Lysine-Phosphatidylcholine Adducts in Kringle V Impart Unique Immunological and Potential Pro-inflammatory Properties to Human Apolipoprotein(a)*

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Lipoprotein(a), Lp(a), an athero-thrombotic risk factor, reacts with EO6, a natural monoclonal autoantibody that recognizes the phosphocephaly (PC) group of oxidized phosphatidylcholine (oxPtdPC) either as a lipid or linked by a Schiff base to lysine residues of peptides/proteins. Here we show that Eo6 reacts with free apolipoprotein(a) apo(a), its C-terminal domain, F2 (but not the N-terminal F1), kringle V-containing fragments obtained by the enzymatic digestion of apo(a) and also kringle V-containing apo(a) recombinants. The evidence that kringle V is critical for EO6 reactivity is supported by the finding that apo(a) of rhesus monkeys lacking kringle V did not react with EO6. Based on the previously established EO6 specificity requirements, we hypothesized that all or some of the six lysines in human kringle V are involved in Schiff base linkage with oxPtdPC. To test this hypothesis, we made use of a recombinant lysine-containing apo(a) fragment, rIII, containing kringle V but not the protease domain. EO6 reacted with rIII before and after reduction to stabilize the Schiff base and also after extensive ethanol/ether extraction that yielded no lipids. On the other hand, delipidation of the saponified product yielded an average of two mol of phospholipids/mol of protein consistent with direct analysis of inorganic phosphorus on the non-saponified rIII. Moreover, only two of the six theoretical free lysine amino groups per mol of rIII were unavailable to chemical modification by 2,4,6-trinitrobenzene sulfonic acid. Finally, rIII, like human apo(a), stimulated the production of interleukin 8 in THP-1 macrophages in culture. Together, our studies provide evidence that in human apo(a), kringle V is the site that reacts with EO6 via lysine-oxPtdPC adducts that may also be involved in the previously reported pro-inflammatory effect of apo(a) in cultured human macrophages.

Lipoprotein(a) (Lp(a)), a recognized risk factor for atherosclerotic cardiovascular disease is made of a lipoprotein particle containing apoB100 linked by a single disulfide bridge to apolipoprotein(a), apo(a), a multikringle structure that varies in size due to differences in the number of kringle (K) type IV-2 repeats. The other kringle, classified from 1 to 10, occur as a single copy each differing in amino acid sequence (1). The function of some of these kringles has been recognized. For instance, the microdomain comprising KIV-5 through KV-8 has been reported to be involved in the first step of non-covalent interaction between apo(a) and apoB100, and KIV-9 via its unpaired cysteine is responsible for the formation of the disulfide bond that stabilizes the interaction between apo(a) and apoB100 (1, 2). Moreover, KIV-10 contains the high affinity lysine binding site that plays a dominant role in the binding of apo(a) to fibrinogen and components of the vascular extracellular matrix (3). For KV, the reported roles have been an attenuating effect on LDL oxidation (4) and stimulation of IL-8 production in human macrophages (5). Of interest, human KV has six lysine residues in contrast with the other kringles that contain no lysine except for KIV-4 and KIV-9 each having one lysine (6).

In apo(a), kringles are joined by linkers that vary in size and amino acid sequence and contain O-glycans that account for most of the carbohydrate content of apo(a), about 33% by weight. An exception is the linker between KIV-4 and KIV-5 that contains no carbohydrates and shown to be the preferential site of cleavage by metalloproteinases and leukocyte and pancreatic elastases (7). Under conditions of limited proteolysis these enzymes cleave apo(a) into two domains, F1 and F2, representing the N- and C-terminal domains, respectively, the latter spanning the region between KIV-5 and the protease domain (PD). F1 and F2 differ markedly in structural properties and function (7).

