SMase) stimulates cytosolic phospholipase A_2 ($cPLA_2$) activation. $cPLA_2$ activity was determined in lysates from THP-1 monocytes treated with 100 μg/ml LDL_{nat} or LDL that had been modified with $sPLA_2$ or *B. cereus* SMase. For LDL modification, LDL was pretreated with $sPLA_2$ (12 h) or SMase (1 h) at 37°C as described in Methods before addition to THP-1 monocytes. Data are expressed as means ± SD of triplicate determinations within separate experiments. Asterisks indicate that values are statistically different from untreated (*) or LDL_{nat}-treated (**) cells.

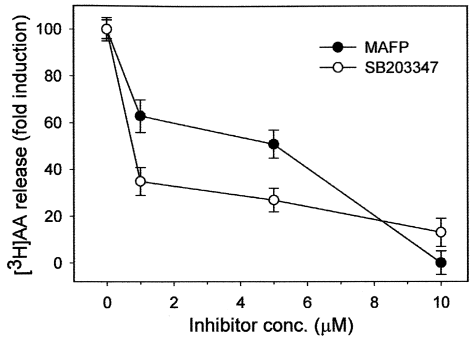

Fig. 3. Effect of the $sPLA_2$ inhibitor SB203347 and the $cPLA_2$/iPLA inhibitor methyl arachidonyl fluorophosphate (MAFP) on LDL-stimulated [^{3}H]AA release. [^{3}H]AA-labeled THP-1 cells were preincubated with the indicated concentrations (conc.) of MAFP or SB203347 for 1 h before the addition of LDL_{nat} (100 μg/ml), further incubation for 30 min, and determination of [^{3}H]AA released to the supernatants as described in Methods. Data are expressed as means ± SD of triplicate determinations within separate experiments.

Lipolytic modification of LDL with $sPLA_2$ or SMase potentiates monocyte AA release

To examine whether the modification of LDL with $sPLA_2$ or SMase affects the AA-inducing capacity of LDL, LDL_{nat} was pretreated with either recombinant human group IIa $sPLA_2$ or *B. cereus* SMase before incubation with THP-1 monocytes for different time periods. Indeed, induction of AA cellular release was markedly enhanced after treatment of LDL with $sPLA_2$ or SMase, repeatedly resulting in up to a 4.5-fold increase in AA release (relative to untreated cells; **Fig. 4**), i.e., a 2- to 3-fold enhancement relative to LDL_{nat}. Importantly, the enhanced AA release observed for $sPLA_2$- or SMase-treated LDL compared with LDL_{nat} correlated with the increased induction of $cPLA_2$ activity by the lipolytically modified LDL (Fig. 2), indicating the possible involvement of $cPLA_2$ in LDL-stimulated AA release.

To investigate the importance of preincubation time of $sPLA_2$ or SMase with LDL for the AA-releasing capacity of LDL, LDL was preincubated with either $sPLA_2$ or SMase for various time periods before incubation with the THP-1 monocytes. We observed that the cellular AA release increased with increasing preincubation times of LDL with $sPLA_2$ or SMase, resulting in maximal release for LDL that had been pretreated with $sPLA_2$ for 15–18 h (**Fig. 5A**). In contrast, the maximal effect of SMase on LDL modification was obtained after 40 min of preincubation with LDL (Fig. 5B). Thus, under our experimental conditions, the SMase-mediated modification of LDL that is responsible for the enhanced AA release occurs far more rapidly than the corresponding $sPLA_2$-induced LDL modification. Also, increasing concentrations of $sPLA_2$ (**Fig. 6A**) or SMase (Fig. 6B) during preincubation with LDL dose-dependently enhanced AA liberation.

To address the question of whether $sPLA_2$ activity is of significance for the AA release stimulated by $sPLA_2$-modified LDL, we used an anti-group IIa activity-neutralizing antibody or the $sPLA_2$ inhibitor SB203347 to block enzyme activity. Addition of an anti-group IIa $sPLA_2$ activity-neutralizing antibody or SB203347 during $sPLA_2$/LDL preincubation completely inhibited the effect of $sPLA_2$ on cellular AA liberation (**Fig. 7A**). Thus, $sPLA_2$ catalytic activity is of crucial significance for the LDL modification that causes AA potentiation. Likewise, the presence of 10 mM EDTA to inhibit SMase during the SMase/LDL preincubation completely abrogated the enhancing effect of SMase-treated LDL on AA liberation (Fig. 7B). Hence, the catalytic activity of SMase is responsible for LDL modification, generating LDL with increased capacity for AA release in human monocytes.

