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Bioactive products generated by group V sPLA₂ hydrolysis of LDL activate macrophages to secrete pro-inflammatory cytokines

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Abstract

Objective—Previous studies have established that hydrolysis of LDL by Group V secretory phospholipase A₂ (GV sPLA₂) generates a modified particle capable of inducing macrophage foam cell formation. The aim of the present study was to determine whether GV sPLA₂-hydrolyzed LDL (GV-LDL) produces pro-atherogenic effects in macrophages independent of cholesterol accumulation.

Methods and Results—J-774 cells incubated with GV-LDL produced more TNF- α and IL-6 compared to cells incubated with control-LDL. Indirect immunofluorescence showed that GV-LDL but not control-LDL induced nuclear translocation of NF κ B. Inhibitors of NF κ B activation, effectively blocked cytokine production induced by GV-LDL. Control-LDL and GV-LDL were separated from albumin present in reaction mixtures by ultracentrifugation. The albumin fraction derived from GV-LDL contained 80% of the FFA generated and was more potent than the re-isolated GV-LDL in inducing pro-inflammatory cytokine secretion. Linoleic acid (18:2) and oleic acid (18:1) were the most abundant FFAs generated, whereas newly formed lyso-PCs contained 14:0 (myristic), 16:1 (palmitic), and 18:2 fatty acyl groups. Experiments with synthetic FFA showed that 18:1 induced J-774 cells to secrete TNF- α and IL-6.

Conclusions—These results indicate that in addition to promoting atherosclerotic lipid accumulation in macrophages, GV sPLA₂ hydrolysis of LDL leads to activation of NF κ B, a key regulator of inflammation.

Keywords

atherosclerosis; inflammation; lipoprotein modification; tumor necrosis factor-alpha; interleukin-6; NF κ B

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1. Introduction

According to current paradigms, the development of atherosclerosis culminates from a gradual increase in lipid deposition and chronic inflammation in the subendothelium [1-2]. The subendothelial retention of atherogenic lipoproteins provides substrate for multiple modifying agents. These modified lipoproteins are subsequently taken up by locally recruited macrophages to form foam cells, which in turn are activated to produce various cytokines that promote and maintain local inflammation.

The secretory phospholipase A₂ (sPLA₂) family of enzymes has been implicated in pro-atherosclerotic processes. The sPLA₂'s hydrolyze the ester bond at the *sn*-2 position of glycerophospholipids to release free fatty acids (FFA) and lyso-phospholipids (lyso-PL) [3-4]. Several lines of evidence suggest that sPLA₂'s play a role in atherosclerosis through their hydrolyzing activities in the arterial intima [5-7]. To date, seven members of the sPLA₂ family have been detected in atherosclerotic lesions [8]. Of these, group V (GV), group X (GX) and recently Group III (GIII) have been shown to effectively hydrolyze LDL phospholipids (PL) *in vitro* [9-11]. Hydrolysis of LDL by sPLA₂ leads to alterations in the conformation of apoB100 on the PL-depleted particle, which destabilizes the particle and promotes aggregation. The structurally altered LDL particle also exhibits enhanced binding to extracellular matrix and cell-surface proteoglycans [12], which is likely due to the exposure of a proteoglycan binding site present on apoB-100 that is normally buried within the LDL particle [13]. In the case of GV sPLA₂-modified LDL (GV-LDL), recent data indicate that syndecan 4, a proteoglycan expressed on the surface of macrophages, mediates uptake of GV-LDL to form foam cells [14]. Thus, based on numerous *in vitro* studies, LDL hydrolysis by sPLA₂ may promote atherosclerosis by enhancing the retention of LDL particles in the subendothelium and by promoting macrophage LDL uptake.

