Apolipoprotein(a) stimulates nuclear translocation of β-catenin: a novel pathogenic mechanism for lipoprotein(a)

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ABSTRACT **Lipoprotein(a) (Lp(a)) is associated with cardiovascular disease risk. This may be attributable to the ability of Lp(a) to elicit endothelial dysfunction. We previously reported that apolipoprotein(a) (apo(a); the distinguishing kringle-containing component of Lp(a)) elicits cytoskeletal rearrangements in vascular endothelial cells, resulting in increased cellular permeability. These effects require a strong lysine-binding site (LBS) in apo(a). We now report that apo(a) induces both nuclear β-catenin–mediated cyclooxygenase-2 (COX-2) expression and prostaglandin E2 secretion, indicating a proinflammatory role for Lp(a). Apo(a) caused the disruption of VE-cadherin/β-catenin complexes in a Src-dependent manner, decreased β-catenin phosphorylation, and increased phosphorylation of Akt and glycogen synthase kinase-3β, ultimately resulting in increased nuclear translocation of β-catenin; all of these effects are downstream of apo(a) attenuation of phosphatase and tensin homologue deleted on chromosome 10 activity. The β-catenin–mediated effects of apo(a) on COX-2 expression were absent using a mutant apo(a) lacking the strong LBS. Of interest, the normal and LBS mutant forms of apo(a) bound to human umbilical vein endothelial cells in a similar manner, and the binding of neither was affected by lysine analogues. Taken together, our findings suggest a novel mechanism by which apo(a) can induce proinflammatory and proatherosclerotic effects through modulation of vascular endothelial cell function.**

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INTRODUCTION

Results from epidemiological studies underscore an association between circulating lipoprotein(a) (Lp(a)) levels and risk for cardiovascular disease (Dubé *et al.*, 2012; Tsimikas and Hall, 2012). Lp(a) contains a low-density-lipoprotein (LDL) moiety, as well as the unique glycoprotein apolipoprotein(a) (apo(a)), which is covalently linked to apolipoproteinB-100 (apoB-100) in LDL by a single disulfide bond (Koschinsky *et al.*, 1993). Apo(a) shares a high degree of homology with the fibrinolytic proenzyme plasminogen and contains contiguous repeats of a sequence that closely resembles the plasminogen kringle IV domain, followed by domains homologous to the kringle V and protease domains of plasminogen (McLean *et al.*, 1987). The kringle IV domains of apo(a) are divided into 10 types (McLean *et al.*, 1987). Apo(a) contains a strong lysine-binding site (LBS) within the kringle IV type 10 (KIV_{10}) sequence, which mediates the binding of apo(a) and Lp(a) to physiological ligands such as fibrin and extracellular matrix proteins (Hughes *et al.*, 1997; Sangrar and Koschinsky, 2000). Moreover, the strong LBS within KIV_{10} of apo(a) was found to be absolutely required for apo(a)-mediated vascular endothelial dysfunction characterized by increased endothelial cell contraction and permeability via a Rho/Rho kinase–dependent pathway (Cho *et al.*, 2008).

Vascular endothelial cells represent the primary target of many physiological and pathological agents, such as thrombin, inflammatory cytokines, oxidized LDL (oxLDL), and Lp(a) (Wojciak-Stothard *et al.*, 1998; Essler *et al.*, 1999; Pellegrino *et al.*, 2004). Many studies

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Abbreviations used ε-ACA, ε-aminocaproic acid; apo(a), apoliproprotein(a); COX-2, cyclooxygenase-2; EC, endothelial cell; GSK-3β, glycogen synthase kinase-3β; HUVEC, human umbilical vein endothelial cell; LBS, lysine-binding site; Lp(a), lipoprotein(a); PGE_2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; PKB/Akt, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

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have shown that Lp(a), through its apo(a) component, exerts different effects on cells involved in the development of atherosclerotic lesions. For example, Lp(a)/apo(a) stimulates vascular smooth muscle cell proliferation and migration by inhibition of transforming growth factor-β (TGF-β) activation (O'Neil *et al.*, 2004). In addition, Lp(a)/apo(a) enhances actin stress fiber formation and loss of cell–cell contact mediated in vascular endothelial cells; this is mediated by the apo(a) component of Lp(a) and leads to increased contraction of vascular endothelial cell monolayers (Pellegrino *et al.*, 2004). Apo(a), through its strong LBS, induces disruption of adherens junctions measured by VE-cadherin dispersion, resulting in increased endothelial cell (EC) permeability (Cho *et al.*, 2008). The composition of adherens junctions, where VE-cadherins associate with α-, β-, and γ-catenins, is a key determinant of the ability of the cell to regulate endothelial permeability and junction stability (Dejana *et al.*, 2008). The effect of apo(a) on the composition of adherens junctions has not yet been investigated and as such formed a primary focus of the present study.

β-Catenin plays an important role in EC function. As a major component of the cell–cell adherens junctions, β-catenin links members of the cadherin family of transmembrane cell–cell adhesion receptors to the actin cytoskeleton (Ozawa *et al.*, 1989). In quiescent cells, β-catenin is associated with VE-cadherin in complexes mainly at the plasma membrane, maintaining β-catenin in the cytoplasm at low levels. When liberated into the cytoplasm, β-catenin either translocates to the nucleus or becomes incorporated into a cytoplasmic complex with adenomatosis polyposis coli (APC), axin/conductin proteins, and glycogen synthase kinase-3β (GSK-3β), the last of which phosphorylates β-catenin on Ser-33, -37/Thr-41 sites; this leads to subsequent ubiquitination and proteasomal degradation of β-catenin (Behrens *et al.*, 1998). If GSK-3β is inactivated by phosphorylation at Ser-9, the degradation of β-catenin is attenuated, and nuclear translocation of β-catenin is favored (Behrens *et al.*, 1998). Within the nucleus, β-catenin functions as an activator of T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to stimulate transcription of variety of growth-related genes, including those encoding c-myc (Shtutman *et al.*, 1999), cyclin D1 (He *et al.*, 1998), and cyclooxygenase-2 (COX-2; Kim *et al.*, 2002; Araki *et al.*, 2003; Barbieri and Weksler, 2007). COX enzymes catalyze the synthesis of prostaglandins (PGs) from arachidonic acid. COX-1 is expressed constitutively in most tissues and appears to be responsible for the production of PGs that control normal physiological functions, including platelet aggregation (Stemme *et al.*, 2000). In contrast, COX-2 is not detected in most normal tissues but is rapidly induced by both inflammatory and mitogenic stimuli, resulting in increased PG synthesis associated with pathological processes, including acute and chronic inflammatory states (Stemme *et al.*, 2000). Prostaglandin E_2 (PGE_2) is a major metabolite produced by COX-2 in EC, which influences vascular tone, regional blood flow, vascular permeability, and remodeling, proliferation, and angiogenesis (Dannenberg *et al.*, 2005; Barbieri and Weksler, 2007).

Several lines of evidence suggest that phosphatase and tensin homologue deleted on chromosome 10 (PTEN) interacts indirectly with β-catenin by binding scaffolding proteins containing a PDZ domain, thus participating in regulating cell–cell junctions and vascular permeability (Kotelevets *et al.*, 2005; Sanchez *et al.*, 2007). PTEN is a lipid phosphatase that specifically dephosphorylates the D3 position of the inositol ring of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3; Maehama and Dixon, 1998). PIP_3 plays a critical role in the regulation of cell survival and growth signaling through activation of phosphatidylinositol-dependent kinases (PDKs) and their downstream target, protein kinase B (PKB)/Akt (Coffer *et al.*, 1998). A number of studies demonstrated that overexpression of PTEN results in abrogation of Akt activity (Maehama *et al.*, 2001). Consistent with this, both the cellular levels of PIP_3 and those of Akt phosphorylation/activity are constitutively elevated in PTEN-deficient mouse embryonic fibroblasts (Sun *et al.*, 1999). Thus PTEN is an upstream negative regulator of Akt through inactivation of PIP_3. The carboxyl-terminal region of PTEN was shown to modulate its protein stability (Vazquez *et al.*, 2006). Moreover, phosphorylation of the carboxyl-terminal residues Ser-380, Thr-382, and Thr-383 results in a sixfold increase in PTEN half-life, which suggests that phosphorylation plays an important role in regulating PTEN function (Vazquez *et al.*, 2006).

On the basis of our previous findings (Pellegrino *et al.*, 2004; Cho *et al.*, 2008) indicating the ability of apo(a) to disrupt adherens junctions, in the present study we address potential consequences of β-catenin release from these junctions relevant to the proatherosclerotic and proinflammatory roles ascribed to Lp(a) in the vasculature.

RESULTS

Apo(a), through its strong LBS, stimulates VE-cadherin/β-catenin complex dissociation

We previously demonstrated that apo(a), via its strong LBS in KIV_{10}, mediates VE-cadherin dispersion and actinomyosin-derived EC contraction, resulting in increased endothelial permeability (Pellegrino *et al.*, 2004; Cho *et al.*, 2008). To illustrate a direct effect of apo(a) on disruption of adherens junctions, we investigated the dissociation of β-catenin from the complex by immunostaining and immunoprecipitation in response to treatment of EC with two different forms of apo(a) (Figure 1): 17K contains all of the domains found in all apo(a) isoforms and, in fact, represents a physiologically relevant isoform (Koschinsky *et al.*, 1991); 17KΔAsp is identical to 17K except for a single–amino acid substitution that abolishes the strong LBS in KIV_{10} (Cho *et al.*, 2008).