There has been much recent emphasis on the pro-inflammatory and pro-atherogenic influences of oxidized lipoproteins with particular focus on oxidized LDL (oxLDL) (8). During the oxidation of LDL, many neo-antigenic determinants are generated, which can elicit autoantibody responses. One of these antibodies, which has received a great deal of attention is the

PtdPC, phosphatidylcholine; oxPtdPC, oxidized phosphatidylcholine; PC, phosphocholine; PL, phospholipids; PVPNC, 1-palmitoyl-2-(5'-oxo)valeroyl-sn-glycero-3-phosphorylcholine; F1, N-terminal fragment of apo(a); F2, C-terminal fragment of apo(a); K, kringle, rIII, recombinant protein containing the signal peptide, fusion kringle 1 and 5 and kringles 9, 10, and V; rK6, recombinant protein containing the signal peptide, fusion kringle 1/5, kringle 6–10, V and the protease domain; PD, protease domain; TNBS, 2,4,6-trinitrobenzene sulfonic acid; IL-8, interleukin 8; EACA, e-amino caproic acid; DTE, dithierythritol; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
monoclonal antibody EO6, an IgM natural antibody cloned from the spleen of apoE-deficient mice (9) along with several other such antibodies, recognizes predominantly the phospholipid moiety of oxidized phosphatidylcholine (PC) moieties of oxLDL (20–22). The oxidized phospholipid (PL) is covalently attached to apoB of oxLDL. In the latter situation, the oxidized PC is linked to the ε amino group of lysine of apoB via a Schiff base with the aldehyde derived from the sn-2 fatty acid of oxPC (10–12). EO6 binds not only to oxLDL but oxidized epitopes in sites of inflammation most notably the atherosclerotic plaque (13). In human plasma, EO6 has been shown to specifically react with epitopes on Lp(a) (14). These observations prompted us to investigate the location and nature of the reactive site(s) in epitopes on Lp(a) (14). These observations prompted us to investigate the location and nature of the reactive site(s) in apo(a). For this purpose we used pure preparations of human Lp(a), apo(a), Lp(a) and the potential involvement of lysines in apo(a). The results of these studies are the subject of this report. 

EXPERIMENTAL PROCEDURES

Materials—Human leukocyte elastase (LE) (EC 3.4.21.37), porcine pancreatic elastase (EC 1.4.3.11) Type V, ε-aminocaproic acid (EACA), (4-amidinophenyl)-methanesulfonic fluoride (APMSF), diisopropyl fluorophosphates (DFP), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and PtdPC-specific phospholipase C (3.1.4.3) from Bacillus cereus (2000 units/mg; P9439) (<20 units/mg sphingomyelinase activity) were from Sigma Chemical Co. Immobilon-P membranes were from Millipore (Bedford, MA) and an enhanced chemiluminescent kit (ECL Western blotting detection kit) from Amersham Biosciences. All tissue culture reagents were obtained from Invitrogen and were of low endotoxin grade.

Preparation of Human Lp(a), Apo(a), Lp(a−), and LDL—The purified fragments were stored in 10 mM PO4, pH 7.4, containing 100 mM EACA and 125 mM trehalose at −80 °C.

Generation of Recombinant Apo(a) Fragments—The 6K recombinant, r6K, was cloned into a pCMV-4 vector driven by a cytomegalovirus promoter as described and provided by Dr. N. O. Davidson (22). It contains the signal sequence, a 333 bp fusion kringles (KIV-1) and 240 bp from KIV-5, followed by 2004 bp of apo(a) sequence containing single copies of KIV-2 through KIV-10, LDL, and a protease domain. The peptide signal sequence was necessary in order to permit secretion of the recombinant protein into the conditioned medium. The rIII recombinant lacking KIV-6, 7, 8, and PD (6K/KIV6–8PD) was prepared by digesting the 6K expression plasmid with ClaI and EcoRV. Subsequently, the 2.4 kb ClaI-EcoRV insert was subjected to digestion with BamHI in order to remove the BamHI-BamHI internal DNA domains coding for apo(a) KIV-6 through 8. The 0.32 kb ClaI-BamHI and the 1.03 kb BamHI-EcoRV DNA fragments were then ligated back into the 6K expression plasmid digested with ClaI and EcoRV (vector part). Both the 6K and the rIII (6K/KIV6–8PD) (Fig. 2) were transfected into human embryonic kidney 293 cells and the clones producing significant amounts of recombinant products (0.5–10 mg/liter of the culture medium) were purified by lysine-Sepharose chromatography.