The possibility that GV sPLA₂ promotes atherosclerotic lipid deposition *in vivo* is supported by gain-of-function and loss-of-function studies carried out in LDL receptor^{-/-} mice [15]. However, in studies in apoE^{-/-} mice, deficiency of GV sPLA₂ had no effect on atherosclerotic lesion area in either male or female mice [16]. The discrepancy in results from the two mouse models may be partially explained by *in vitro* studies showing that GV sPLA₂ modification of LDL from LDL receptor^{-/-} mice enhances the capacity of the particle to promote macrophage foam cell formation, whereas GV sPLA₂ modification of LDL from apoE^{-/-} mice lacked this pro-atherogenic effect. Interestingly, apoE × GV sPLA₂ double knock-out mice had significantly less collagen deposition in lesions compared to apoE^{-/-} mice despite similar atherosclerotic lipid area. Thus, GV sPLA₂ activity in the arterial intima may lead to two independent processes: 1) macrophage foam cell formation through the generation of a structurally altered particle; and 2) altered gene expression through the generation of bioactive lipid mediators.

The objective of this study was to investigate whether GV-LDL produces inflammatory effects in macrophages independent of cholesterol accumulation. Although there is data suggesting that PLA₂s and their lipolytic products, namely lyso-PL and FFA, modulate inflammation and thus have an effect on atherosclerosis development, the current literature is controversial. For example, Curfs et al. have reported that GX sPLA₂ has anti-inflammatory effects *in vitro*, however its overexpression *in vivo* triggers lung pathology consistent with massive inflammatory cell deposition [17]. Studies showing that saturated FFA but not unsaturated fatty acids activate NFκB in macrophages by stimulating TLR4 [18-19] have been recently questioned [20]. Lyso-PCs have been implicated in pro-inflammatory responses in neural tissue [21] but on the other hand have been shown to abrogate effects of lipopolysaccharide (LPS) in neutrophils [22].

In the current study we show that lipolytic products released from GV-LDL induce NF κ B activation in macrophages, and consequently, the secretion of pro-inflammatory cytokines. Thus, this study provides an additional mechanism by which sPLA₂ may promote atherogenic processes.

Experimental procedures

2.1 Isolation and modification of LDL

LDL (density 1.019-1.063) was isolated from the plasma of healthy volunteers by sequential ultracentrifugation and stored at 4 °C under argon gas. For LDL hydrolysis, mouse GV sPLA₂ was partially purified from conditioned media of COS-7 cells infected with a replication-defective adenoviral vector encoding this enzyme as described previously [10]. Phospholipase activity of partially purified enzyme was determined using a colorimetric assay with 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) as substrate (1 unit generates 1 nmol of FFA in 20 minutes) [10]. LDL particles (1 mg/ml) were incubated overnight in hydrolysis buffer (0.1 M HEPES, 0.1 M NaCl, 1 mM CaCl₂, 20 mg/ml fatty acid-free BSA and 0.01% butylated hydroxytoluene) under argon gas at 37 °C in the presence of 500 U/ml GV sPLA₂ (GV-LDL). LDL hydrolysis was quantified by measuring the amount of FFA released in the solution [10]. As control, LDL was incubated overnight in hydrolysis buffer in the absence of the enzyme (control-LDL). For the experiments described here, incubation with GV sPLA₂ resulted in the hydrolysis of ~ 60% of LDL-PL. As reported previously [23], there was no evidence that GV sPLA₂ hydrolysis led to apoB degradation or alterations in the charge of LDL particles. To remove large aggregates, GV-LDL hydrolysis mixtures were passed through a 0.1- μ m filter. Details regarding density gradient ultracentrifugation of control-LDL and GV-LDL and the characterization of fractions derived from control-LDL and GV-LDL are provided in Supplemental Materials.