After a brief, 15-min, serum starvation, confluent human umbilical vein endothelial cells (HUVECs) displayed organized β-catenin and VE-cadherin as slender lines along the margin of the cells (Figure 2A), representing their localization in adherens junctions. Treatment with 200 nM 17K caused time-dependent dispersion of both β-catenin and VE-cadherin and the formation of intercellular gaps (Figure 2A). Consistent with an important role of the strong LBS in KIV_{10} of apo(a) in mediating EC responses (Cho *et al.*, 2008),

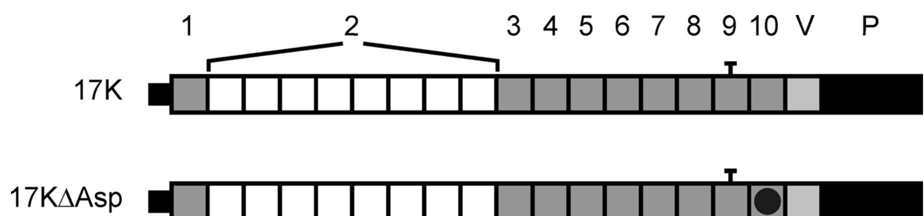
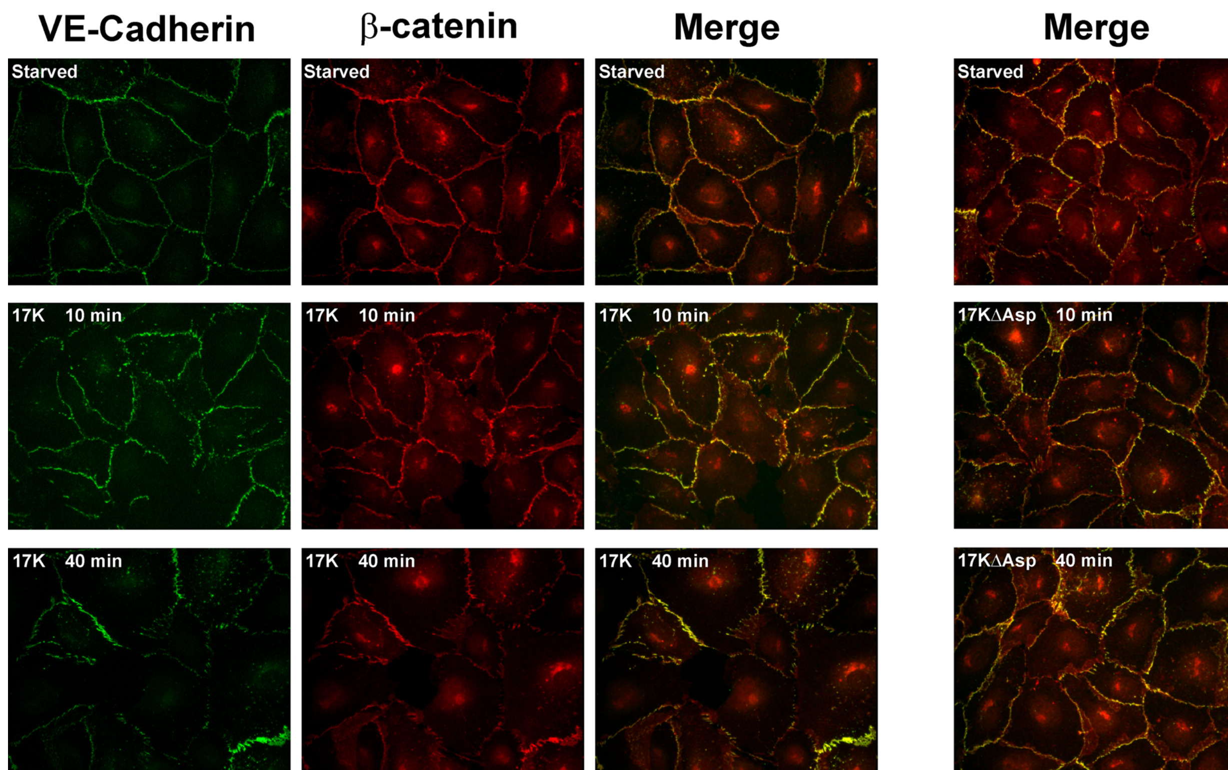


FIGURE 1: Recombinant apo(a) variants used in this study. Schematic diagram shows the topology of the r-apo(a) variants. 17K represents a physiologically relevant apo(a) isoform and contains all of the kringle domains present in all apo(a) isoforms. The numbering of KIV types is provided at the top. A dot in KIV_{10} of 17KΔAsp presents a single–amino acid substitution, which abolishes strong lysine-binding sites. The bar over KIV_9 represents an unpaired cysteine, which forms a single disulfide bond with apolipoproteinB-100.



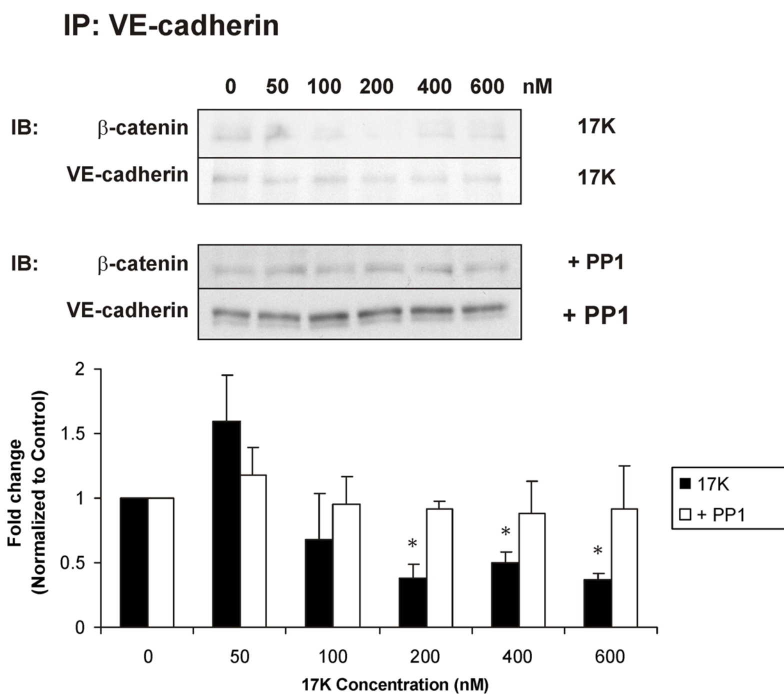


FIGURE 2: A strong lysine-binding site in KIV_{10} is required for stimulation of VE-cadherin/β-catenin complex dissociation. (A) HUVECs were serum starved for 15 min and then treated with 200 nM of r-apo(a) variants (17K or 17KΔAsp) for the indicated times. Cells were then fixed, permeabilized, and stained for VE-cadherin and β-catenin, as indicated, as described in *Materials and Methods*. (B) HUVECs were pretreated with 10 μM PP1 for 1 h; control cells were not pretreated with PP1. Then, all cells were either treated or not treated with 200 nM 17K for 10 min. Cell lysates were prepared and subjected to immunoprecipitation with VE-cadherin; immunoprecipitates were subjected to Western blot analysis using either β-catenin or VE-cadherin antibodies in order to assess the extent of association of these two proteins. The intensity of the respective bands was measured densitometrically; the graph shows mean band density (normalized to the control) ± SE of three independent experiments, with the asterisks representing significant differences ($p < 0.05$) compared with the control.

17KΔAsp did not alter β-catenin or VE-cadherin dissociation or the gap formation in the EC monolayer. To definitively assess dissociation of β-catenin from adherens junctions, we performed a quantitative analysis using immunoprecipitation. We immunoprecipitated VE-cadherin in lysates of treated cells and then subjected the immunoprecipitated material to Western blot analysis using anti–β-catenin antibodies. These analyses demonstrated that 17K, in a concentration-dependent manner, induced a marked decrease of β-catenin in the VE-cadherin complex (Figure 2B); 17K concentrations of 200 nM or greater resulted in a significant increase in β-catenin complex dissociation. Therefore 200 nM apo(a) was used for subsequent experiments.

The tyrosine phosphorylation of VE-cadherin and other components of adherens junctions is associated with weak junctions and impaired barrier function. Many studies have reported that permeability-increasing agents such as histamine (Shasby *et al.*, 2002), tumor necrosis factor-α (Angelini *et al.*, 2006), and vascular endothelial cell growth factor (Esser *et al.*, 1998) induce the tyrosine phosphorylation of VE-cadherin and its binding partners, β-catenin, plakoglobin, and p120. Although the mechanism of VE-cadherin complex phosphorylation has not been fully clarified, Src plays a major role in phosphorylating tyrosine residues of proteins in the complex (Dejana *et al.*, 2008). To investigate whether Src is involved in dissociation of β-catenin induced by 17K, we incubated HUVECs with a potent pharmacological inhibitor of Src (PP1) before treatment with apo(a). Indeed, PP1 effectively blocked 17K-mediated dissociation of β-catenin from the VE-cadherin complex (Figure 2B).

Apo(a), through its strong LBS, mediates β-catenin nuclear accumulation

After dissociation from membrane-associated adherens junctions into the cytoplasm, β-catenin either translocates to the nucleus or becomes incorporated into an APC complex that favors protein degradation (Ozawa *et al.*, 1989; Behrens *et al.*, 1998). Exposing HUVECs to 17K stimulated translocation of β-catenin to the nucleus (Figure 3A). Concomitantly, the level of β-catenin in the cytoplasmic extract decreased as the time of 17K treatment increased. On the other hand, 17KΔAsp showed no effect on β-catenin nuclear translocation. Interleukin-1β (2 ng/ml) and plasminogen (400 nM) were used as positive and negative controls, respectively, and showed the expected increase and lack of effect on β-catenin translocation.

Incubation of HUVECs with a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002) or ε-aminocaproic acid (ε-ACA), a lysine analogue, before 17K treatment completely abolished the 17K-mediated effects. Treatment with the Src inhibitor PP1 resulted in a reduction in 17K-mediated nuclear translocation of β-catenin, indicating that tyrosine phosphorylation by Src is at least partially involved in the process of β-catenin trafficking (Figure 3B). Of interest, the Rho kinase inhibitor Y27632 did not prevent 17K-mediated β-catenin translocation in the present study (unpublished data), although it was a potent inhibitor for 17K-mediated Rho/Rho kinase–dependent increase in EC contraction and permeability in our previous study (Pellegrino *et al.*, 2004; Cho *et al.*, 2008). These findings, collectively, indicate that 17K uses a Rho/Rho kinase–independent signaling pathway involving PI3K to mediate β-catenin nuclear translocation.

