Received: 24 June 2022 | Revised: 29 December 2022 | Accepted: 27 January 2023

DOI: 10.1096/fj.202201001RR

RESEARCH ARTICLE

The FASEB Journal

O-glycan structures in apo(a) subunit of human lipoprotein(a) suppresses the pro-angiogenic activity of galectin-1 on human umbilical vein endothelial cells

Vasantha Kalaivani | **Mahesh S. Krishna** | **Asokan Aneesh Kumar** | **Gopika Satheesh** | **Abdul Jaleel**

Diabetes Biology Laboratory, Division of Cardiovascular Diseases and Diabetes Biology, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India

Correspondence
Vasantha Kalaivani and Abdul Jaleel, Diabetes Biology Laboratory, Division of Cardiovascular Diseases and Diabetes Biology, Rajiv Gandhi Centre for Biotechnology, Poojappura, Thycaud P.O., Thiruvananthapuram, Kerala 695014, India.
Email: kalaivaniv@rgcb.res.in and jaleel@rgcb.res.in

Funding information
DST | Science and Engineering Research Board (SERB), Grant/Award Number: PDF/2016/002807

Abstract

Apolipoprotein(a) [apo(a)] is a highly polymorphic O-glycoprotein circulating in human plasma as lipoprotein(a) [Lp(a)]. The O-glycan structures of apo(a) subunit of Lp(a) serve as strong ligands of galectin-1, an O-glycan binding pro-angiogenic lectin abundantly expressed in placental vascular tissues. But the pathophysiological significance of apo(a)-galectin-1 binding is not yet been revealed. Carbohydrate-dependent binding of galectin-1 to another O-glycoprotein, neuropilin-1 (NRP-1) on endothelial cells activates vascular endothelial growth factor receptor 2 (VEGFR2) and mitogen-activated protein kinase (MAPK) signaling. Using apo(a), isolated from human plasma, we demonstrated the potential of the O-glycan structures of apo(a) in Lp(a) to inhibit angiogenic properties such as proliferation, migration, and tube-formation in human umbilical vein endothelial cells (HUVECs) as well as neovascularization in chick chorioallantoic membrane. Further, in vitro protein-protein interaction studies have confirmed apo(a) as a superior ligand to NRP-1 for galectin-1 binding. We also demonstrated that the protein levels of galectin-1, NRP-1, VEGFR2, and downstream proteins in MAPK signaling were reduced in HUVECs in the presence of apo(a) with intact O-glycan structures compared to that of de-O-glycosylated apo(a). In conclusion, our study shows that apo(a)-linked O-glycans prevent the binding of galectin-1 to NRP-1 leading to the inhibition of galectin-1/neuropilin-1/VEGFR2/MAPK-mediated angiogenic signaling pathway in endothelial cells. As higher plasma Lp(a) level in women is an independent risk factor for pre-eclamsia, a pregnancy-associated vascular complication, we propose that apo(a) O-glycans-mediated inhibition of the pro-angiogenic activity of galectin-1 may be one of the underlying molecular mechanism of pathogenesis of Lp(a) in pre-eclampsia.

Abbreviations: Apo(a), apolipoprotein(a); Con A, concanavalin A; DOA, de-O-glycosylated apo(a); DSA, de-sialylated apo(a); Gal-1, galectin-1; +H Apo(a), heat-inactivated apo(a); HRP, horse-radish peroxidase; HUVEC, human umbilical vein endothelial cells; Lp(a), lipoprotein(a); MAPK, mitogen-activated protein kinase; N-glycan, N-linked glycan; NRP-1, neuropilin-1; O-glycan, O-linked glycan; PNA, peanut agglutinin; T-antigen, Thomsen-Friedenreich antigen; VEGFR2, vascular endothelial growth factor receptor-2.

KEYWORDS
angiogenesis, apo(a), galectin-1, lipoprotein(a), neuropilin-1, O-glycan