2.2 Cell culture

J-774 macrophage-like cells were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine. Cells were incubated for the indicated times with 100 μ g/ml control-LDL or GV-LDL or 2 mg/ml albumin-enriched fraction derived from control-LDL and GV-LDL. Throughout the experiments 100 ng/ml lipopolysaccharide (LPS) was used as positive control. When indicated, cells were pre-incubated for 1 h with 20 μ M of the specific NF κ B inhibitor SN50 or a control peptide (Calbiochem) or 10 μ M 6-amino-4-phenethylaminoquinazoline, an inhibitor of NF κ B transcriptional activation (Calbiochem) [24]. All reagents besides LPS were tested for endotoxin contamination using the limulus amoebocyte lysate assay (Cambrex) and were below the limits of detection (< 0.1 ng/ml). For treatments with oleic and linoleic acid, preparation of experimental media was carried out as described by Toborek et al. [25]. Stock solutions of high purity (>99%) fatty acids (Nu-Chek-Prep, Elysian, MN) were prepared in hexane. NaOH (6 N, or 30 $\times$ molarity of FFA) was used for saponification to convert the FFA into a water-soluble form. The desired amount of FFA was aliquoted, mixed with 6 M NaOH, and dried under argon gas. The residue was dissolved in experimental medium containing 5% FBS. The pH was then adjusted to 7.4 with 1.2 M HCl and the medium was sterilized through a syringe-driven filter unit.

2.3 Real-Time RT- PCR

Total RNA was isolated from J-774 cells using the TriReagent (Molecular Research Center, Inc.). RNA (1-2 μ g) was reverse transcribed into cDNA using the Reverse-transcription System (Promega). After 4-fold dilution, 5 μ l was used as a template for real-time PCR. Amplification was done for 40 cycles using Power SYBR Green PCR master Mix Kit (Applied Biosystems)

and DNA Engine Optical 2 System (MJ Research Inc.). Quantification was performed in duplicate using the standard curve method and normalizing to 18S. The primers used for various genes are as follows (gene: primers): mTNF- α forward: 5'-GGCAGGTCTACTTTGGAGTCATTG-3', reverse: 5'-GGTAGAAGGACACAGACTGG-3'; mIL-6 forward: 5'-CAACGATGATGCACTTGCAG-3', reverse: 5'-GTAGCTATGGTACTCCAGAAG-3'; m18S RNA, forward: 5'-GCTGGAATTACCGCGGCT-3'; reverse: 5'-CGGCTACCACATCCAAGGAA-3'.

2.4 TNF- α and IL-6 measurements

TNF- α and IL-6 were quantified using enzyme linked immunosorbent assay (ELISA) kits (R&D Systems).

2.5 Indirect immunofluorescence

Cells were grown on glass cover-slips for confocal microscopy. After the treatments, J-774 cells were fixed in 10% formaldehyde for 10 min, permeabilized with 0.1 M Na-citrate for 15 min and then subjected to indirect immunofluorescent staining using mouse anti-p65 IgG (Santa Cruz). Alexa Fluor-488 labeled goat anti-mouse IgG (Molecular Probes) was used as secondary antibody at a dilution of 1:200. As control, cells were incubated in the presence of non-specific primary antibody. Cellular cytoskeleton was visualized with rhodamine phalloidin staining (R415, Molecular Probes) at room temperature for 1 h. After incubations, cells were washed extensively and mounted on slides using Fluorescence Protecting Medium containing DAPI (Vectashield, Vector Laboratories, Burlingame, CA). Confocal microscopy was performed at the University of Kentucky Imaging Facility using a Leica laser scanning confocal microscope with argon (488 nm) and krypton (568 nm) lasers. Nuclear localization of NF κ B was quantified in at least 50 randomly chosen cells using ImageProTM software. All areas with positive staining for p65 were selected and fluorescent area associated with the nucleus (defined by DAPI staining) was expressed as % of the total fluorescent area in cells (defined by phalloidin staining).

2.6 Quantitation of phospholipids and fatty acids by HPLC ESI tandem Mass spectrometry

Lipids and fatty acids were extracted from LDL and BSA fractions using acidified organic solvents [26] with addition of 50 pmol C17 lysoPC as an internal recovery standard. After evaporation to dryness material was dissolved in 1 ml 4:1 MeOH:CHCl₃ and an aliquot removed for phosphorous determination after wet digestion in perchloric acid. Half of the remaining material was used for phospholipid analysis. Molecular species of PC and lyso-PC were quantified by reverse phase HPLC electrospray ionization tandem mass spectrometry using an ABI 4000 Q-Trap hybrid linear ion trap triple quadrupole mass spectrometer operated in positive mode with ion source and instrument settings optimized using a set of internal standards [27-28]. Molecular species specific precursor product transitions were monitored and calibration accomplished by reference to a set of synthetic standards obtained from Avanti Polar Lipids that were independently quantified by phosphorous analysis.