17K, but not 17KΔAsp, induces COX-2 expression in HUVECs in a manner dependent on PI3K

β-Catenins present in the nucleus act as a transcription cofactor with the TCF/LEF family of DNA-binding proteins to modify expression of many inflammatory genes, including COX-2 (Kim *et al.*, 2002; Araki *et al.*, 2003; Barbieri and Weksler, 2007). To determine whether apo(a) influences COX-2 expression, we exposed HUVECs to 17K and analyzed COX-2 expression by Western blot analysis. As shown in Figure 4A, 200 nM 17K induced COX-2 protein expression in a time-dependent manner. 17K increases COX-2 expression to a maximal extent at 7 h of stimulation, whereas 17K concentrations <100 nM did not influence COX-2 expression (unpublished data). After 7 h of 17K treatment, COX-2 expression declined yet remained at a level far above

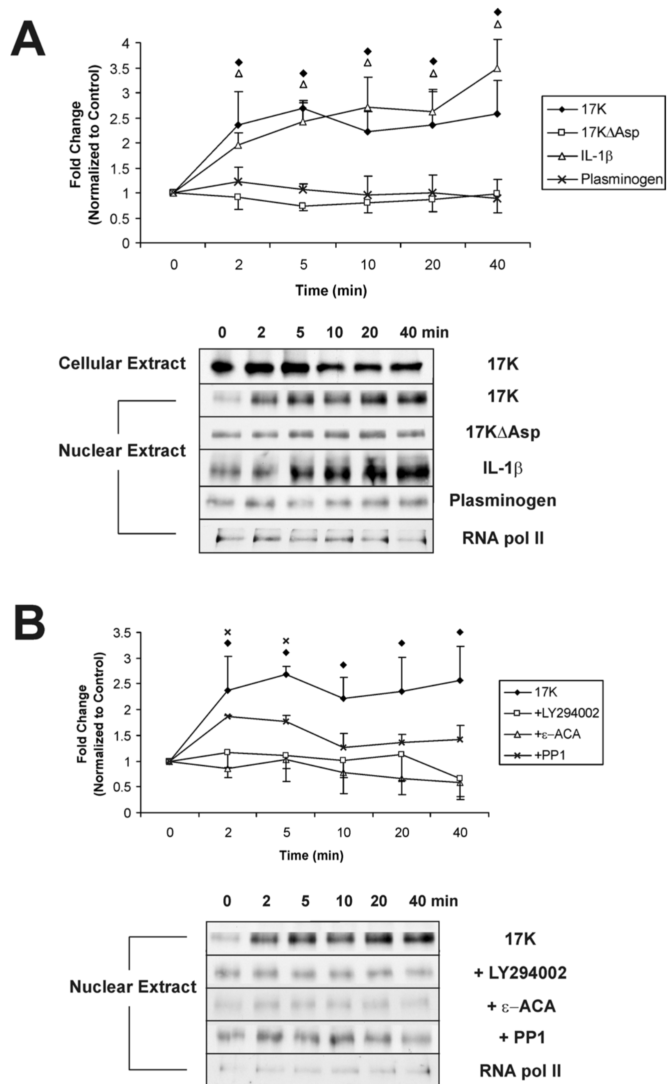


FIGURE 3: A strong lysine-binding site in KIV_{10} enhances nuclear β-catenin translocation in HUVECs. (A) HUVECs were serum starved for 15 min and then treated with 200 nM 17K or 17KΔAsp for the indicated times. Total nuclear proteins were harvested and subjected to Western blot analysis using anti–β-catenin or anti–RNA polymerase II antibodies. IL-1β and plasminogen were used as positive and negative control, respectively. (B) Cells were pretreated with LY294002 (5 μM, 1 h) and PP1 (10 μM, 1 h) and then were incubated with 17K (200 nM) for the indicated time periods (0–40 min). In some experiments, ε-ACA (80 mM) was included with the 17K. Graphs show mean band density (normalized to the control) ± SE of three independent experiments; representative Western blots are shown below the graphs. Symbols above the plots represent significant differences ($p < 0.05$) compared with the control for different treatments represented by the corresponding plot symbols in each panel (as shown in the graph legends).

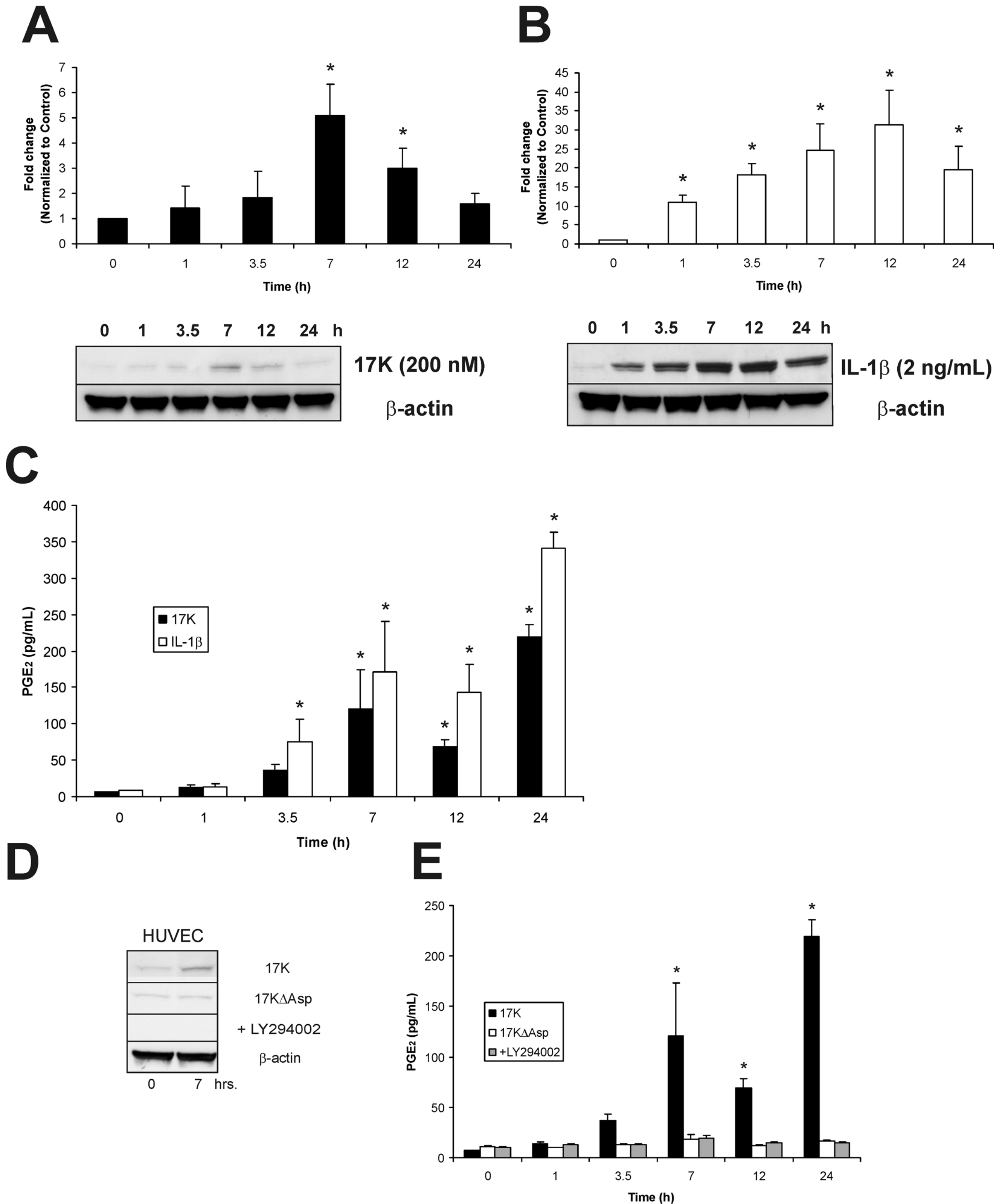


FIGURE 4: 17K induces COX-2 activation and PGE_2 secretion. (A, B) HUVECs were treated with serum-depleted medium containing 17 K (200 nM) or IL-1β (2 ng/ml) for the up to 24 h. At specific time points, cell lysates were prepared and subjected to Western blot analysis using COX-2 antibody while the corresponding media were collected to measure PGE_2 levels. (C) HUVECs were treated for 7 h with 200 nM 17K or 17KΔAsp or with 200 nM 17K and 5 μM LY294002 (after a 1-h pretreatment with LY294002). Cell lysates were prepared and subjected to Western blot analysis using COX-2 antibody (D), and the corresponding media were collected to measure PGE_2 levels (E). Graphs show mean band density (normalized to the control) ± SE of three independent experiments, and representative Western blots of three independent experiments are shown. The asterisks represent significant differences ($p < 0.05$) compared with controls.