To partially address the binding mechanisms by which $sPLA_2$-/SMase-modified LDL promotes cellular AA release, we used different strategies aimed at modulating cellular LDL binding or LDL degradation. Cell surface proteoglycans have been implicated in the binding and subsequent internalization and degradation of atherogenic lipoproteins (28, 29). To test if heparan sulfate- or chondroitin sulfate-containing proteoglycans are involved in AA release stimulated by $sPLA_2$- or SMase-modified

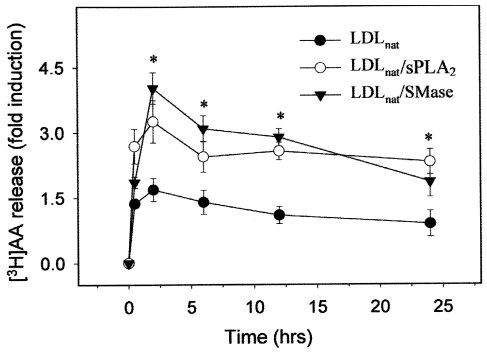

Fig. 4. Pretreatment of LDL_{nat} with $sPLA_2$ or SMase potentiates LDL-elicited [^{3}H]AA mobilization in THP-1 monocytes. LDL_{nat} (100 μg/ml) was incubated at 37°C for 12 h with 1 μg/ml human recombinant group IIa $sPLA_2$, for 1 h with 350 mU/ml *B. cereus* SMase, or without enzymes. Samples of nontreated LDL (LDL_{nat}) and treated LDL (LDL_{nat}/$sPLA_2$ and LDL_{nat}/SMase) were subsequently incubated with THP-1 monocytes for the indicated time periods before determination of extracellular [^{3}H]AA release as described in Methods. Data are expressed as means ± SD of triplicate determinations within separate experiments. Asterisks indicate that the values for LDL_{nat}/$sPLA_2$- and LDL_{nat}/SMase-treated cells are statistically different from those for LDL_{nat}-treated cells.

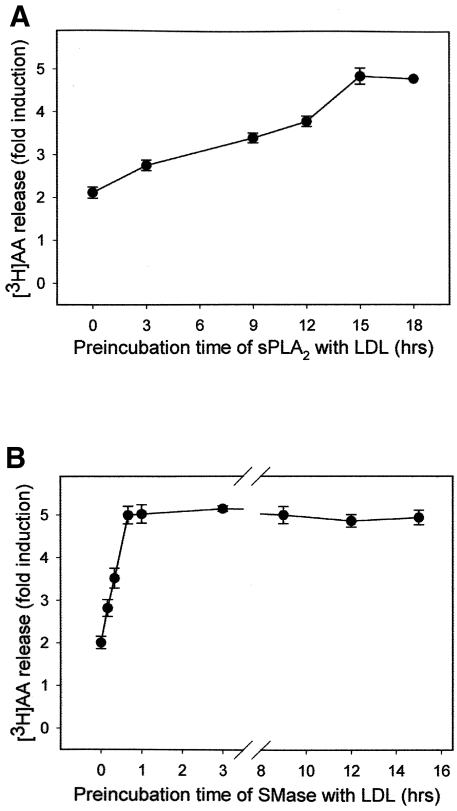

Fig. 5. Preincubation time of LDL with $sPLA_2$ or SMase markedly affects [^{3}H]AA liberation. LDL_{nat} (100 μg/ml) was preincubated at 37°C for the indicated time periods with either 1 μg/ml human recombinant group IIa $sPLA_2$ (A) or 500 mU/ml *B. cereus* SMase (B) before addition to THP-1 monocytes, treatment for 30 min, and determination of extracellular [^{3}H]AA release as described in Methods. Data are expressed as means ± SD of triplicate determinations within separate experiments.