Fatty acids were quantified as 3-acyloxymethyl-1-methylpyridinium iodide (AMMP) derivatives using a modification of the method described by W-C Yang *et al.* [28]. In brief, the remaining half of the extracted material was evaporated to dryness, derivatized by reaction with 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide methyl pyridinium and the AMPP derivatives analysed by HPLC ESI tandem mass spectrometry using an ABI 4000 Q-Trap hybrid linear ion trap triple quadrupole mass spectrometer operated in positive mode with ion source and instrument settings optimized using a set of AMPP derivatized fatty acid standards. Fatty acid molecular species were quantified using specific precursor product ion transitions. Calibration was accomplished by reference to a set of

synthetic fatty acid AMPP derivatives prepared from accurate mass standards obtained from NuCheck Prep. Product ion spectra were generated for the most abundant species to determine the position of double bonds.

2.7 Statistical analysis

Data are expressed as mean $\pm$ SE. Results were analyzed by one or two-way ANOVA followed by Tukey's or Bonferroni's post-test, respectively. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 GV sPLA₂-modified LDL promote TNF- α and IL-6 secretion by J-774 macrophages

In previous studies we determined that human LDL hydrolyzed by Group V sPLA₂ induces macrophage foam cell formation to a significantly greater extent than native LDL [10]. In this study we investigated whether GV-LDL has other pro-atherosclerotic effects on macrophages in addition to intracellular lipid accumulation. J-774 macrophage-like cells were incubated with 0.1 mg/ml GV-LDL or control-LDL, and then cells and media were collected at selected intervals to quantify the expression of inflammatory cytokines. Incubations with GV-LDL led to a significant, time-dependent increase in both TNF- α and IL-6 mRNA expression compared to baseline values, whereas control-LDL lacked this effect (Fig. 1A, B). Accompanying these increases in mRNA expression were significant ~ 11 - and 6.5-fold increases in the amount of TNF- α and IL-6, respectively, in media from macrophages incubated with GV-LDL for 8 h compared to control LDL (Fig. 1C, D). In contrast, addition of equivalent amounts of GV sPLA₂ in the absence of LDL did not significantly affect cytokine secretion (Fig. 1C, D grey bars). Similar effects on TNF- α secretion also occurred when mouse peritoneal macrophages were incubated with GV-LDL (supplemental Fig. 1).

3.2 GV-LDL induces nuclear translocation of NF κ B

NF κ B is a transcription factor mediating the inflammatory responses triggered by various stimuli. A required step for NF κ B activation is its transport to the nucleus, which occurs subsequent to the phosphorylation and ubiquitination of the inhibitor of NF κ B, I κ B. To investigate whether GV-LDL induces cytokine expression through NF κ B activation, the intracellular localization of the NF κ B p65 subunit was assessed in J-774 cells after 1 h treatment with either control-LDL or GV-LDL. For comparison, J-774 cells were treated with 100 ng/ml LPS. As expected, analysis by confocal microscopy revealed that the bulk of p65 is present in the nucleus of J-774 cells after treatment with LPS, consistent with activation of NF κ B (Fig. 2A, upper left panel, arrows). Interestingly, cells treated with GV-LDL also showed nuclear localization of p65 (Fig. 2A, upper right). In contrast, there was no evidence for translocation of p65 to the nucleus of cells treated with control-LDL (Fig. 2A, lower left). The use of a non-specific primary antibody confirmed the specificity of the immunofluorescence in these experiments (Fig. 2A, lower right). Quantification of nuclear p65-positive fluorescence (expressed as the percent of total cellular fluorescence) confirmed nuclear translocation of NF κ B in cells treated with either LPS or GV-LDL, but not control-LDL (Fig. 2B).