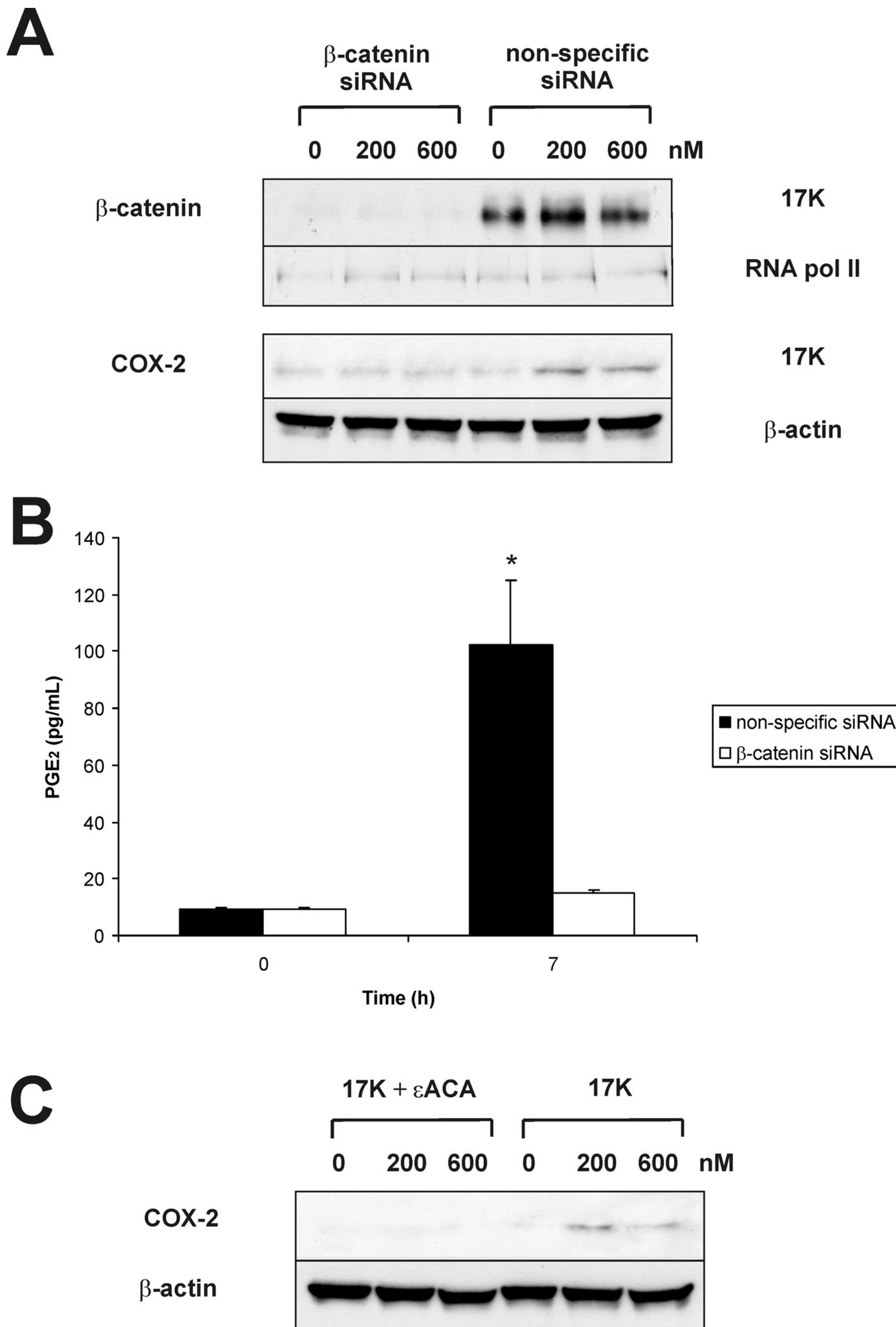


FIGURE 5: The role of β-catenin in regulation of COX-2 expression by apo(a). (A) HUVECs were transfected with β-catenin siRNA or nonspecific siRNA as described in *Materials and Methods* and then stimulated for 7 h with 17K. Representative Western blots for β-catenin nuclear translocation and COX-2 expression in three independent experiments are analyzed. (B) COX activity as measured by secretion of PGE_2 into culture media of cells treated for 0 or 7 h with apo(a) that had been transfected with β-catenin siRNA or nonspecific siRNA. Shown are the means ± SE of three independent experiments, each performed in duplicate. The asterisks represent significant differences ($p < 0.05$) compared with the control. (C) Representative Western blot (of three independent experiments) showing the effect of 17K treatment on COX-2 expression in the presence or absence of ε-ACA (80 mM).

that observed in the control (Figure 4A). Interleukin-1β (IL-1β; 2 ng/ml) was used as positive control (Figure 4B) and resulted in a maximal extent of COX-2 inhibition that was approximately sixfold higher than that observed with 17K. Consistent with its lack of effect on β-catenin nuclear translocation, 17KΔAsp failed to increase COX-2 expression (unpublished data). Treatment with LY294002 completely inhibited 17K-mediated effects on COX-2 expression in HUVECs (Figure 4C), whereas expression of the constitutively expressed enzyme COX-1 was not modified after treatment with 17K (unpublished data).

An increase in COX-2 protein levels in endothelial cells leads to up-regulation of PGE_2 production and secretion to induce inflammatory and atherogenic effects (Sanchez *et al.*, 2007). Both 17K and IL-1β up-regulated PGE_2 release to the media in HUVECs (Figure 4D). 17KΔAsp did not affect PGE_2 release, in agreement with its lack of effect on COX-2 expression (Figure 4E). Furthermore, LY294002 completely abolished PGE_2 release mediated by 17K (Figure 4E).

Apo(a)-mediated accumulation of β-catenin in the nucleus is required for the effect of apo(a) on COX-2 expression and PGE_2 production

To determine whether β-catenin was directly involved in regulating COX-2 expression by apo(a), we first transfected HUVECs with β-catenin small interfering RNA (siRNA) or with a nonspecific siRNA and then exposed them to different concentrations of apo(a) (200 or 600 nM). Transfection of HUVECs with siRNA directed against β-catenin markedly decreased levels of nuclear β-catenin compared with cells transfected with nonspecific siRNA (Figure 5A). Treatment of the cells transfected with nonspecific siRNA with 17K resulted in an increase in nuclear β-catenin, whereas treatment of the cells transfected with β-catenin siRNA did not result in the appearance of nuclear β-catenin. Accordingly, transfection of the cells with β-catenin siRNA abolished the 17K-mediated increase in COX-2 expression, whereas the nonspecific siRNA did not (Figure 5A). Moreover, the β-catenin siRNA completely abolished the 17K-dependent increase in PGE_2 secretion (Figure 5B). These results are an indication that increased COX-2 expression leading to enhanced PGE_2 secretion as a consequence of 17K treatment is directly mediated by 17K-induced nuclear translocation of β-catenin. Consistent with previous findings, the 17K-mediated increase in COX-2 expression was also completely abolished by treatment of cells with ε-ACA (Figure 5C).

Apo(a) reduces β-catenin degradation to facilitate β-catenin nuclear translocation

β-Catenin accumulation in the nucleus requires not only its release from membrane complexes with VE-cadherin (Figure 2B), but also decreased degradation in the cytoplasm by the ubiquitin/proteasome

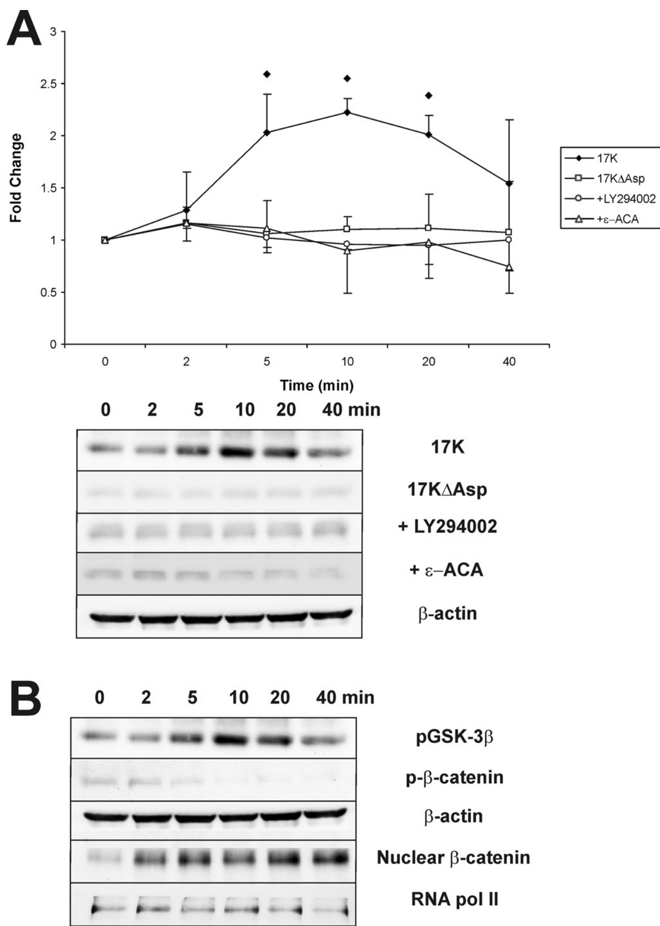


FIGURE 6: Strong lysine-binding site in KIV_{10} in apo(a) mediates inactivation of GSK-3β, which attenuates phosphorylation of β-catenin and enhances nuclear β-catenin accumulation. HUVECs were stimulated with either 17K or 17KΔAsp. In some experiments, cells were preincubated with LY294002 (5 μM) for 1 h or were treated with ε-ACA (80 mM) along with 17K. (A) Total cellular proteins were harvested and subjected to Western blot analysis using specific anti–phospho-GSK-3β (Ser-9) or β-actin antibodies. The graph shows the normalized band densitometry (mean ± SE) obtained from three independent experiments, where asterisks represent significant increases ($p < 0.05$) compared with the control. Representative Western blots are shown below the graph. (B) Representative Western blots (of three independent experiments) showing phosphorylated GSK-3β (p-GSK-3β), phosphorylated β-catenin (p-β-catenin on Ser-33, -37/Thr-41), and nuclear β-catenin in the lysates of 17K-stimulated HUVECs are shown at indicated time points.

pathway involving GSK-3β (Ozawa *et al.*, 1989; Behrens *et al.*, 1998). Phosphorylation of GSK-3β at Ser-9, which inhibits its kinase activity, results in an increased accumulation of β-catenin in the nucleus (Behrens *et al.*, 1998). Therefore we assessed whether apo(a) could influence the extent of phosphorylation of GSK-3β at this inhibitory site. Treatment of HUVECs with 200 nM 17K resulted in a significant increase in GSK-3β at Ser-9 in a time-dependent manner, with a maximum effect at 10 min (Figure 6A). Consistent with other findings in this study, the 17K-mediated increase in phosphorylation of GSK-3β was completely abrogated by εACA and LY294002, and 17KΔAsp resulted in no changes in phosphorylation state of GSK-3β compared with the control (Figure 6A).