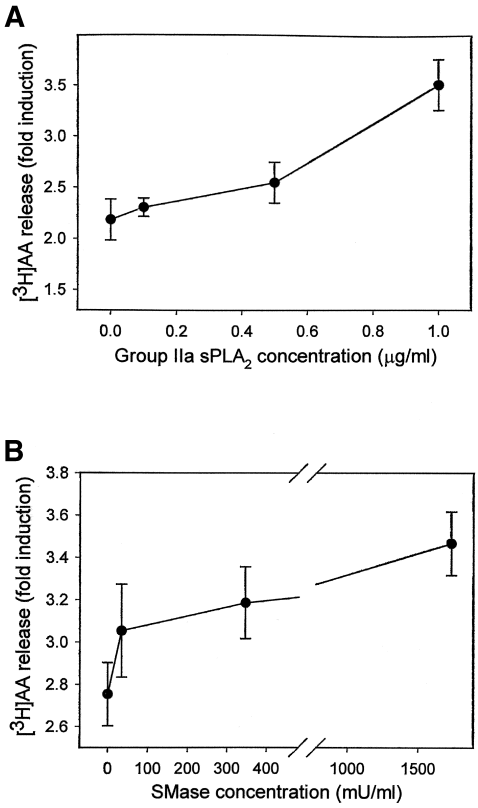

Fig. 6. Effect of $sPLA_2$ and SMase concentration during LDL preincubation on [^{3}H]AA mobilization. LDL_{nat} (100 μg/ml) was incubated with the indicated concentrations of either human recombinant group IIa $sPLA_2$ (A) or *B. cereus* SMase (B) at 37°C for 12 or 1 h, respectively, before addition to THP-1 monocytes and treatment for 30 min. Extracellular release of [^{3}H]AA was quantified as described in Methods. Data are expressed as means ± SD of triplicate determinations within separate experiments.

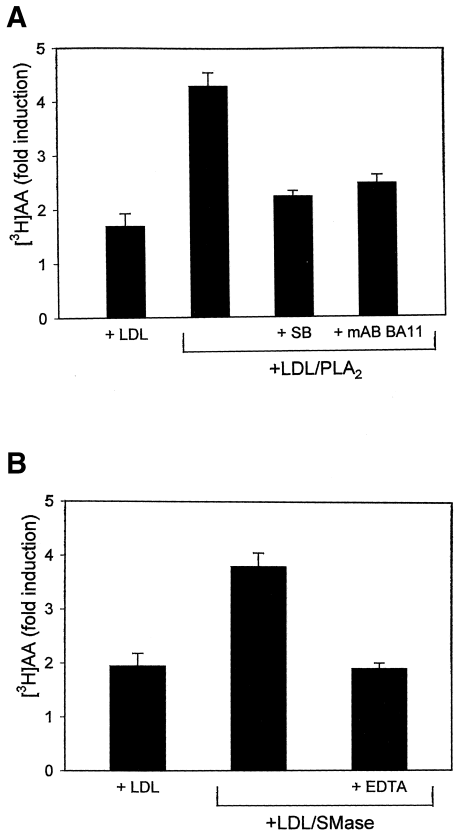

Fig. 7. Presence of the sPLA2 inhibitor SB203347 or EDTA during LDL preincubation with $sPLA_2$ or SMase blocks LDL-induced [^{3}H]AA release. A: LDL_{nat} (100 μg/ml) was preincubated at 37°C for 12 h with 1 μg/ml human recombinant group IIa $sPLA_2$ in the presence or absence of the $sPLA_2$ inhibitor SB203347 (SB; 10 μM) or the anti-$sPLA_2$ activity-neutralizing antibody BA11 (mAB BA11; 30 μg/ml) before addition to THP-1 monocytes and treatment for 30 min. B: LDL_{nat} (100 μg/ml) was preincubated at 37°C for 1 h with 500 mU/ml *B. cereus* SMase in the presence or absence of EDTA (10 mM) before addition to THP-1 monocytes and treatment for 30 min. Extracellular release of [^{3}H]AA was quantified as described in Methods. Data are expressed as means ± SD of triplicate determinations within separate experiments.

LDL, we investigated whether the preincubation of cells with heparinase or chondroitinase ABC affected AA liberation. Heparinase and chondroitinase ABC lyase had no effects on AA release (**Table 1**). To examine if the CD36 scavenger receptor is involved in LDL-induced AA liberation, THP-1 cells were preincubated with an anti-CD36 blocking antibody before incubation with SMase-treated LDL. In the presence of the anti-CD36 antibody, AA liberation was reduced to 83% of that of SMase/LDL-stimulated cells (Table 1). Thus, we suggest that the CD36 scavenger receptor in part mediates AA release in response to SMase-modified LDL. In contrast, pretreatment of the cells with chloroquine or cytochalasin D to inhibit lysosomal activity and actin-dependent endocytosis did not significantly reduce LDL-induced AA release.