Delipidation of Apo(a)—Lipids were extracted from purified apo(a) at 4 °C for 8 h with 95% ethanol/ethyl ether, 3:2 v/v. The precipitated apo(a) was subjected to a further extraction with ethyl ether for 18 h at 4 °C followed by four washes of ethyl ether.

Protein Modification—NaCNBH3 reduction of Lp(a) and apo(a), its derived fragments and the recombinants was carried out by a method previously described with minor modifications (11). Briefly, Lp(a), apo(a), and fragments, present in 0.05 μL HEPES buffer, pH 7.0, were incubated with 0.15 mm NaCNBH3 at a final concentration of 20 μM at room temperature for 2 h. The reaction was stopped by extensive dialysis against 10 mM PO4 buffer, pH 7.0. Under these conditions, Schiff base adducts are selectively reduced to stable amines.
Properties of Kringle V of Apolipoprotein(a)

The EO6 Reactive Site Resides in Apo(a)—In human plasma EO6 reacts with Lp(a) (14). To define the EO6 reactivity of the protein, we carried out Western blot analyses of 4% SDS-PAGE on Lp(a), Lp(a-), apo(a), and authentic LDL isolated from the same subject. Both Lp(a) and apo(a) were EO6 reactive as well as oxLDL, used as a positive control. In contrast, both authentic LDL and Lp(a-)/apo(a-) were unreactive (Fig. 3A). Of note, the immunoblots performed with EO6 detected mainly the associated forms of apo(a), i.e. dimer and trimer (Fig. 3A, lane 1) probably an expression of a high abundance of epitopes in the aggregated species. In contrast, the anti-apo(a) blots exhibited the monomeric form (Fig. 3A, lane 6). Aggregation was likely attributable to the presence of kringle IV-9 containing an unpaired cysteine. Because of differences in sensitivity, the detection in the EO6 system required a 50-fold larger gel loading of apo(a) than in the anti-apo(a) system. The concentration dependence of the aggregation was demonstrated by the fact that a load of 5 µg of apo(a) under the same electrophoretic conditions (presence of SDS, non-reduced) resulted in the appearance of dimer-trimer species upon Coomassie Blue staining (data not shown).

The EO6 Reactive Site Resides in the C-terminal Domain of Apo(a)—To define the EO6 reactive site, apo(a) was subjected to limited proteolysis by pancreatic elastase. In keeping with previous findings (21), this procedure generated two major fragments, F1, and F2, representing the N- and C-terminal domains, respectively (Fig. 1). By Western blot analyses of 4% SDS-PAGE, EO6 detected only F2 (Fig. 3B). As noted for apo(a) in Fig. 3A, the mass of F2 required for EO6 detection was 5 µg, and aggregated forms were also seen.

The EO6 Reactive Site Resides in the KV-PD Region of F2—In these studies we subjected apo(a) to a more extensive digestion by leukocyte elastase and the resulting fragments were purified to homogeneity (5). Of them, only F7 (KV-PD) reacted with EO6 (Fig. 3C), as compared with the lack of reactivity by F5 and F6 (KIV-5 through KIV-10). The absence of aggregation noted for F7 can be explained by the fact that it lacks kringle IV-9 containing the free cysteine. The results in Fig. 3 were corroborated in a direct ELISA binding assay (Fig. 4). Fragment F7 (KV-PD) was less reactive by ELISA than by Western blot analyses. This could be due to less efficient platelet efficiency due to its smaller size.