We then explored the hypothesis that exposure of EC to apo(a) modulates β-catenin degradation by diminishing phosphorylation of β-catenin on Ser-33, -37/Thr-41 since these sites are constantly phosphorylated by active GSK-3β. 17K treatment of EC indeed decreased the phosphorylation of β-catenin on these sites after 5 min (Figure 6B), a time period during which 17K significantly increased inactivating phosphorylation of GSK-3β (Figure 6A) and increased the amount of β-catenin in the nucleus (Figure 6B). These data suggest that apo(a) facilitates the entry of β-catenins into the nucleus by modulating disruption of adherens junction, as well as by preventing the degradation of released β-catenin in the cytosol.

Apo(a) increases Akt activity through inhibition of PTEN

Protein kinase B (PKB/Akt), a serine/threonine kinase located downstream of PI3K, has been implicated as phosphorylating GSK-3β at the Ser-9 inhibitory site (Cross *et al.*, 1995). Treatment of HUVECs with 17K significantly increased Akt phosphorylation at Ser-473 in a regulatory domain within 5–10 min (Figure 7A). Phosphorylation of Akt at Thr-308 in a catalytic domain is also required to mediate full activation of this kinase; 17K significantly enhanced phosphorylation at Thr-308 after 10 min of treatment (Figure 7B). To demonstrate that 17K-mediated activation of Akt is responsible for the accumulation of β-catenin in the nucleus and increased COX-2 expression, we pretreated HUVECs with the PI3K inhibitor LY294002 for 1 h before the apo(a) treatment. The rapid increase of Akt and GSK-3β phosphorylation was entirely suppressed by LY294002 treatment, consistent with its effects on 17K-mediated changes in β-catenin phosphorylation, β-catenin nuclear translocation, COX-2 expression, and PGE_2 secretion (Figures 3, 4, and 6).

The activation of PTEN may prevent phosphorylation of tyrosine residues on β-catenin and PI3K signaling events that are required to modulate permeability of endothelial monolayers (Vazquez and Sellers, 2000 COMP: Link to Vazquez and Sellers, 2000; Vogelmann *et al.*, 2005). PTEN is negatively regulated by phosphorylation of residues Ser-380 and Thr-382/-383 (Vazquez *et al.*, 2006). Therefore we assessed the effect of apo(a) treatment of cultured HUVECs on PTEN phosphorylation. Ten minutes of 17K treatment resulted in increased phosphorylation of Ser-380 and Thr-382/-383 in HUVECs (Figure 8). Consistent with a recent study (Barbieri *et al.*, 2008) and our findings, interleukin-1β (2 ng/ml) increased phosphorylation of PTEN at the Ser-380, Thr-382/-383 sites, whereas 17KΔAsp had no effect on PTEN phosphorylation (Figure 8). Taken together, these data support the hypothesis that apo(a)-induces activation of a PI3K/Akt/GSK-3β–dependent pathway PI3K through inhibition of its negative regulator PTEN and enhances β-catenin nuclear translocation, with subsequent effects on COX-2 expression and PGE_2 secretion from the cells.

To assess whether the effects of apo(a) on HUVECs could be correlated with binding of apo(a) to these cells, we performed a binding experiment using fluorescently labeled 17K, 17KΔAsp, and plasminogen (Figure 9). Of interest, there was no difference in binding observed between the two apo(a) variants. In addition, the binding of neither apo(a) variant was diminished by the addition of ε-ACA, although, as expected, the binding of plasminogen was markedly diminished by this lysine analogue.

DISCUSSION

There is strong evidence to suggest that elevated concentrations of plasma Lp(a) (>25 mg/dl) are a risk factor for a variety of cardiovascular diseases, including coronary heart disease, ischemic stroke, and venous thrombosis (Dubé *et al.*, 2012; Tsimikas and Hall, 2012). The ability of Lp(a) to induce vascular inflammation and endothelial dysfunction appears to be a common link among these diseases. Studies suggest roles for apo(a) as a proatherosclerotic factor, with

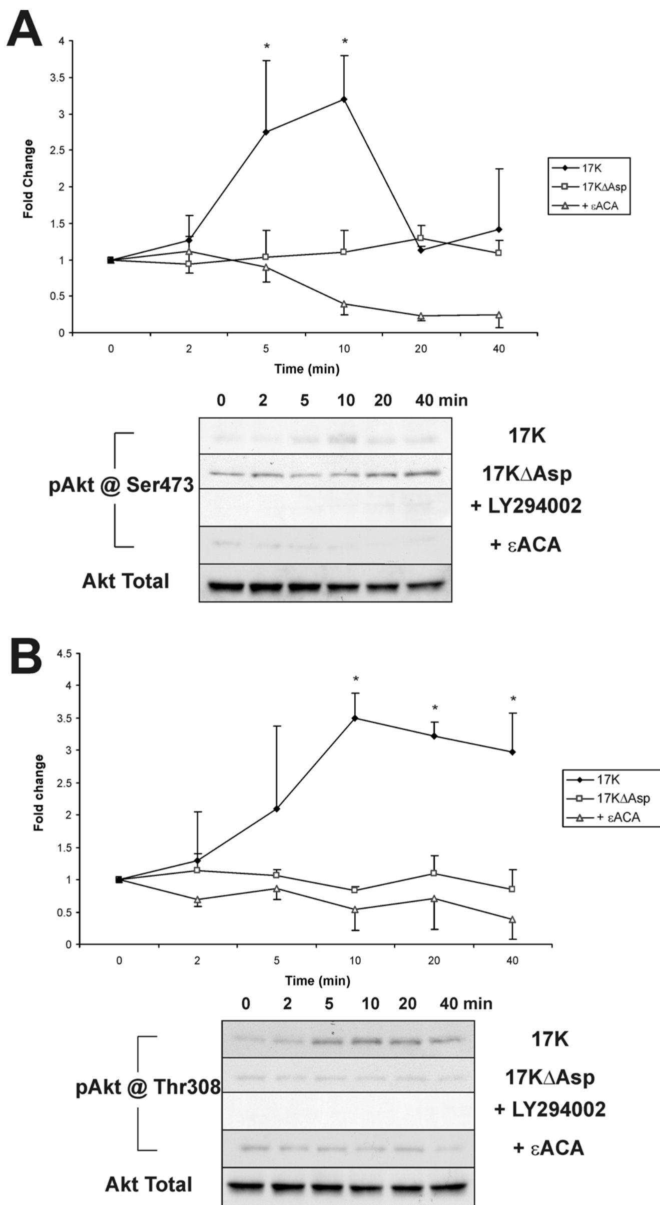


FIGURE 7: Apo(a) activates the PI3K/Akt pathway in manner dependent upon the strong lysine binding in KIV_{10}. HUVECs were serum starved for 15 min and treated with 200 nM 17K or 17KΔAsp or with 17K plus ε-ACA (80 mM) for the indicated time periods. Total cellular proteins were harvested and subjected to Western blot analysis using specific anti–phospho-Akt (Ser-473) antibody (A) or anti–phospho-Akt (Thr-308) antibody (B). In some experiments, cells were pretreated with LY294002 (5 µM) for 1 h. The graphs show the band intensities (means ± SE; normalized to total Akt as determined by Western blot analysis) obtained from three independent experiments, where asterisks represent significant increases ($p < 0.05$) compared with the control. Representative Western blots are shown below the graphs.

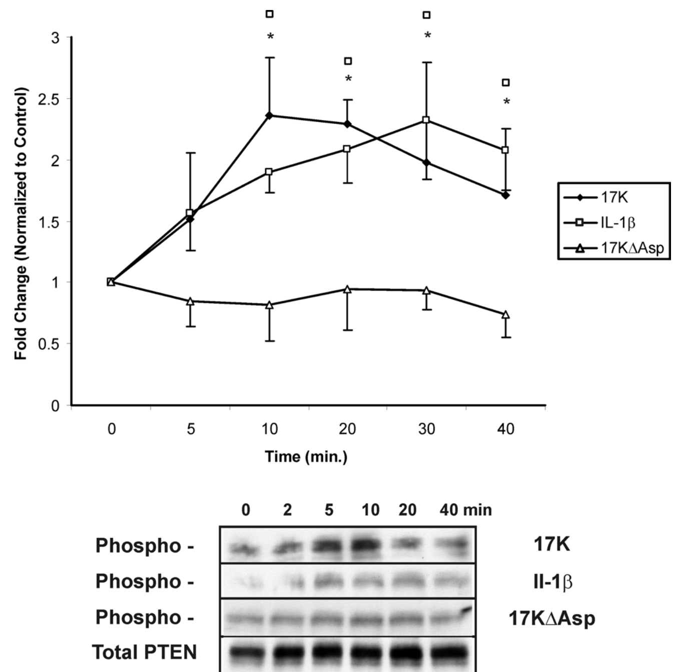


FIGURE 8: Apo(a) inhibits PTEN activity through its phosphorylation of serine/threonine residues. HUVECs were serum starved for 15 min and treated with 200 nM 17K or 200 nM 17KΔAsp for the indicated time periods. Total cellular proteins were harvested and subjected to Western blot analysis using specific anti–phospho-PTEN (Ser-380, Thr-382/-383) antibody. Il-1β (2 ng/ml) was used as a positive control. The graphs show the band intensities (means ± SE; normalized to total PTEN as determined by Western blot analysis) obtained from three independent experiments, where asterisks represent significant increases after 17K treatment ($p < 0.05$) compared with the control; squares show significant increases after IL-1β treatment ($p < 0.05$) compared with control. Representative Western blots are shown below the graphs.

most of the pathological effects by which it contributes to vascular dysfunction mediated through the unique glycoprotein moiety, apo(a) (Koschinsky and Marcovina, 2009). In this regard, we previously demonstrated that the apo(a) component of Lp(a) induces cytoskeleton rearrangements that promote an increase in EC contraction and permeability (Pellegrino *et al.*, 2004). Because LDL and plasminogen have no effect in this regard (Pellegrino *et al.*, 2004), it is clear that the apo(a) portion of Lp(a) is the active moiety. Therefore the activation of apo(a)-induced inflammatory responses in the vasculature is expected to be important for the cascade of events that culminates in the formation of atherosclerotic lesions.