1 | INTRODUCTION

Lipoprotein(a) [Lp(a)] is a distinctive circulating plasma lipoprotein present only in humans and old-world monkeys.[1] It consists of a single subunit of low-density lipoprotein to which a unique glycoprotein known as apolipoprotein(a) [apo(a)] is covalently attached.[2,3] Apo(a) is structurally similar to plasminogen and composed of multiple numbers of specialized protein structures known as "kringles".[2] Two types of kringle structures, kringle IV and kringle V are present in apo(a). A single copy of the kringle V domain and 10 different sub-types of the kringle IV domain, which are named kringle IV type 1 to type 10 are present in apo(a). Further, the number of kringle IV type 2 domain in apo(a) varies according to the polymorphism of apo(a) gene.[1] Both carbohydrate analysis and peptide sequence analysis have shown that the interkringle regions of apo(a) kringle IV type 2 domain are glycosylated with both N-and O-linked glycan structures.[2,3] Compared to the N-glycans, apo(a) is heavily enriched with core-1-type O-glycan structures with a predominance of a clinically important disaccharide, Gal β 1→3 GalNAc, also known as Thomsen-Friedenreich antigen (T-antigen) with a terminal sialic acid group.[2] As the number of kringle IV type 2 domain increases, the interkringle region increases and so the number of O-glycan also increases, thereby making apo(a) one of the most polymorphic O-glycoprotein (molecular weight ranges from 300 to 900 kDa) in human population.[4] Interestingly, the plasma of a single individual contains two different Lp(a) isoforms.[5] Moreover, the peculiar apo(a) gene polymorphism is the reason for a large variation in plasma Lp(a) level, ranging from 0.1 mg/dL to 1000 mg/dL.[5]

Very few studies have addressed the role of apo(a)-O-glycans in physiologic or pathologic functions of Lp(a). The heavily O-glycosylated structures of apo(a) make Lp(a) a strong ligand for galectin-1, an evolutionarily conserved, pro-angiogenic, O-glycan binding animal lectin abundantly expressed in vascular tissues such as heart and placenta.[6–9] Galectin-1 is secreted as a monomer or a dimer with two identical carbohydrate-binding domain. Galectin-1 is present on both the intracellular and extracellular side of all cell membranes as well as in the extracellular matrices.[10] A few studies have observed an increased expression of galectin-1, followed by its translocation to the outer cell membrane and an increase in membrane-bound galectin-1 in activated endothelial cells.[11,12] Galectin-1 performs both intracellular and extracellular functions, in which the former is independent of its lectin activity while the latter is mainly dependent on it.[10] Basically, galectin-1 is a β-galactoside binding lectin,[9] which can also recognize N-acetyl lactosamine (LacNAc) residues present in core-2 O-glycans and complexed branched N-glycans.[10] Similarly, galectin-1 is shown to bind Galβ1→3GalNAc residue (Thomsen–Friedenreich antigens or T-antigens) exclusively present in core-1 O-glycans.[13] Interestingly, P.S. Appukuttan and his group revealed that the T antigen structure in O-linked glycoproteins is far superior to N-acetyl lactosamine structures as ligands for galectin-1.[6,13]

The binding of galectin-1 to cell-surface glycoconjugates plays a significant role in diverse cellular functions during various stages of pregnancy.[14–19] On the female reproductive tract and placenta, galectin-1 binds a cell-surface expressed O-glycoprotein, known as neuropilin-1 (NRP-1) via its highly conserved carbohydrate-binding domain.[20] The O-glycan-mediated binding of galectin-1 to NRP-1 further activates another angiogenic protein, vascular endothelial growth factor receptor 2 (VEGFR2). In fact, galectin-1 can directly bind to the LacNAc residues in VEGFR2.[21] As a result, the downstream proteins of mitogen-activated protein kinases (MAPK) signaling pathway get activated eventually leading to the activation of angiogenic processes such as proliferation, migration, and tube formation in endothelial cells.[14,21–26] An earlier report demonstrated that the inhibition of galectin-1–stimulated angiogenesis with anginex, a designed anti-angiogenic peptide, leads to impaired angiogenesis and poor placentation and provokes pre-eclampsia-like symptoms with intrauterine fetal growth restriction in mice.[25] Pre-eclampsia is a vascular disease of the placenta that arises during pregnancy in association with hypertension and proteinuria.[27] Poor placentation with impaired vascular remodeling and altered angiogenesis of maternal spiral arteries leading to intrauterine growth restriction is one of the major pathological features of pre-eclampsia.[27,28] Numerous studies have provided strong evidence for the association of galectin-1 dysregulation and pre-eclampsia[26,29] and also an increased fetal death was observed in a galectin-1 knockout mice model.[18] Recently, researchers have shown an elevated plasma level of Lp(a) (>30 mg/dL) in women as an independent risk factor for pre-eclampsia.[30–32] The placenta of pre-eclamptic women was found to contain an increased amount of Lp(a) deposits which is a marker for chronic vascular damage,[33,34] suggesting a significant role of Lp(a) in pre-eclampsia pathology. Despite the reports on the association of Lp(a) in pre-eclampsia, the underlying

molecular mechanism of Lp(a) in the pathogenesis of pre-eclampsia is still unknown.