3.3 NF κ B inhibition reduces cytokine secretion induced by GV-LDL

To provide additional evidence that GV-LDL induces cytokine secretion through activation of NF κ B, we incubated cells with GV-LDL in the presence of two different NF κ B inhibitors: SN50, a cell-permeable inhibitory peptide of NF κ B translocation [29], or 6-amino-4-phenethylaminoquinazoline (quinazoline), an inhibitor of NF κ B transcriptional activation [24]. Cells were pre-incubated for 1 h with SN50 or control peptide, or quinazoline or vehicle, followed by 8 h incubations with 0.1 mg/ml control-LDL or GV-LDL in the presence of the

same treatment (Fig. 3). Whereas inhibition of NF κ B activation had no effect on the amount of TNF- α secreted by cells incubated with control-LDL, the increase in TNF- α induced by GV-LDL was significantly blocked in cells incubated with either SN50 (Fig. 3A) or quinazoline (Fig. 3B).

3.4 Albumin-bound lipolytic products present in GV-LDL promote cytokine secretion by J-774 cells

It was of interest to determine whether GV-LDL leads to NF κ B activation through actions of the structurally altered LDL particle or, alternatively, lipid products generated from the hydrolysis of LDL-PL. In our studies, LDL was hydrolyzed in the presence of physiologic albumin concentrations (2%). Under these conditions, FFA liberated by sPLA₂ are transferred from LDL to albumin [23,30]. We therefore considered density gradient ultracentrifugation a reasonable method to effectively separate the hydrolyzed LDL particle from its lipolytic products (Supplemental Fig. 2). We tested the ability of re-isolated LDL (top fractions after density-gradient centrifugation) and the albumin/FFA-enriched fraction (bottom fractions after density-gradient centrifugation) to induce cytokine release in J-774 cells. In order for responses to be directly compared, cells were incubated with either 0.1 mg/ml of the re-floated LDL or 2.0 mg/ml of the albumin-containing fraction, concentrations corresponding to those used prior to density-gradient ultracentrifugation (see Fig. 1). Although the re-floated GV-LDL was somewhat more effective in stimulating cytokine release compared to the re-floated control-LDL, this fraction was clearly less potent compared to the albumin fraction derived from GV-LDL or a mixture of the bottom and top fractions derived from GV-LDL (Fig. 4A,C). TNF- α and IL-6 secretion induced by the albumin-enriched fraction was significantly inhibited by quinazoline, suggesting a role for NF κ B in the pro-inflammatory effect (Fig. 4 B,D).

3.5 Albumin-bound 18:1 present in GV-LDL promote cytokine secretion by J-774 cells

Our data indicate that lipolytic products present in GV-LDL promote macrophage inflammatory responses. Thus, it was of interest to identify the FFA and lyso-PC species that are generated when LDL is hydrolyzed by GV sPLA₂. Lipid extracts from control-LDL and GV-LDL were analyzed by HPLC ESI tandem Mass spectrometry as described in Experimental Procedures. The newly formed FFA and lyso-PC species (defined as the molecular species present on re-isolated GV-LDL or the albumin-enriched fraction derived from GV-LDL after subtracting the constitutively present species on the corresponding fractions from control-LDL) are shown in Fig. 5 A and B. The most abundant newly formed FFA species were 18:2 (linoleic acid) and 18:1 (oleic acid, Fig. 5A). As expected, most of the FFA was associated with the albumin fraction. The most abundant newly formed lyso-PC species contained 14:0 (myristic), 16:1 (palmitic), and 18:2 fatty acyl groups. The MS/MS analysis identified 18:1 as delta 9 and 18:2 as delta 9 delta 12. Interestingly, lyso-PC species were approximately equally distributed between LDL particles and albumin (Fig. 5B).