We hypothesized, on the basis of our previous findings that apo(a) induces disruption of adherens junctions (Pellegrino *et al.*, 2004; Cho *et al.*, 2008), that this could, in turn, release β-catenin for subsequent nuclear translocation and induction of gene expression, including the proinflammatory COX-2. Accordingly, we now demonstrate that 17K apo(a) treatment of EC enhances the disruption of the VE-cadherin/β-catenin complex and nuclear translocation of β-catenin, resulting in up-regulation of COX-2 expression and PGE_2 secretion in ECs. Our findings thus suggest another novel mechanism by which apo(a) contributes to atherogenesis other than through activation of Rho/Rho kinase–dependent pathway as we previously described (Pellegrino *et al.*, 2004). PGE_2 is a potent regulator of vascular inflammation and affects numerous mechanisms that have been implicated in carcinogenesis and atherogenesis, including stimulation of cell proliferation and motility, while inhibiting immune surveillance and apoptosis (Krysan *et al.*, 2008).

Our experiments defined the intracellular pathway stimulated by apo(a) that culminates in changes in COX-2 expression (Figure 10). In addition to disruption of VE-cadherin/β-catenin complexes, treatment of vascular EC with apo(a) attenuates the phosphorylation of β-catenin at Ser-33,-37/Thr-41 by GSK-3β (Figure 6), thus causing a

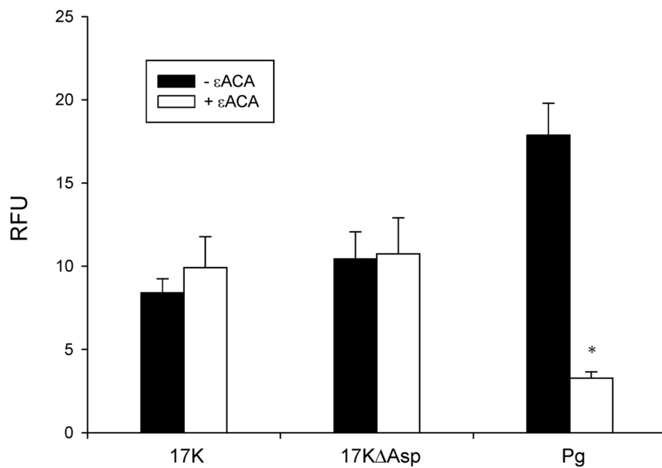


FIGURE 9: Binding of r-apo(a) to HUVECs. Fluorescently labeled 17K, 17KΔAsp, and plasminogen (Pg; 400 nM) were incubated with HUVECs for 1 h at 37°C in the presence or absence of 200 mM ε-ACA. After washing, the amount of fluorescently labeled ligand bound was measured using a plate-reading fluorimeter. RFU, relative fluorescence units. The asterisk denotes a significant ($p < 0.01$) difference in binding in the presence of ε-ACA compared with the absence of ε-ACA.

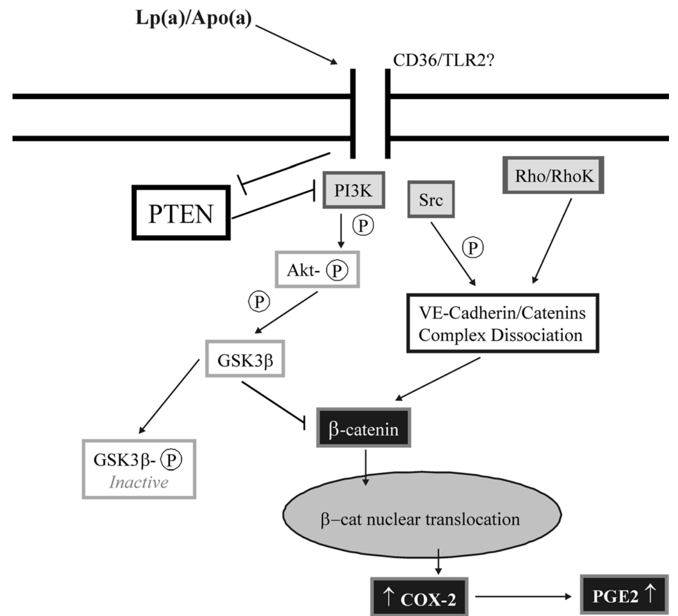


FIGURE 10: Novel signaling pathways involved in apo(a)-mediated effects on vascular endothelial cells. Apo(a), through its strong LBS in KIV_{10}, binds to a putative receptor (possibly a complex of CD36 and TLR2), resulting in activation of a Src- and Rho/Rho kinase–dependent pathway to increased VE-cadherin/β-catenin complex dissociation, leading to an increase in vascular permeability in cultured EC. Apo(a) also induces a PI3K/Akt/GSK-3β signaling pathway, independent of the Rho/Rho kinase pathway, to increase β-catenin nuclear translocation, and hence COX-2 expression, and PGE_2 secretion. Furthermore, apo(a) mediates loss of cell–cell contact and stimulates β-catenin translocation by disrupting the VE-cadherin/β-catenin complex through inhibition of PTEN.

decrease in the degradation of cytoplasmic β-catenin. Akt is a critical regulator of PI3K-mediated inactivation of GSK-3β (Cross *et al.*, 1995). On agonist stimulation, the PH domain of Akt binds to the lipid products of PI3K, and Akt is recruited to the plasma membrane. It is then sequentially phosphorylated at Thr-308 and Ser-473 by upstream kinases referred to as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK2, respectively, to yield a fully activated kinase (Hemmings, 1997; Downward, 1998). We show that apo(a) increases phosphorylation of both sites for a full activation of Akt (Figure 7), which can then phosphorylate GSK-3β at Ser-9 (Figure 6). Ser-9 phosphorylation completely abolishes the activity of GSK-3β. This favors β-catenin nuclear translocation by reducing the degradational signal mediated by the phosphorylation of β-catenin at serine/threonine residues by GSK-3β. Accordingly, addition of a pharmacological inhibitor of PI3K, LY294002, decreased Akt activation and GSK-3β inactivation mediated by apo(a), enhanced phosphorylation and degradation of β-catenin, reduced nuclear β-catenin accumulation, and decreased COX-2 expression and PGE_2 secretion. Our findings are consistent with a recent study in mouse cardiac endothelial cells demonstrating that tobacco smoke–induced nuclear translocation of β-catenin to induce COX-2 expression is mediated by a PI3K/Akt/GSK-3β pathway (Barbieri and Weksler, 2007).

We demonstrate here for the first time that apo(a) inactivates PTEN by causing phosphorylation of serine/threonine residues of the PTEN tail (Figure 8), suggesting a direct pathway for modulation of PTEN activity by apo(a). Casein kinase-2 (CK2) is one of the major kinases that reduce catalytic activity of PTEN with consequent activation of PI3K/Akt pathway. CK2-mediated phosphorylation also stabilizes PTEN in a monomeric "closed" conformation with low affinity for β-catenin/scaffolding protein complexes (Vazquez *et al.*, 2001). Our present findings suggest that induction of serine/threonine phosphorylation and inactivation of PTEN modulated by apo(a) may be regulated through CK2 since active Src increases phosphorylation and catalytic activity of this kinase (Donella-Deana *et al.*, 2003; Hildesheim *et al.*, 2005). Indeed, we show that Src is implicated in apo(a)-mediated disruption of adherens junctions and in reduction of β-catenin nuclear accumulation (Figure 2B). Further investigation of the effect of apo(a) in EC, focusing on the involvement of CK2, would give us a better understanding of how apo(a) activates the PI3K/Akt pathway through modulation of PTEN.

Our research thus far has demonstrated that the strong LBS in apo(a) kringle IV type 10 is absolutely required for apo(a)-mediated changes in cytoskeletal rearrangements and enhanced EC contraction and permeability (Pellegrino *et al.*, 2004; Cho *et al.*, 2008). Consistent with this finding, we show here that β-catenin–mediated effects of apo(a) on increased COX-2 expression, which results in PGE_2 secretion, were not evident when apo(a) lacking the strong LBS in KIV type 10 (17KΔAsp) was used. Furthermore, 17KΔAsp failed to increase PTEN phosphorylation or phosphorylation of any PI3K downstream effectors that lead to inactivation of GSK-3β. In addition, treatment with ε-ACA, a lysine analogue, completely abolished all of the apo(a)-induced effects, consistent with a role for the strong LBS in apo(a). Plasminogen, which shares a striking homology with apo(a) and contains an LBS with similar lysine affinity to that in apo(a) KIV type 10 (McLean *et al.*, 1987), was found to have no effect on β-catenin nuclear translocation (Figures 2 and 3), consistent with our previous results (Pellegrino *et al.*, 2004; Cho *et al.*, 2008). Therefore it is clear that the LBS in apo(a) is able to mediate effects that plasminogen with its multiple LBS cannot. However, whether apo(a) works through known plasma membrane receptors or binds to a novel receptor to exert its function is not known and is under investigation by our group.

As a first step toward identifying the receptors(s) to which apo(a) binds, we studied the binding of apo(a) to HUVECs. We made the surprising finding that removal of the strong LBS in KIV_{10} of apo(a)

was not required for binding and that the binding of both apo(a) variants was insensitive to the addition of the lysine analogue ε-ACA. These data clearly indicate that signaling by apo(a) can be dissociated from cell binding of apo(a) per se. Many different receptors have been proposed for apo(a), including Mac-1 on macrophages (Sotiriou *et al.*, 2006) and plasminogen receptors on hepatocytes (Tam *et al.*, 1996). Of note, none of these are signaling receptors in their own right. A possible way to reconcile these findings comes from the work of the Tabas group, who found that apo(a) acts through CD36/Toll-like receptor 2 (TLR2) to promote apoptosis in macrophages undergoing ER stress (Seimon *et al.*, 2010). Of interest, TLR2 has been shown to operate through activation of both PI3K and Src (Arbibe *et al.*, 2000; Janssens and Beyaert, 2002; Manukyan *et al.*, 2009) and as such is a plausible candidate for the apo(a) signaling receptor on HUVECs. Of note, it is the oxidized phospholipids that are covalently attached to apo(a) that appear to be the active signaling molecules in the context of ER-stressed macrophages. It is tempting to speculate that the same holds true for proinflammatory apo(a) signaling in endothelial cells.