Effect of $sPLA_2$- or SMase-modified LDL on TNF-α secretion

LDL promotes immune reactions by modulating the production of various cytokines and the expression of vascular adhesion molecules in the arterial wall (1). Therefore, we examined whether the enhanced AA mobilization observed for $sPLA_2$- or SMase-modified LDL correlated with the increased production of the proinflammatory mediator TNF-α. Indeed, LDL premodified with $sPLA_2$ or SMase augmented TNF-α secretion from THP-1 cells, resulting in a 2- to 3-fold increase compared with LDL_{nat} (**Fig. 8**).

DISCUSSION

Subendothelial retention of LDL with subsequent oxidation and lipolytic remodeling of LDL are considered to be critical events during early atherosclerosis development. Human $sPLA_2$ and SMase have recently been implicated in the lipolytic modification of LDL in vitro, and roles for these enzymes in the LDL modification, aggregation, and atherogenic lipid accumulation in vivo have been suggested (4–9, 29). However, the molecular mechanisms underlying the atherogenic effects of lipolytically

TABLE 1. Mechanisms involved in SMase/LDL-enhanced [^{3}H]AA

Treatment	[^{3}H]AA Released
SMase/LDL (100 mg/ml)	100 ± 4
Plus chondroitinase ABC (2 U/ml)	94 ± 8
Plus chondroitinase ABC (0.5 U/ml)	105 ± 1
Plus heparinase (0.5 U/ml)	101 ± 9
Plus anti-CD36 (30 mg/ml)	83 ± 6[a]
Plus IgG_{2a}	102 ± 2

AA, arachidonic acid; SMase, sphingomyelinase. THP-1 cells were preincubated at 37°C with chondroitinase ABC or heparinase for 2 h or for 30 min with anti-CD36 receptor-blocking antibody. Thereafter, SMase-treated LDL (incubated with 500 mU/ml *B. cereus* SMase for 40 min at 37°C) was added to cells and incubation was continued for 2 h. Extracellular release of [^{3}H]AA was determined as described in Methods. Results are expressed as percentages of the [^{3}H]AA release induced by SMase-treated LDL in the absence of additional treatments and represent means ± SD of triplicate determinations. The results are representative of two independent experiments.

[a] $P < 0.05$.

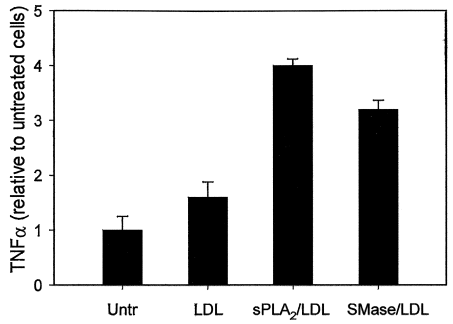

Fig. 8. Effect of $sPLA_2$- and SMase-modified LDL on tumor necrosis factor-α (TNF-α) secretion. THP-1 cells were incubated for 12 h with 100 μg/ml LDL_{nat}, LDL treated with recombinant human group IIa $sPLA_2$, or LDL modified with *B. cereus* SMase. Determination of TNF-α in the extracellular medium was performed as described in Methods. Untr, untreated. Data are expressed as means ± SD of triplicate determinations within separate experiments. Asterisks indicate that values are statistically different from those of LDL_{nat}-treated cells.

modified LDL are poorly understood. Therefore, we addressed the question of whether the LDL modified by human group IIa $sPLA_2$ or bacterial SMase may contribute to the AA mobilization and proinflammatory activation of human monocytes.

Interestingly, we found that modification of LDL by human group IIa $sPLA_2$ or bacterial SMase markedly enhances the AA-inducing potential of LDL, which is accompanied by increased TNF-α secretion compared with LDL_{nat}. Importantly, no oxidative changes in LDL (measured as thiobarbituric acid-reactive substances and conjugated dienes) were detected after dialysis and treatment with SMase or $sPLA_2$ for 1 or 12 h, respectively. Likewise, incubation of LDL, SMase-treated LDL, or $sPLA_2$-treated LDL with THP-1 cells for 2 h did not alter the oxidative status of LDL. Collectively, these findings suggest that AA release in response to LDL or enzymatically modified LDL is not attributable to oxidative changes in LDL, as have been reported previously to occur (30), and indicate that LDL modifications produced by $sPLA_2$ and SMase hydrolysis bring about AA liberation. Hence, although it has been found that prior $sPLA_2$ treatment may render LDL more susceptible to oxidation (31), LDL oxidation appears not to mediate AA release in our system. Our results show that lipolytically modified LDL rapidly induces AA mobilization, entailing maximal release after 2 h of LDL exposure. Cholesterol loading of the cells does not occur during this incubation period. Also, because pretreatment of the cells with chloroquine (to inhibit lysosomal activity) had no significant effect on the AA mobilization induced by lipolytically modified LDL (data not shown), these results indicate that AA release occurs independently of LDL internalization and degradation. Hence, more rapid LDL signaling mechanisms appear to participate in the observed cellular AA liberation. In this regard, it is noteworthy that oxidized LDL or ligand binding to the macrophage scavenger receptor A rapidly (within minutes) stimulates kinase activities (32–34). Thus, such early LDL signaling events may mediate the $cPLA_2$ activation and AA release observed in our study. Pretreatment of the cells with chloroquine or cytochalasin D to inhibit lysosomal activity and actin-dependent endocytosis did not significantly reduce LDL-induced AA release, indicating that AA release occurs independently of LDL internalization and degradation.