To investigate whether either 18:1 or 18:2 comprise the bioactive component of GV-LDL, J-774 cells were incubated with 100 μ M albumin-conjugated 18:1 or 18:2, concentration comparable to the amount of the FFA present in the GV-LDL mix. Incubations with 18:1 resulted in significantly increased TNF- α and IL-6 secretion (Fig. 5C, D). Although 18:2 resulted in modestly increased TNF- α and IL-6 secretion, this effect was not significant.

4. Discussion

Several lines of evidence suggest that GV sPLA₂ modification of LDL results in structural alterations of the lipoprotein particle that promote atherosclerotic lipid accumulation [8-10, 31-32]. In this study we provide novel evidence that products generated through LDL hydrolysis activate NF κ B, a transcription factor and key regulator of inflammation that has

been implicated in atherosclerotic processes [33]. Our data show that incubations with GV-LDL, but not control LDL, result in nuclear translocation of the p65 subunit of NF κ B and increased expression of NF κ B target genes, namely TNF- α and IL-6. Importantly, SN50, a cell-permeable peptide that inhibits nuclear translocation of the transcription factor active complex [29], and 6-amino-4-phenethylaminoquinazoline, an inhibitor of NF κ B transcriptional activation [24], effectively blocked TNF- α and IL-6 upregulation by GV-LDL. Analysis of data from all of our studies using the cell-permeable peptides suggested these reagents activate macrophages to some extent. Nevertheless, the effect of GV-LDL on macrophage activation was clearly blunted when nuclear translocation of NF κ B was inhibited. In addition, treatments with quinazoline, an inhibitor of NF κ B transcriptional activation, also blocked the pro-inflammatory effect of GV-LDL without elevating baseline cytokine secretion. The conclusion that inflammatory cytokine induction was due to a specific product of LDL hydrolysis and not an experimental artifact such as endotoxin contamination was substantiated by control experiments in which preparations of LDL ("control-LDL") were subjected to identical manipulations as GV-LDL, except that GV sPLA₂ was omitted from hydrolysis reactions. We also verified that all reagents besides LPS used in this study were endotoxin-free, and that incubating cells with equivalent amounts of GV sPLA₂ by itself did not evoke NF κ B activation in macrophages.

After density gradient ultracentrifugation, the bioactive component of GV-LDL was recovered in a FFA-enriched albumin fraction and not the re-floated GV-LDL. Thus, the effect of GV-LDL on NF κ B-mediated inflammatory responses does not appear to be dependent on binding/uptake of the modified LDL particle, but is rather mediated by bioactive lipids generated through LDL hydrolysis. Analysis by HPLC ESI tandem mass spectrometry demonstrated that considerable amounts of both 18:1 and 18:2 were generated when LDL was hydrolyzed by GV sPLA₂, confirming previously published studies [34]. We further defined these FFA species as 18:1 Δ 9 and 18:2 Δ 9 Δ 12. Although newly formed lyso-PC species were also present on GV-LDL (containing primarily 14:0, 16:1, and 18:2), it seems unlikely that these represent the bioactive component since they partitioned approximately equally between the FFA-enriched albumin fraction and the re-floated GV-LDL. Notably, additional studies showed that treatments with 18:1 alone at concentrations approximating the amount present in unfractionated GV-LDL reproduced the effect of GV-LDL. Interestingly, evidence suggests that dietary monounsaturated fatty acids lead to cholesteryl oleate enrichment of LDL, which has been implicated in atherosclerotic processes in both experimental animals and in humans (recently reviewed in [35]). Our studies would suggest that oleate-enrichment of LDL phospholipids may also be detrimental. Other studies have reported pro-inflammatory activity of non-saturated fatty acids, namely 18:2, on endothelial cells [36]. These authors pointed to a pathway for NF κ B activation involving phosphatidylinositol 3-kinase/amino kinase terminal (PI3K/Akt), extracellular signal regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK).