Studies on Rhesus Monkey Apo(a)—Rhesus monkey apo(a) lacks KV while retaining the PD (24). By both Western blot analysis (not shown) and ELISA (Fig. 4A), rhesus apo(a) did not react with EO6. This observation is consistent with the thesis that KV of apo(a) is responsible for the EO6 reactivity found on apo(a).

Studies with Recombinants—In these studies we wanted to establish whether the information on KV obtained with natural products could also apply to apo(a) recombinants. For this purpose, we used two recombinants, r6K and rIII that have been described in Fig. 2. These two recombinants share the signal peptide, the fusion kringle IV-1/5, KIV-9, KIV-10 and KIV but not the PD. Both r6K and rIII reacted with EO6 by Western blot analyses (Fig. 3D) and ELISA (Fig. 4) confirming that KV is the apo(a)-reacting site. KIV-1/5, KIV-9 and KIV-10 are excluded as the reactive site based on the experiments.

Other Analyses—Inorganic phosphorous (P) was quantified using the microtechnique described by Bartlett (23) by first digesting the sample with 10 x H2SO4, at 160 °C for 18 h. Phospholipid values were obtained by multiplying the inorganic phosphate results by 25. Protein determinations were performed by the Bio-Rad DC protein assay using BSA as a standard.

RESULTS

Identification of the EO6 Reactive Site on Apo(a)

Through KIV-8 and the PD. The kringle numbers correspond to those in the complete growth medium in the presence of 100 nM phorbol myristate acetate (50 µM) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin, and gentamicin (50 µg/ml) and 50 µM dexamethasone at 37 °C, 5% CO2. Experiments were conducted at a cell density of 1 x 106 cells/ml and incubated in the complete growth medium in the presence of 100 nM phorbol 12-myristate-13-acetate for 72 h. After washing in serum-free medium and a further incubation for 16 h, the cells were exposed to 200 µg of apo(a) or rIII for 24 h at 37 °C. At the end of the incubation, the amount of IL-8 released in the culture media was assessed by ELISA (BIO-SOURCE International, Camarillo, CA) according to the manufacturer's instructions. All the measurements were conducted in triplicate. The endotoxin content of both apo(a) and rIII, was determined by using the Limulus amoebocyte lysate assay. The amount of endotoxin for both proteins was extremely small (less than 0.25 pg/µg of protein).
depicted in Fig. 3C showing that only F7 of the fragments F5, F6, and F7 is reactive. It should be noted that on the Western blots both recombinants showed aggregates due to the relatively high loading (5 μg) on the gels. Only monomers were present in the anti-apo(a) blots.

Identification of the Determinants in KV Reacting with EO6

Having obtained evidence that KV is the EO6 reactive site in apo(a), we next directed our attention to the lysine residues based on the previous observations that EO6 binds to the PC group of oxidized phospholipids that are covalently bound to lysines of proteins or peptides by a Schiff base between the aldehyde of oxPtdPC and the epsilon amino group of the lysine (11, 12). In order to stabilize any Schiff bases present we reduced both apo(a) and rIII with NaCNBH₃ to form a stable secondary amine (see "Experimental Procedures") and performed a Western blot analysis. These two stabilized products exhibited the same EO6 reactivity as their untreated counterparts (Fig. 5A).