The LDL molecular properties that are changed by $sPLA_2$ or SMase treatment and that cause the enhanced AA mobilization for lipolytically treated LDL relative to LDL_{nat} are unknown, but they may involve altered physiochemical properties [e.g., aggregation, as is known to occur under experimental conditions similar to those used in our experiments (4, 6)] or structural changes in apolipoprotein B [as reviewed in ref. (35)]. One alternative mechanism for the induction of AA release by enzymatically modified LDL could be through LDL-derived lipid mediators generated by $sPLA_2$ or SMase, such as lysophosphatidylcholine and ceramide. However, we found that lysophosphatidylcholine and ceramide induce AA release in THP-1 cells more rapidly than LDL (maximal within 10–20 min, in contrast to 2 h for LDL treatment). Hence, the AA-releasing capacity of enzymatically modified LDL is probably not mediated by lysophosphatidylcholine and ceramide.

We observed that the preincubation time necessary to obtain LDL with enhanced AA-releasing capacity was extended for human $sPLA_2$ treatment compared with that of bacterial SMase. These results are in accordance with previous reports showing that LDL hydrolysis by $sPLA_2$ is slower than that by *B. cereus* SMase, which is also reflected by slower LDL aggregation in response to the $sPLA_2$ treatment (29, 36). Also, SMase induces both aggregation and fusion, whereas $sPLA_2$ induces only aggregation in the absence of proteoglycans. Hence, the preincubation times of either lipolytic enzyme with LDL necessary to induce AA release appear to parallel the different LDL hydrolytic rates and aggregation and/or fusion of LDL particles induced by $sPLA_2$ or SMase, which suggests that LDL aggregation and/or fusion is important for the enhanced AA

release. In an attempt to explore this possibility, we separated LDL treated with agarose-coupled bee venom $sPLA_2$ into native-sized and aggregated LDL particles [by gel filtration chromatography (6)] before incubation of these distinct LDL species with THP-1 cells. However, we found that $sPLA_2$-treated native-sized particles stimulated AA release to a similar extent as did $sPLA_2$-induced aggregated LDL; therefore, further investigations are needed to resolve this issue.

Interestingly, $sPLA_2$ or SMase treatment of LDL is known to affect LDL interaction with cells (5, 7), but the exact mechanisms involved in cellular binding of $sPLA_2$- or SMase-modified LDL are unknown. Cellular binding of LDL may generally be mediated by different LDL receptors or by distinct cell surface proteoglycans, such as heparin sulfate- or chondroitin sulfate-containing proteoglycans (28, 29, 37). We found that preincubation of THP-1 cells with heparinase or chondroitinase ABC did not significantly affect AA release induced by LDL. In contrast, the presence of an anti-CD36 receptor-blocking antibody partially reduced AA liberation induced by lipolytically modified LDL, which suggests that the CD36 receptor contributes to AA release. However, the exact mechanisms by which lipolytically remodeled LDL contributes to AA release should be addressed in detail in future studies.