There is a structural similarity of apo(a) to the naturally occurring anti-angiogenic proteins such as plasminogen and angiostatin,[2] and the anti-angiogenic property of Lp(a) has been demonstrated.[35–37] However, the Lp(a)- receptor protein on endothelial cells and the exact molecular mechanism of its anti-angiogenicity are not completely known. Interestingly, an earlier study demonstrated an exclusively O-glycan-mediated in situ binding of exogenously added human Lp(a) to galectin-1 expressed on human mammary arterial cells.[6] Hence, we hypothesize that the O-glycan-mediated binding of apo(a) subunit of Lp(a) to galectin-1 on the vascular tissues may have a detrimental effect on galectin-1/NRP-1/VEGFR-2/MAPK-mediated angiogenic signaling pathway. Using native apo(a) isolated from human plasma, we have demonstrated an apo(a) O-glycan-mediated inhibition of galectin-1-stimulated angiogenic processes in cultured human umbilical vein endothelial cells (HUVECs) and in chick chorioallantoic membrane.

2 | MATERIALS AND METHODS

2.1 | Materials

Out-dated human plasma was collected from the Blood bank, Ananthapuri Hospitals and Research Institute, Thiruvananthapuram, Kerala, India after getting approval (IHEC/1/2019/02) from the Institute Human Ethical Committee. Human Umbilical Vein Endothelial Cells (HUVECs) were received from Central Cell and Tissue Repository of Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India. Fertilized hen eggs were purchased from a local hatchery, Thiruvananthapuram, Kerala, India. The lectins, jacalin, peanut agglutinin (PNA), and concanavalin A (con A), used in this study were a kind gift from Dr. P. S. Appukuttan, Senior Grade Professor & Former Head of Department of Biochemistry, Sree Chitra Tirunal Institute for Science and Technology (SCTIMST), Thiruvananthapuram, Kerala, India. Lectins were subsequently conjugated with horse-radish peroxidase type VI (HRP, P8375-5U; Sigma-Aldrich, USA) as described previously.[38]

2.2 | Isolation of apolipoprotein(a) [apo(a)] from human plasma

Apo(a) was isolated from human plasma as reported earlier.[39] Briefly, human plasma was taken in Optiseal polyallomer centrifuge tubes after adjusting its density to 1.24 g/mL with solid potassium bromide (KBr) and subjected to ultracentrifugation at 535000×*g* for 7 h at 4°C using an NVT90 rotor in a Beckman Coulter Vacuum Ultracentrifuge (Life Sciences, USA). Lp(a) containing lipid sample was collected from the top 20% layer, pooled, and dialyzed in a 10 kDa cut-off dialysis tubing cellulose membrane (D9777; Sigma-Aldrich, USA) against 20 mM potassium phosphate buffer, pH 7.4 containing 150 mM sodium chloride (PBS) at 4°C for overnight. Apo(a) was separated from Lp(a) by reducing the dialyzed lipids with 4 mM di-thiothreitol (DTT) for 15 min at 37°C. The density of the reduced lipid solution was again adjusted to 1.24 g/mL with KBr and subjected to one more ultracentrifugation as above. The separated apo(a) protein was collected from the bottom 30% layer using a syringe with a long needle and subjected to dialysis overnight in PBS at 4°C. The amount of total protein in apo(a) samples were estimated using Bio-Rad protein assay reagent (Bio-Rad, USA) according to the manufacturer's instruction.

2.3 | Glycosylation modifications of apo(a)

In this study, we have tried both chemical and enzymatic methods to modify apo(a) O-glycans.[39] To remove the entire O-glycan structures (de-O-glycosylation), a specified amount of apo(a) sample was incubated with 1 M sodium borohydride in 0.05 N sodium hydroxide solution for 16 h at 37°C in a water bath and later, dialyzed overnight against PBS. To remove the terminal sialic acid group (desialylation), a specified amount of apo(a) sample was incubated in 0.1 N sulfuric acid for 1 h at 80°C in a water bath followed by overnight dialysis in PBS. For enzymatic removal of apo(a) O-glycans, native apo(a) was treated with O-glycosidase (Cat.No:11347101001, Sigma-Aldrich, USA) as described previously.[6] To get heat-inactivated apo(a), a specified amount of apo(a) sample was incubated in 150 mM PBS (pH 7.4) buffer at 90°C in a water bath. After 5 min, heat-inactivated apo(a) was kept in ice and later subjected to a gentle spin-down at 1000×*g* for 1 min.