Phospholipids in Apo(a) and rIII—The EO6 reactivity observed on the proteins subjected to Western blot analysis in the presence of SDS implies a covalent association of PC containing oxPL with the reactive fragments of apo(a). In these studies we wanted to determine whether there were PLs in apo(a) and in rIII and, if so, their mode of association. As a first step, we subjected NaCNBH₃ reduced apo(a) and rIII to an extensive delipidation procedure with ethanol/ether followed by exhaustive ethyl ether washes (see "Experimental Procedures"). This procedure should remove all non-covalent lipids. The combined organic extracts from each sample contained no PL as assessed by P₄ analyses. Both the apo(a) and rIII precipitates readily dissolved in aqueous buffers and retained the same EO6 reactivity as the untreated product as assessed by Western blot analyses (Fig. 5A) and by ELISA (Fig. 4). As a second step, we considered a covalent association between PL and apo(a)/rIII. The covalent attachment of oxidized PL to the apo(a) is probably via a fatty aldehyde in the sn-2 position linked via a Schiff base to the lysine of the apo(a). Saponification of this complex...
should separate the fatty acid and fatty aldehyde from the glycerophosphorylcholine backbone which should now be water soluble. The NaCNBH₃ reduced Schiff base is resistant to such hydrolysis. Accordingly, apo(a) and rIII were saponified with 1 N NaOH and titrated to neutrality with 6 N HCl followed by an exhaustive ethanol/ether extraction as above. The saponified apo(a)/rIII contained no Pi, and also failed to react with EO6 (Fig. 5A). The water soluble extract contained 1.7 ± 0.5 mol and 1.5 ± 0.6 mol of phospholipid P₃/mol apo(a) and rIII, respectively.

Friedman et al. (12) have shown that the PC head group of oxPL was essential for EO6 recognition and that oxidized PL containing other head groups such as phosphorylserine (PS) did not yield EO6 reactivity. Based on this observation, we treated apo(a) and rIII with phospholipase C, an enzyme that specifically recognizes PtdPC. The results in Fig. 5B show that after phospholipase C digestion, EO6 reactivity was no longer present. Taken together the results indicated that PL, and in particular, PC as part of an oxPtdPC are covalently attached to apo(a)/rIII and that they are critical for EO6 reactivity.

Amino Group Determinations in Apo(a)/rIII—The lysine residues involved in aldehydic interaction with oxPtdPC form an imine that upon reduction with NaCNBH₃ yield a secondary amine. Thus, determinations of free amines should indicate the number of amino groups not linked to oxPtdPC. Based on the reactivity of apo(a)/rIII with TNBS, we calculated that there were an average of 2 mol of Lys/mol of protein likely involved in adduct formation. Note that this agrees well with the estimate of about 1.7 mol of P/mol apo(a) released by saponification.

**Effect of Apo(a) and rIII on the Secretion of IL-8 into THP-1-conditioned Medium**

We showed previously that apo(a) stimulates the secretion of IL-8 from THP-1 macrophages (5). In the current work we compared the effect of rIII with that of apo(a). As shown in Fig. 6, both apo(a) and rIII stimulated IL-8 secretion. Notably, rIII was 1.5–2-fold more potent than apo(a). Both apo(a) and rIII contain a single copy of KV and 2 mol of oxPtdPC/mol of protein suggesting that these elements could have been solely responsible for the effect. This conclusion is supported by our previous finding that apo(a) fragments F5 and F6 (see Fig. 1) that lack KV and are EO6 unreactive do not stimulate IL-8 production. Based on our previous finding that apo(a) fragments F5 and F6 (see Fig. 1) that lack KV and are EO6 unreactive do not stimulate IL-8 production. This interpretation is in agreement with our previous finding that F2, the C-terminal fragment of apo(a), elicits a higher production and secretion of IL-8 than the intact parent apo(a) (5).

**DISCUSSION**

The current studies have shown that KV is a critical element in the recognition of apo(a) by EO6, an autoantibody shown to react with oxPtdPC either in its free form or linked by a Schiff base to lysine residues of peptides or proteins. The role of KV in this reaction is supported by the observations that only KV-containing products, either natural or recombinants, reacted

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**Fig. 4. Chemiluminescent immunoassay for EO6 antibody binding.** The indicated antigens at 5 µg/ml in 1% BSA-PBS were plated overnight at 4 °C on 96-well microtiter plates. The wells were washed three times with PBS, blocked with 1% BSA-PBS and washed again three times with PBS. Monoclonal antibody, EO6, was applied and incubated for 1 h at room temperature. After three washes, alkaline phosphatase-labeled goat anti-mouse IgM in 1% BSA-PBS was applied and incubated for 1 h at room temperature. The bound EO6 was determined using LumiPhos (530) substrate. The amount of bound antibody was expressed as relative light units (RLU/100 ms. Each bar is the mean of triplicate determinations. Lp(a), apo(a), LDL, F2, KV-PD, r6K, and rIII were obtained as described under “Experimental Procedures.” Rh apo(a) was isolated from purified rhesus Lp(a) by mild reduction with 1.25 mM DTE as described for human apo(a). d apo(a), apo(a) that was exhaustively delipidated with ethanol/ether as described under “Experimental Procedures.”