Our findings show that the $cPLA_2$/$iPLA_2$ inhibitor MAFP and the $sPLA_2$ inhibitor SB203347, but not the $iPLA_2$-specific inhibitor BEL, attenuated the LDL-elicited AA release (by 80–100% at 5–10 μM). Also, we found that $cPLA_2$ activity in monocytes was promoted by LDL lipolytically modified with $sPLA_2$ or SMase. Moreover, we simultaneously measured AA and OA release in THP-1 monocytes and found that OA release, regulated by $sPLA_2$s (because $iPLA_2$ is probably not involved in the observed fatty acid release, as discussed previously), which show no fatty acid preference, largely paralleled AA release upon exposure to $sPLA_2$- or SMase-treated LDL (data not shown). By the use of macrophages from $cPLA_2$ (−/−) mice and the $iPLA_2$-specific inhibitor BEL, it has been found that $cPLA_2$, but not $iPLA_2$, mediates AA release in response to oxidized LDL (30). Hence, based on these observations, we propose that both $sPLA_2$s and the AA-selective $cPLA_2$, but probably not $iPLA_2$, participate in LDL-stimulated AA release in THP-1 monocytes. The necessity of both $sPLA_2$ and $cPLA_2$ in AA liberation could be attributable to a sequential interplay between the enzymes leading to maximal AA release, as reported in response to inflammatory stimuli in other cells, leading to the activation of the immunomodulatory transcription factor nuclear factor-κB (22, 24). Several closely related $sPLA_2$ isoforms are expressed in THP-1 monocytes (38), but which of these that participate in LDL-elicited AA release is uncertain.

In conclusion, we demonstrate that lipolytic modification of LDL with $sPLA_2$ or SMase markedly potentiates LDL-induced monocyte AA release and $cPLA_2$ activation, accompanied by increased TNF-α secretion. Importantly, as both $sPLA_2$ and SMase are present in human atherosclerotic tissue, bind to arterial proteoglycans, and are implicated in inflammation (6, 7, 9, 10, 39), our results should be of physiological relevance. AA mobilization plays a crucial role in inflammatory signaling. Thus, $sPLA_2$- or SMase-modified LDL may contribute to atherosclerosis through the induction of AA and AA-derived lipid mediators that could exert autocrine and/or paracrine control of cellular processes, such as inflammation, chemotaxis, apoptosis, proliferation, and differentiation, at sites of lipoprotein retention in the arterial wall. Therefore, our results suggest novel atherogenic effects of lipolytically modified LDL.

This work was supported by Grants 123641/310 from the Norwegian Research Council (M.W.A.), 1997/68 from the Norwegian Public Health program (B. J.), and 6228 from the University of Trondheim (B.J.) and by the Programme Molécules et cibles Thérapautiques 2000 (INSERM-Centre National de la Recherche Scientifique) (E.N.).

REFERENCES

1. Ross, R. 1999. Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340:** 115–126.
2. Klouche, M., S. Rose-John, W. Schmiedt, and S. Bhakdi. 2000. Enzymatically degraded, nonoxidized LDL induces human vascular smooth muscle cell activation, foam cell transformation, and proliferation. *Circulation.* **100:** 1799–1805.
3. Aviram, M., and I. Maor. 1992. Phospholipase A2-modified LDL is taken up at enhanced rate by macrophages. *Biochem. Biophys. Res. Commun.* **185:** 465–472.
4. Öörni, K., J. K. Hakala, A. Annila, M. Ala-Korpela, and P. T. Kovanen. 1998. Sphingomyelinase induces aggregation and fusion, but phospholipase A2 only aggregation, of low density lipoprotein (LDL) particles. Two distinct mechanisms leading to increased binding strength of LDL to human aortic proteoglycans. *J. Biol. Chem.* **273:** 29127–29134.
5. Tabas, I., Y. Li, R. W. Brocia, S. W. Xu, T. L. Swenson, and K. J. Williams. 1993. Lipoprotein lipase and sphingomyelinase synergistically enhance the association of atherogenic lipoproteins with smooth muscle cells and extracellular matrix. A possible mechanism for low density lipoprotein and lipoprotein(a) retention and macrophage foam cell formation. *J. Biol. Chem.* **268:** 20419–20432.
6. Hakala, J. K., K. Öörni, M. O. Pentikainen, E. Hurt-Camejo, and P. T. Kovanen. 2001. Lipolysis of LDL by human secretory phospholipase A(2) induces particle fusion and enhances the retention of LDL to human aortic proteoglycans. *Arterioscler. Thromb. Vasc. Biol.* **21:** 1053–1058.
7. Marathe, S., G. Kuriakose, K. J. Williams, and I. Tabas. 1999. Sphingomyelinase, an enzyme implicated in atherogenesis, is present in atherosclerotic lesions and binds to specific components of the subendothelial extracellular matrix. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2648–2658.
8. Camejo, E. H., S. Andersen, R. Standal, B. Rosengren, P. Sartipy, E. Stadberg, and B. Johansen. 1997. Localization of secretory nonpancreatic phospholipase A2 in normal and atherosclerotic arteries: activity of the isolated enzyme on low density lipoproteins. *Arterioscler. Thromb. Vasc. Biol.* **17:** 300–309.
9. Leitinger, N., A. D. Watson, S. Y. Hama, B. Ivandic, J. H. Qiao, J. Huber, K. F. Faull, D. S. Grass, M. Navab, A. M. Fogelman, F. C. de Beer, A. J. Lusis, and J. A. Berliner. 1999. Role of group II secretory phospholipase A2 in atherosclerosis. 2. Potential involvement of biologically active oxidized phospholipids. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1291–1298.
10. Kugiyama, K., Y. Ota, K. Takazoe, Y. Moriyama, H. Kawano, Y. Miyao, T. Sakamoto, H. Soejima, H. Ogawa, H. Doi, S. Sugiyama, and H. Yasue. 1999. Circulating levels of secretory type II phospholipase A(2) predict coronary events in patients with coronary artery disease. *Circulation.* **100:** 1280–1284.
11. Marathe, S., D. L. Tribble, G. Kuriakose, M. La Belle, B. M. Chu,