Several NF κ B activation pathways have been identified in cells, with LPS being one of the most potent activators of both classic and alternative pathways (reviewed in [33]). Hence, LPS has been widely used as a stimulatory agent to study the effects of various molecules, including GV sPLA₂ on inflammation. Using an siRNA approach, Ruiperez et al. showed that endogenous GV sPLA₂ potentiates the effect of LPS on COX-2 activation in P388D1 macrophages [37]. In contrast, our studies were carried out in the absence of any other pro-inflammatory stimuli. While the amount of cytokine secretion induced by GV-LDL is relatively modest compared to maximal responses produced by LPS, the co-localization of GV sPLA₂ and high concentrations of LDL in the vessel wall could provide an important mechanism for initiating macrophage activation.

A previous study by Karabina *et al.* investigated the effect of incubating human endothelial cells with LDL particles hydrolyzed by GX sPLA₂ [38]. According to this report, GX sPLA₂ hydrolysis results in the formation of an altered LDL particle that is capable of activating the MAP kinase pathway in endothelial cells, which leads to increased arachidonic acid release and increased expression of major adhesion molecules, including ICAM-1 and VCAM-1. Although re-isolation of GX-LDL by ultracentrifugation did not attenuate its ability to induce AA release, the authors did not rule out the possibility that hydrolytic products generated through the action of GX sPLA₂ act alone or in combination with the modified LDL to induce biological responses. Whether lipolytic products were effectively removed from the re-isolated GX-LDL was not directly determined. Since hydrolysis reactions were carried out in the presence of sub-physiological concentrations of BSA (0.0125%), it seems likely that under the conditions of the study potentially bioactive lipids liberated by sPLA₂ did indeed accumulate in the hydrolyzed LDL particle, as previously described by Kleinman *et al.* [30].

According to a previously published report, human recombinant GIB and GX sPLA₂ induce TNF- α and IL-6 secretion from human alveolar macrophages through a mechanism that is independent of their phospholipase activity [39]. These findings contrast to the current study, where incubations with GV sPLA₂ in the absence of LDL failed to evoke cytokine secretion. Differences in cell types and the amount of sPLA₂ used in the two studies likely account for the discrepant results. The authors of the previous study speculated that sPLA₂-induced cytokine production may be mediated through high affinity binding to the M-type sPLA₂ receptor, which is highly expressed in human alveolar macrophages. Based on results from RT-PCR analysis, J-774 cells express little/no M-type receptor mRNA (our unpublished data). Furthermore, the effect of GIB and GX on cytokine secretion did not become statistically significant until relatively high concentrations were used (1 μ g/ml). We estimate based on silver staining of partially purified enzyme that J-774 cells were exposed to <1 ng/ml GV sPLA₂ in the current study.

Our finding that GV sPLA₂ hydrolysis of LDL leads to increased pro-inflammatory responses through NF κ B activation differs from lipoprotein lipase action on triglyceride-rich lipoproteins, which appears to effectively limit inflammatory responses through the production of ligands for PPARs [40-41]. Although sPLA₂'s can generate lipid mediators (FFAs, prostaglandins) that are potential PPAR ligands, evidence that this occurs is controversial [40,42-43]. In the case of lipoprotein-associated phospholipase A₂ (Lp-PLA₂) the enzyme effectively hydrolyzes truncated acyl chains present on oxidatively modified LDL to release lyso-phosphatidylcholine and oxidized FFAs [44]. Thus, different FFA-generating lipases may induce distinct responses s generated. Our finding that hydrolytic products, likely 18:1, liberated from LDL by GV sPLA₂ induce macrophages to secrete inflammatory cytokines may have important implications for innate immunity and chronic inflammation in atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary

GV-LDL	LDL hydrolyzed by Group V sPLA ₂
GV sPLA ₂	group V secretory phospholipase A ₂
LDL	low-density lipoprotein
control-LDL	LDL incubated in hydrolysis buffer in the absence of enzyme
lyso-PL	phospholipid
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
PC	phosphatidylcholine
PL	phospholipid