**Fig. 5. Immunoblots of apo(a) and rIII after either saponification or phospholipase C digestion.** A, Western blots of 4–12% SDS-PAGE run without βME and probed with EO6 monoclonal antibodies. Apo(a) and rIII were reduced with NaCNBH₃, delipidated and then saponified with 1 N NaOH for 1 h at 37 °C. The reaction mixtures were neutralized with 6 N HCl, and 5 µg of protein were applied per lane. Lane 1, apo(a); lanes 2 and 3, delipidated apo(a) and rIII before saponification, respectively, lanes 4 and 5, apo(a) and rIII after saponification, respectively. B, Western of 4–12% native gel probed with EO6 monoclonal antibodies. Apo(a), rIII, and oxLDL were incubated with phospholipase C for 4 h at 37 °C, and LDL was oxidized with 10 µM Cu²⁺ for 18 h as described under “Experimental Methods.” Lanes 1 and 2, apo(a), before and after PLC digestion; lanes 3 and 4, rIII, before and after PLC digestion; lanes 5 and 6, oxLDL, before and after PLC digestion.
with EO6 and by the absence of reactivity in rhesus monkey apo(a) that lacks KV. In human apo(a), KV contains 6 lysines in contrast to the other kringles that are lysine-free except for the single lysine in KIV-9 and KIV-4, the latter kringle located in the F1 domain that we now show does not react with EO6.

By amino group analyses we also found that only four of the six lysines in KV reacted with the TNBS reagent suggesting that the other two were covalently linked, likely by a Schiff base to oxPtdPC. This conclusion is supported by the finding that nearly two mol PL/mol protein could be extracted from either apo(a) or rIII only after saponification and that such extraction caused these two proteins to lose EO6 reactivity. Moreover, reactivity was also lost upon cleavage of these proteins by phospholipase C. As EO6 does not react with oxPL containing PE or PS headgroups, this provides further support that the PL cleaved contained the PC headgroup. In the current study, the conclusion derived from the chemical analyses that only two of the six lysines in KV were involved in adduct formation received support from a molecular modeling approach that permitted the identification of Lys-12 and -42 as the likely candidates for linkage with oxPL, this based on freedom of constraints and location on the KV surface (see legend to Fig. 7). Verification of these assignments is now in progress using HPLC with tandem mass spectrometry (LC/MS) and site-directed mutagenesis. It should be noted that only 2 mol of reacting Lys were the constituents of an apo(a) with a mass of 289,000, as a glycated protein.

The finding that a recombinant product generated by human embryonic kidney cells reacted with EO6, like the apo(a) isolated from human plasma, indicates that adduct modification of the lysines of KV can occur in tissues other than the liver and does not require the presence of an apoB-containing lipoprotein. Indeed, EO6 reacts with 1-palmitoyl-2-(5'-oxo)valeroyl-sn-glycero-3-phosphorylcholine (POVPC) modified BSA and even POVPC-lysine (12). This points to the possibility that apo(a) is able to link to oxPtdPC of cell membrane origin. Apo(a) is exclusively made in the liver and generally thought to covalently bind to apoB100-containing lipoproteins, mainly LDL, at the time of secretion from the hepatocyte membrane (2). Although we cannot rule out that lysine-modifying ox-PtdPCs in apo(a) may derive from LDL, we favor the hepatocyte membrane as a source of them. First, neither native LDL, nor native apoB reacts with EO6. Second, based on the observations made in three laboratories, apo(a) partially prevents LDL from undergoing oxidation (4, 25, 26). Third, Lp(a) has a higher concentration of platelet-activating factor acetylhydrolase than authentic LDL (27), a situation that would promote a decrease rather than accumulation of oxPtdPC. According to this concept, the transport of oxPtdPCs in apo(a) would be dependent on their ability to link to the reactive lysines in KV. This would be in keeping with the hydrophilic nature of apo(a), its lack of binding to unmodified PL (28) and the high correlation between plasma concentrations of Lp(a) and EO6 reactivity (14). Along these lines it would be important to determine whether the