In conclusion, we have characterized a signal transduction pathway by which apo(a), through its strong LBS in KIV_{10}, increases activation of PI3K/Akt/GSK-3β pathways and thus stimulates accumulation of NFκB and β-catenin in the nucleus, the latter of which up-regulates COX-2 expression with a concomitant increase in secretion of PGE_2. It will be interesting to assess whether apo(a) is able to modulate the expression of other genes involved in the atherosclerotic process through this pathway. In addition, apo(a) mediates Src-dependent disruption of VE-cadherin/β-catenin complexes, which would contribute to EC permeability. Taken together, our results suggest novel mechanisms by which apo(a) can contribute to the initiation and development of cardiovascular diseases.

MATERIALS AND METHODS

Expression and purification of recombinant apo(a)

A 17-kringle (17K)-containing form of recombinant apo(a) (17K r-apo(a)), as well as a mutant form of 17K r-apo(a) (17KΔAsp) containing an Asp → Ala substitution at amino acid position 56 that abolishes the strong LBS in the KIV_{10} domain (Figure 1), were constructed and expressed as previously described (Koschinsky *et al.*, 1991; Sangrar *et al.*, 1997). Both r-apo(a) species were purified from the conditioned medium (CM) of stably expressing human embryonic kidney 293 cell lines by lysine–Sepharose (Amersham Biosciences, Piscataway, NJ) affinity chromatography as previously reported (Sangrar *et al.*, 1997).

Cell culture

Primary HUVECs were obtained from Clonetics (San Diego, CA) and grown in complete medium (endothelial basal medium EBM-2 and endothelial growth medium EGM-2 [Clonetics] containing 2% fetal calf serum) as specified by the manufacturer. Cells were used at passages 3–6.

Double immunofluorescence

For double-immunofluorescence studies, cells were plated onto gelatin-precoated (1 h, 0.1% gelatin [Fisher Scientific, Pittsburgh, PA] at 37°C) glass coverslips at a density of 25,000 cells/well in 24-well tissue culture dishes and grown to near confluence. Before treatment with r-apo(a), cells were serum starved for 15 min at 37°C in EBM-2 (Clonetics). This solution was then replaced with fresh EBM-2 containing 200 nM apo(a) variants, and the cells were incubated at 37°C for 10 or 40 min. Cells were then prepared for double immunofluorescence as follows. Cells were fixed with 3.7% paraformaldehyde solution in phosphate-buffered solution (PBS) for 5 min, washed once with PBS, and permeabilized with 1.4% formaldehyde containing 0.1% Nonidet P-40 (NP-40) in PBS for 1.5 min, and then washed three times with PBS. Cells were incubated for 1 h at room temperature with both an anti-VE-cadherin monoclonal antibody (Research Diagnostics, Flanders NJ) and an anti–β-catenin antibody raised in rabbits (Sigma-Aldrich, St. Louis, MO), each diluted 1:350 in saponin buffer (0.1% saponin, 20 mM KPO_4, 10 mM 1,4-piperazinediethanesulfonic acid, 5 mM ethylene glycol tetraacetic acid, 2 mM $MgCl_2$, pH 6.8). After three washes with PBS, cells were stained for 1 h with 1:500-diluted goat anti–mouse Alexa 488–conjugated antibody and goat anti–rabbit Alexa 568–conjugated antibody (Molecular Probes, Eugene, OR) in saponin buffer in the dark. After three washes with PBS, coverslips were mounted on slides using an antifade mounting solution (Dako, Carpinteria, CA) and examined using a Zeiss Axiovert S100 inverted fluorescence microscope equipped with a 40× oil immersion lens (Carl Zeiss, Jena, Germany). Images were captured using a high-sensitivity Cooke SensiCam and SlideBook software (Intelligent Imaging Innovations, Denver, CO).

Protein preparation and analysis

For Western blotting of total cellular extracts, HUVECs were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 1% [vol/vol] NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF) and added to 6× SDS sample buffer as previously described (Cho *et al.*, 2008). Samples were subjected to SDS–PAGE using polyacrylamide gels of an appropriate percentage, and resolved proteins were transferred to Immobilon P membranes (Millipore, Billerica, CA) in transfer buffer (25 mM Tris, 192 mM glycine, 10% [vol/vol] methanol). The membranes were blocked in 4% (wt/vol) milk in TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% [vol/vol] Tween 20) for 30 min and then incubated overnight at 4°C with primary antibody in blocking buffer against either anti–COX-2 (polyclonal antibody; Cayman Chemical Company, Ann Arbor, MI) or COX-1 (monoclonal antibody; Cayman Chemical Company); anti–phospho-Akt (Ser-473 and Thr-308), total Akt, anti–phospho-GSK-3β (Ser-9), anti–phospho-β-catenin (Ser-33, -37/Thr-41), anti–phospho-PTEN (Ser-380, Thr-382/-383), total PTEN, anti–phosphorylated (Ser-32) IκBα, and total IκBα from Cell Signaling Technology (Beverly, MA); and β-actin (Sigma-Aldrich). A detailed protocol for each antibody was followed as described in the corresponding manufacturer's protocol. Blots were subsequently incubated with horseradish peroxidase (HRP)–conjugated donkey anti–mouse, goat anti–rabbit, and bovine anti–goat (Santa Cruz Biotechnology, Santa Cruz, CA) immunoglobulin G (IgG), as appropriate, and visualized using enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham Biosciences) and exposed to x-ray film. Densitometric analysis of the bands was performed using Corel Photopaint, version 11 (Corel, Mountain View, CA).

For the preparation of nuclear extracts, cells were suspended in hypotonic buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.6, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM PMSF, and 1 mM each of pepstatin, aprotinin, and leupeptin) for 10 min in ice and vortexed for 10 s. Nuclei were pelleted by centrifugation and washed with buffer A before suspension in buffer B (10 mM HEPES, pH 7.6, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM, 0.5 mM PMSF, 1 μM each of pepstatin, aprotinin, and leupeptin, and 5% [vol/vol] glycerol) for 30 min on ice. The supernatants containing nuclear proteins

were collected by centrifugation at 12,000 × g for 20 min (Barbieri and Weksler, 2007). Samples were added to SDS-sample buffer and subjected to SDS–PAGE using the appropriate percentage polyacrylamide gel. Membranes were probed using anti–β-catenin (BD Transduction Laboratories, Lexington, KY), anti-NFκB (Cell Signaling Technology), or anti–RNA polymerase II (Millipore) monoclonal antibodies in 4% (vol/vol) milk in TBST. Washed membranes were incubated with HRP-conjugated anti–mouse IgG secondary antibody (Santa Cruz Biotechnology) and developed using ECL reagents.

Inhibitors, including a Rho kinase inhibitor (Y27632), a PI3 kinase inhibitor (LY294002), and an Src inhibitor (PP1 Analog II), were purchased from Calbiochem (La Jolla, CA) and used to pretreat the cells as described in the figure legends.

Immunoprecipitation

HUVECs were grown in six-well tissue culture plates and lysed in immunoprecipitation buffer (62.5 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.1% Tween 20, 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor cocktail) for 30 min on ice. A monoclonal antibody against VE-cadherin (1 µg; Hycult Biotechnology, Plymouth Meeting, PA) was added to protein A–Sepharose beads (20 µl; Sigma-Aldrich) and incubated with gentle rocking overnight at 4°C. The protein A–Sepharose beads were pelleted by brief centrifugation and washed with immunoprecipitation buffer; 0.3 mg of cell lysate proteins was added to the protein A–Sepharose beads for 2 h at 4°C, after which the beads were washed and the pellet then resuspended in 20 µl of 2× Laemmli buffer. Twenty-microliter aliquots of immunoprecipitated protein were separated on 7% polyacrylamide SDS–PAGE gels and immunoblotted with antibodies to VE-cadherin or β-catenin (BD Transduction Laboratories).

Transfection of siRNA

Approximately 25,000 cells/well of six-well plates were grown to 50–70% confluence. Transfection with siRNAs was performed using transfection reagent and medium supplied by Santa Cruz Biotechnology, used in a final β-catenin siRNA duplex or control siRNA concentration of 50 nM. After 7 h of incubation, an equal volume of fresh complete medium was added without removing the transfection mixture, according to the manufacturer's protocol. After 18 h, the transfected cells were starved for 15 min in EBM-2 before treatment with apo(a).

Prostaglandin E_2 assay

HUVECs were grown to confluence in six-well tissue culture plates. Cells were starved for 15 min in EBM-2 and treated with 200 nM recombinant apo(a) (17K or 17KΔAsp) or 2 ng/ml interleukin-1β. CM was collected at different time points, and the corresponding cells were lysed with lysis buffer. The lysates were subjected to Western blot analysis using COX-2 and COX-1 antibodies as described. CM samples were immediately frozen at −70°C for PGE_2 assay the following day. PGE_2 levels in the CM were measured using a PGE_2 enzyme immunoassay kit (Cayman Chemical Company) according to the manufacturer's specifications.

Cell-binding assays

Purified apo(a) was fluorescently labeled using Alexa Fluor 594 protein labeling kit (Invitrogen, Carlsbad, CA). The degree of labeling for all apo(a) preparations was 2.8–3 moles of dye per mole of protein. Human plasminogen labeled with fluorescein isothiocyanate was purchased from Molecular Innovations (Novi, MI). HUVECs, grown in 96-well plates with opaque well walls were washed three times with Hank's balanced salt solution (HBSS)–0.4% bovine serum albumin (BSA) and fluorescently labeled r-apo(a) or plasminogen at a concentration of 400 nM and in the presence or absence of 200 mM ε-ACA, was incubated with cells for 1 h at 37°C. After incubation, cells were washed once with HBSS–0.4% BSA, and the fluorescence was measured at an excitation wavelength of 590 nm and an emission wavelength of 617 nm using a plate-reading fluorescence spectrometer; a 610-nm cutoff filter was placed in the emission light path.