Fig.1. GV-LDL promotes TNF-alpha and IL-6 secretion from J-774 cells

J-774 cells were incubated with 100 μ g/ml control-LDL, GV-LDL, or the equivalent amount of GV sPLA₂ present in 100 μ g/ml GV-LDL for the indicated times. (A, B) RNA was isolated from cells and TNF- α and IL-6 mRNA expression was quantified by qRT-PCR and normalized to 18S. Results are expressed as fold increase compared to baseline levels. (C, D) TNF- α and IL-6 protein in the media were quantified by ELISA using commercially available kits. Data are presented as mean $\pm$ SE and are representative of 3 independent experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to control-LDL.

Fig. 2. GV-LDL induces NFκB nuclear translocation

(A) J-774 cells were incubated with 100 ng/ml LPS, 100 μg/ml GV-LDL or 100 μg/ml control-LDL for 1h and then fixed in 10% formaldehyde. NFκB was visualized by indirect immunofluorescence using mouse anti-p65 IgG as primary antibody (green). The lower right panel shows cells immunostained with a non-specific primary IgG. Cell cytoskeleton was visualized with rhodamine phalloidin (red) and nuclei with DAPI (blue). Arrows indicate nuclear localization of p65. (B) Nuclear NFκB was quantified in 50 randomly selected cells per group and expressed as the amount of p65-positive fluorescence associated with the nucleus as a percent of total cellular fluorescence. ** indicates $p < 0.01$ compared to control-LDL, # indicates $p < 0.01$ compared to GV-LDL.

Fig. 3. The induction of inflammatory cytokines by GV-LDL is dependent on NFκB activation
(A) J-774 cells were pre-incubated for 1 h with 20 μM control peptide or the NFκB specific inhibitor SN50. Cells were then incubated for 8h with 100 μg/ml control-LDL or GV-LDL in the presence of the same peptide, and the amount of TNF-α in the media was quantified. **(B)** J-774 cells were pre-incubated for 1 h with 10 μM 6-amino-4-phenethylaminoquinazoline (quinazoline) or vehicle, and then incubated under the same treatment conditions for 8h with 100 μg/ml control-LDL or GV-LDL. Data are presented as mean±SE of triplicate determinations and are representative of 3 independent experiments. ** indicates $p < 0.01$ compared to cells incubated in the presence of inhibitor.

Fig. 4. The albumin-bound lipolytic products promote cytokine secretion by J-774 cells
J-774 cells were incubated for 8h with 100 μ g/ml re-floated control-LDL or GV-LDL (top), or 2 mg/ml albumin-enriched fractions derived from control-LDL or GV-LDL (bottom), or a mixture of the two fractions (recombined), and the amount of (A) TNF- α and (C) IL-6 protein present in the media was quantified by ELISA. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to the corresponding fraction from control-LDL. (B, D) J-774 cells were incubated for 8 h with the bottom fraction derived from GV-LDL (100 μ g/ml) in the presence of vehicle or 10 μ M quinazoline. * indicates $p < 0.05$ compared to vehicle. Data are presented as mean $\pm$ SE and are representative of at least 3 experiments.

Fig. 5. Analysis of FFA and lyso-PC generated after hydrolysis of LDL

(A, B) Lipids were extracted from 100 μ l aliquots of the top (LDL) and bottom (BSA) fractions derived from control-LDL and GV-LDL after ultracentrifugation and analyzed for molecular species of FFA and lyso-PL by HPLC ESI tandem Mass spectrometry. Newly formed FFA and lyso-PC are defined as the molecular species present on re-isolated GV-LDL or the albumin-enriched fraction derived from GV-LDL after subtracting the species present on the corresponding fractions from control-LDL. (C, D) J-774 cells were incubated for 8h with 100 μ M of albumin-bound 18:1 or 18:2 and the amount of TNF- α and IL-6 secreted into the media was quantified by ELISA. Data are averaged from 2 separate experiments, each performed in triplicate. * indicates $p < 0.05$, ** indicates $p < 0.01$.