![Fig. 6. Effect of apo(a) and rIII on the secretion of IL-8 by THP-1 macrophages.](image)

The cells were incubated with apo(a) or rIII, each at 200 nM for 24 h at 37 °C. The medium was collected, and IL-8 determined by ELISA. The data are representative of three independent experiments each conducted in duplicate. The bars on each column represent ± S.D. Control, conditioned medium only, with no protein additives.

![Fig. 7. Amino acid sequence and spatial model of KV.](image)

Left panel, amino acid sequence (30) organized into three loops linked by disulfide bonds. The numbering begins with Cys in position 1. The six Lys in positions 7, 12, 13, 42, 48, and 70 are shown as blue-filled circles. The amino acid residues preceding Cys-1 and following Cys-80 are portions of the linker regions joining KIV-10 to Cys-1 and Cys-80 to PD. Right panel, spatial model of KV based on the coordinates derived from the crystal structure of human apo(a) KIV-10 (31). The six Lys are shown by space-filled atoms. Lys-48 and -70 form salt bridges with the carboxyl side chains of Glu-47 and Asp-73, respectively (shown in smaller space-filled atoms). Lys-13 makes hydrogen bonds with the main chain carbonyl of Asp-76. Lys-7 being located in the inner surface groove is excluded from interactions. Lys-12 and -42 protrude from the kringle surface and appear to be free of constraints and may be the likely candidates for covalent linkage to oxPL. Hydrogen in white; carbon in green; nitrogen in blue; oxygen in red.
number of lysines in KV undergoing modification may vary as a function of the oxPtdPC concentration in its immediate microenvironment. However, it should be appreciated that these studies have only examined the relationship of EO6 reactivity to the apo(a) of Lp(a). Thus, the possibility exists that some EO6 reactivity may be found in the lipid phase of the intact Lp(a).

The biological importance of the oxPtdPC adducts in human apo(a) is unclear. From the cardiovascular standpoint, various mechanisms have been suggested to explain the athero-thrombogenic potential of Lp(a) and distinct domains in apo(a) have been identified for each reported function (1). Recently, we have shown that apo(a), its C-terminal domain and one of the fragments comprising the KV-PD region stimulated the production and secretion of IL-8 in human THP-1 macrophages grown in culture (5). In those studies we also showed that IL-8 stimulation was abrogated in the presence of a monoclonal antibody specific for KV. In the current work we now show that recombinant rIII that contains KV but not PD also stimulates IL-8 production. This suggests that KV may be the effector in the reaction and that oxPtdPC adducts may have a role, taking into consideration that oxPtdPC by itself has a pro-inflammmatory function (29). However, the current study points to the fact that additional elements can cause apo(a) to stimulate IL-8. Obviously, additional mechanistic investigations are warranted. Overall, on a background of an innate immune response our studies have unveiled in KV an interesting association between oxidative and pro-inflammatory events that along with the previously suggested mechanism (1) may play a role in the cardiovascular pathogenicity of Lp(a)/apo(a). In the context of our current findings, we need to consider the possibility that other components of apo(a) may modulate the accessibility of the lysines of KV for adduct formation with oxPL. We have a pointer to this possibility in the relatively modest reactivity of KV-PD with EO6 compared with rIII (Fig. 4). Further work is clearly necessary to resolve this issue.

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