REFERENCES

Angelini DJ, Hyun SW, Grigoryev DN, Garg P, Gong P, Singh IS, Passaniti A, Hasday JD, Goldblum SE (2006). TNFalpha increases tyrosine phosphorylation of vascular endothelial cadherin and opens the paracellular pathway through fyn activation in human lung endothelia. Am J Physiol Lung Cell Mol Physiol 291, L1232–L1245.

Araki Y, Okamura S, Hussain SP, Nagashima M, He P, Shiseki M, Miura K, Harris CC (2003). Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways. Cancer Res 63, 728–734.

Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG (2000). Toll-like receptor 2-mediated NFκB activation requires a Rac1-dependent pathway. Nat Immunol 1, 533–540.

Barbieri SS, Ruggiero L, Tremoli E, Weksler BB (2008). Suppressing PTEN activity by tobacco smoke plus interleukin-1β modulates dissociation of VE-cadherin/β-catenin complexes in endothelium. Arterioscler Thromb Vasc Biol 28, 732–738.

Barbieri SS, Weksler BB (2007). Tobacco smoke cooperates with interleukin-1β to alter β-catenin trafficking in vascular endothelium resulting in increased permeability and induction of cyclooxygenase-2 expression in vitro and in vivo. FASEB J 21, 1831–1843.

Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W (1998). Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β. Science 280, 596–599.

Cho T, Jung Y, Koschinsky ML (2008). Apolipoprotein(a), through its strong lysine-binding site in KIV_{10}, mediates increased endothelial cell contraction and permeability via a Rho/Rho kinase/MYPT1-dependent pathway. J Biol Chem 283, 30503–30512.

Coffer PJ, Jin J, Woodgett JR (1998). Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J 335, 1–13.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.

Dannenberg AJ, Lippman SM, Mann JR, Subbaramaiah K, DuBois RN (2005). Cyclooxygenase-2 and epidermal growth factor receptor: pharmacologic targets for chemoprevention. J Clin Oncol 23, 254–266.

Dejana E, Orsenigo F, Lampugnani MG (2008). The role of adherens junctions and VE-cadherin in the control of vascular permeability. J Cell Sci 121, 2115–2122.

Donella-Deana A *et al.* (2003). Tyrosine phosphorylation of protein kinase CK2 by Src-related tyrosine kinases correlates with increased catalytic activity. Biochem J 372, 841–849.

Downward J (1998). Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 10, 262–267.

Dubé JB, Boffa MB, Hegele RA, Koschinsky M (2012). Lipoprotein(a): more interesting than ever after 50 years. Curr Opin Lipidol 23, 133–140.

Esser S, Lampugnani MG, Corada M, Dejana E, Risau W (1998). Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci 111, 1853–1865.

Essler M, Retzer M, Bauer M, Heemsker JW, Aepfelbacher M, Seiss W (1999). Mildly oxidized low density lipoprotein induces contraction of human endothelial cells through activation of Rho/Rho kinase and inhibition of myosin light chain phosphatase. J Biol Chem 274, 30361–30364.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW (1998). Identification of c-MYC as a target of the APC pathway. Science 281, 1509–1512.

Hemmings BA (1997). PtdIns(3,4,5)P3 gets its message across. Science 275, 628–630.

Hildesheim J, Salvador JM, Hollander MC, Fornace AJ Jr (2005). Casein kinase 2- and protein kinase A-regulated adenomatous polyposis coli and β-catenin cellular localization is dependent on p38 MAPK. J Biol Chem 280, 17221–17226.

Hughes SD, Lou XJ, Ighani S, Verstuyft J, Grainger DJ, Lawn RM, Rubin EM (1997). Lipoprotein(a) vascular accumulation in mice. In vivo analysis of the role of lysine binding sites using recombinant adenovirus. J Clin Invest 100, 1493–1500.

Janssens S, Beyaert R (2002). A universal role for MyD88 in TLR/IL-1R-mediated signaling. Trends Biochem Sci 27, 474–482.

Kim SJ, Im DS, Kim SH, Ryu JH, Hwang SG, Seong JK, Chun CH, Chun JS (2002). Beta-catenin regulates expression of cyclooxygenase-2 in articular chondrocytes. Biochem Biophys Res Commun 296, 221–226.

Koschinsky ML, Côté GP, Gabel B, van der Hoek YY (1993). Identification of the cysteine residue in apolipoprotein(a) that mediates extracellular coupling with apolipoprotein B-100. J Biol Chem 268, 19819–19825.

Koschinsky ML, Marcovina SM (2009). Lipoprotein(a). In: Clinical Lipidology: A Companion to Braunwald's Heart Disease, ed. CM Ballantyne, Philadelphia: Saunders (Elsevier), 130–143.

Koschinsky ML, Tomlinson ZE, Zioncheck TF, Schwartz K, Eaton DL, Lawn RM (1991). Apolipoprotein(a): expression and characterization of a recombinant form of the protein in mammalian cells. Biochemistry 30, 5044–5051.

Kotelevets L, van Hengel J, Bruyneel E, Mareel M, van Roy F, Chastre E (2005). Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. FASEB J 19, 115–117.

Krysan K, Lee JM, Dohadwala M, Gardner BK, Reckamp KL, Garon E, St John M, Sharma S, Dubinett SM (2008). Inflammation, epithelial to mesenchymal transition, and epidermal growth factor receptor tyrosine kinase inhibitor resistance. J Thorac Oncol 3, 107–110.

Maehama T, Dixon JE (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273, 13375–13378.

Maehama T, Taylor GS, Dixon JE (2001). PTEN and myotubularin: novel phosphoinositide phosphatases. Annu Rev Biochem 70, 247–279.

Manukyan M, Nalbant P, Luxen S, Hahn KM, Knaus UG (2009). RhoA GTPase activation by TLR2 and TLR3 ligands: connecting via Src to NFκB. J Immunol 182, 3522–3529.

McLean JW, Tomlinson JE, Kuang WH, Eaton DL, Chen EY, Fless GM, Scanu AM, Lawn RM (1987). cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. Nature 330, 132–137.

O'Neil CH, Boffa MB, Hancock MA, Pickering JG, Koschinsky ML (2004). Stimulation of vascular smooth muscle cell proliferation and migration by apolipoprotein(a) is dependent on inhibition of transforming growth factor-β activation and on the presence of kringle IV type 9. J Biol Chem 279, 55187–55195.

Ozawa M, Baribault H, Kemler R (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J 8, 1711–1717.

Pellegrino M, Furmaniak-Kazmierczak E, LeBlanc JC, Cho T, Cao K, Marcovina SM, Boffa MB, Côté GP, Koschinsky ML (2004). The apolipoprotein(a) component of lipoprotein(a) stimulates actin stress fiber formation and loss of cell-cell contact in cultured endothelial cells. J Biol Chem 279, 6526–6533.

Sanchez T, Skoura A, Wu MT, Casserly B, Harrington EO, Hla T (2007). Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN. Arterioscler Thromb Vasc Biol 27, 1312–1318.

Sangrar W, Gabel BR, Boffa MB, Walker JB, Hancock MA, Marcovina SM, Horrevoets AJ, Nesheim ME, Koschinsky ML (1997). The solution phase interaction between apolipoprotein(a) and plasminogen inhibits the binding of plasminogen to a plasmin-modified fibrinogen surface. Biochemistry 36, 10353–10363.

Sangrar W, Koschinsky ML (2000). Characterization of the interaction of recombinant apolipoprotein(a) with modified fibrinogen surfaces and fibrin clots. Biochem Cell Biol 78, 519–525.

Seimon TA *et al*. (2010). Atherogenic lipids and lipoproteins trigger CD36-TLR2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress. Cell Metab 12, 467–482.

Shasby DM, Ries DR, Shasby SS, Winter MC (2002). Histamine stimulates phosphorylation of adherens junction proteins and alters their link to vimentin. Am J Physiol Lung Cell Mol Physiol 282, L1330–L1338.

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999). The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96, 5522–5527.

Sotiriou SN *et al*. (2006). Lipoprotein(a) in atherosclerotic plaques recruits inflammatory cells through interaction with Mac-1 integrin. FASEB J 20, 559–561.

Stemme V, Swedenborg J, Claesson H, Hansson GK (2000). Expression of cyclo-oxygenase-2 in human atherosclerotic carotid arteries. Eur J Vasc Endovasc Surg 20, 146–152.

Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X, Wu H (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci USA 96, 6199–204.

Tam SP, Zhang X, Koschinsky ML (1996). Interaction of a recombinant form of apolipoprotein[a] with human fibroblasts and with the human hepatoma cell line HepG2. J Lipid Res 37, 518–533.

Tsimikas S, Hall JL (2012). Lipoprotein(a) as a potential causal genetic risk factor of cardiovascular disease. J Am Coll Cardiol 60, 716–721.

Vazquez F, Grossman SR, Takahashi Y, Rokas MV, Nakamura N, Sellers WR (2001). Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J Biol Chem 276, 48627– 48630.

Vazquez F, Matsuoka S, Sellers WR, Yanagida T, Ueda M, Devreotes PN (2006). Tumor suppressor PTEN acts through dynamic interaction with the plasma membrane. Proc Natl Acad Sci USA 103, 3633–3638.

Vazquez F, Sellers WR (2000). The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling. Biochim Biophys Acta 1470, M21–M35.

Vogelmann R, Nguyen-Tat MD, Giehl K, Adler G, Wedlic D, Menke A (2005). TGFβ-induced downregulation of E-cadherin-based cell-cell adhesion depends on PI3-kinase and PTEN. J Cell Sci 118, 4901– 4912.

Wojciak-Stothard B, Entwistle A, Garg R, Ridley AJ (1998). Regulation of TNFalpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. J Cell Physiol 176, 150–165.