E. L. Gong, A. Johns, K. J. Williams, and I. Tabas. 1999. Sphingomyelinase transgenic and knockout mice: direct evidence that SMase is atherogenic in vivo. *Circulation.* **100 (Suppl.):** 3665.
12. Wong, M. L., B. Xie, N. Beatini, P. Phu, S. Marathe, A. Johns, P. W. Gold, E. Hirsch, K. J. Williams, J. Licino, and I. Tabas. 2000. Acute systemic inflammation up-regulates secretory sphingomyelinase in vivo: a possible link between inflammatory cytokines and atherogenesis. *Proc. Natl. Acad. Sci. USA.* **97:** 8681–8686.
13. Six, D. A., and E. A. Dennis. 2000. The expanding superfamily of phospholipase A(2) enzymes: classification and characterization. *Biochim. Biophys. Acta.* **1488:** 1–19.
14. Schissel, S. L., X. Jiang, J. Tweedie-Hardman, T. Jeong, E. H. Camejo, J. Najib, J. H. Rapp, K. J. Williams, and I. Tabas. 1998. Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. *J. Biol. Chem.* **273:** 2738–2746.
15. Qiu, Z. H., M. A. Gijon, M. S. de Carvalho, D. M. Spencer, and C. C. Leslie. 1998. The role of calcium and phosphorylation of cytosolic phospholipase A2 in regulating arachidonic acid release in macrophages. *J. Biol. Chem.* **273:** 8203–8211.
16. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
17. Watanabe, N., S. Kamei, A. Ohkubo, M. Yamanaka, S. Ohsawa, K. Makino, and K. Tokuda. 1986. Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer. *Clin. Chem.* **32:** 1551–1554.
18. Yagi, K. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15:** 212–216.
19. Esterbauer, H., M. Dieber-Rotheneder, G. Striegl, and G. Jurgens. 1990. Biochemical, structural, and functional properties of oxidized low-density lipoprotein. *Chem. Res. Toxicol.* **3:** 77–92.
20. Yang, H. C., M. Mosior, C. A. Johnson, Y. Chen, and E. A. Dennis. 1999. Group-specific assays that distinguish between the four major types of mammalian phospholipase A2. *Anal. Biochem.* **269:** 278–288.
21. Flo, T. H., O. Halaas, E. Lien, L. Ryan, G. Teti, D. T. Golenbock, A. Sundan, and T. Espevik. 2000. Human Toll-like receptor 2 mediates monocyte activation by Listeria monocytogenes, but not by group B streptococci or lipopolysaccharide. *J. Immunol.* **164:** 2064–2069.
22. Anthonsen, M. W., A. Solhaug, and B. Johansen. 2001. Functional coupling between secretory and cytosolic phospholipase A2 modulates TNF-α- and IL1β-induced NF-κB activation. *J. Biol. Chem.* **276:** 30527–30536.
23. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **65:** 55–63.
24. Balsinde, J., and E. A. Dennis. 1996. Distinct roles in signal transduction for each of the phospholipase a(2) enzymes present in p388d(1) macrophages. *J. Biol. Chem.* **271:** 6758–6765.
25. Liu, S., J. P. Laliberte, I. Street, and Z. Huang. 1995. Methyl arachidonyl flourophosphonate, a potent irreversible cPLA2 inhibitor blocks the mobilization of arachidonic-acid in human platelets, neutrophils and differentiated U937 cells. *FASEB J.* **9:** A1310.
26. Street, I. P., H. K. Lin, F. Laliberte, F. Ghomashchi, Z. Wang, H. Perrier, N. M. Tremblay, Z. Huang, P. K. Weech, and M. H. Gelb. 1993. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A2. *Biochemistry.* **32:** 5935–5940.
27. Marshall, L. A., R. H. Hall, J. D. Winkler, A. Badger, B. Bolognese, A. Roshak, P. L. Flamberg, C. M. Sung, M. Chabotfletcher, J. L. Adams, and R. J. Mayer. 1995. SB 203347, an inhibitor of 14 kDa phospholipase A(2), alters human neutrophil arachidonic acid release and metabolism and prolongs survival in murine endotoxin shock. *J. Pharmacol. Exp. Ther.* **274:** 1254–1262.
28. Williams, K. J., and I. V. Fuki. 1997. Cell-surface heparan sulfate proteoglycans: Dynamic molecules mediating ligand catabolism. *Curr. Opin. Lipidol.* **5:** 243–245.
29. Sartipy, P., G. Camejo, L. Svensson, and E. Hurt-Camejo. 1999. Phospholipase A(2) modification of low density lipoproteins forms small high density particles with increased affinity for proteoglycans and glycosaminoglycans. *J. Biol. Chem.* **274:** 25913–25920.
30. Panini, S. R., L. Yang, A. E. Rusinol, M. S. Sinensky, J. V. Bonventre, and C. C. Leslie. 2001. Arachidonate metabolism and the signaling pathway of induction of apoptosis by oxidized LDL/oxysterol. *J. Lipid Res.* **42:** 1678–1686.
31. Neuzil, J., J. M. Upston, P. K. Witting, K. F. Scott, and R. Stocker. 1998. Secretory phospholipase A2 and lipoprotein lipase enhance 15-lipoxygenase-induced enzymic and nonenzymic lipid peroxidation in low-density lipoproteins. *Biochemistry.* **37:** 9203–9210.
32. Hsu, H. Y., D. P. Hajjar, K. M. F. Khan, and D. J. Falcone. 1998. Ligand binding to macrophage scavenger receptor-A induces urokinase-type plasminogen activator expression by a protein kinase-dependent signaling pathway. *J. Biol. Chem.* **273:** 1240–1246.
33. Martens, J. S., N. E. Reiner, P. Herrera-Velit, and U. P. Steinbrecher. 1998. Phosphatidylinositol 3-kinase is involved in the induction of macrophage growth by oxidized low density lipoprotein. *J. Biol. Chem.* **273:** 4915–4920.
34. Maschberger, P., M. Bauer, J. Baumann-Siemons, K. J. Zangl, E. M. Negrescu, A. J. Reininger, and W. Siess. 2000. Mildly oxidized LDL rapidly stimulates via activation of the lysophosphatidic acid receptor Src family and Syk tyrosine kinases and Ca2+ influx in human platelets. *J. Biol. Chem.* **275:** 19159–19166.
35. Sartipy, P., and E. Hurt-Camejo. 1999. Modification of plasma lipoproteins by group IIA phospholipase A(2): possible implications for atherogenesis. *Trends Cardiovasc. Med.* **9:** 232–238.
36. Schissel, S. L., J. Tweedie-Hardman, J. H. Rapp, G. Graham, K. J. Williams, and I. Tabas. 1996. Rabbit aorta and human atherosclerotic lesions hydrolyze the sphingomyelin of retained low-density lipoprotein. Proposed role for arterial-wall sphingomyelinase in subendothelial retention and aggregation of atherogenic lipoproteins. *J. Clin. Invest.* **98:** 1455–1464.
37. Mahley, R. W., and Z. S. Ji. 1999. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *J. Lipid Res.* **40:** 1–16.
38. Oestvang, J., M. W. Anthonsen, and B. Johansen. 2003. Role of secretory phospholipase A2 enzymes in lysophosphatidylcholine-stimulated arachidonic acid release. *FEBS Lett.* **555:** 257–262.
39. Sartipy, P., B. Johansen, G. Camejo, B. Rosengren, G. Bondjers, and E. H. Camejo. 1996. Binding of human phospholipase A2 type II to proteoglycans: differential effect of glycosaminoglycans on enzyme activity. *J. Biol. Chem.* **271:** 26307–26314.