2.4 | HUVEC culture

HUVEC was cultured in EGM™ 2 Endothelial Cell Growth Medium-BulletKit™ (EGM-2, Lonza, Switzerland, CC-3162) with added growth supplements, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B at 37°C under 5% CO_2 in a CO_2 incubator. Approximately, 75–80% confluent HUVECs within passages 4–6 were used for all

the experiments. Prior to treatment, cells were starved overnight by keeping them in the same media conditions without FBS. The control cultures were also kept in EGM-2 without any treatment. Cell morphology was observed and images were taken by an inverted microscope (Nikon Eclipse Ti, USA, magnification, ×10).

2.5 | Measurement of HUVEC proliferation—MTT assay

The effect of apo(a) O-glycans on cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. Briefly, serum-starved 75-80% confluent HUVECs cultured in a 96-well culture plate (0.5×10^4 cells/well) were treated with 10 μg/mL each of native apo(a), de-O-glycosylated apo(a) [DOA], de-sialylated apo(a) [DSA] and 50 mM lactose for 18 h. After that, a 10 μL MTT (5 mg/mL; Sigma-Aldrich, USA) solution was added to each well containing 90 μL PBS, and the wells were incubated for 4 h at 37°C. Then, the supernatant was decanted, and 100 μL of MTT solubilization solution (4 mM HCl and 0.1% NP-40 in isopropanol) was added to each well. The plate was wrapped with aluminum foil and placed on an orbital shaker (Spinix Orbital shaker, Tarsons, India) for 2 h at room temperature. Subsequently, absorbance in each well was measured at 570 nm (A 570) with a microplate reader (Infinite M200, Tecan Inc., USA). The effect of apo(a) O-glycans on the viability of HUVEC was expressed as the % of viable cells using the following formula: % of viable cells = [A 570 of treated cells] × 100/[A 570 of control cell].

2.6 | Measurement of HUVEC migration: Wound-scratch assay

A wound was made on the 100 % confluent, serum-deprived HUVEC monolayer by a single scratch using a sterile 10-μL pipette tip. After washing twice with PBS, cells were incubated for 12 h with 10 μg/mL each of native apo(a), de-O-glycosylated apo(a), and 50 mM lactose. The wounded area was photographed at the beginning and after incubation. The rate of cell migration was quantified after the indicated time intervals using image J software as relative wound density in which the ratio of the occupied area in the wound gap to the total area of the initial wound gap was measured and expressed as a percentage.

To check the effect of apo(a)-linked O-glycans on the pro-angiogenic activity of galectin-1, fully confluent, serum-deprived, scratch-wounded HUVEC monolayer was treated with 2 μg/mL recombinant human galectin-1 protein (Cat.No: NBP1-30230, Novus Biologicals, USA) pre-incubated with (a) PBS 7.4, (b) 10 μg/mL de-O-glycosylated apo(a) [DOA], (c) 10 μg/mL native apo(a), (d) 50 mM lactose, (e) 10 μg/mL heat-inactivated apo(a) [+H apo(a)], and (f) 1 μg/mL polyclonal rabbit anti-human galectin-1 antibody (10290-RP01-400; SinoBiologicals, China). After an incubation period of 12 h at 37°C under 5% CO_2, images of the cells that migrated into the wounded area were photographed as described above.

2.7 | Measurement of HUVEC tube formation

The effect of native and glycosylation-modified apo(a) on tube formation of HUVECs was assayed by capillary-like structure (CLS) formation assay and Matrigel tube formation assay. In the CLS formation assay, a 100% confluent HUVEC monolayer was treated with native and de-O-glycosylated apo(a) (10 μg/mL), and images of CLS formation were taken before and after 18 h of incubation. For the Matrigel tube formation assay, ECM Gel from Engelbreth-Holm-Swarm murine sarcoma (E1270; Sigma-Aldrich, USA) was pipetted into a 24-well plate (100 μL/well) and polymerized at 37°C for 2 h. HUVECs (1 mL, 4×10^4 cells/well) mixed with 10 μg/mL each of native apo(a), de-O-glycosylated apo(a), and 50 mM lactose in serum-deprived EGM-2 media were seeded into Matrigel pre-coated wells and incubated for 6 h and photographed before and after incubation. The number of CLS formed and the number of tubes formed were calculated by manual counting. A three-branch point area was considered as one tubular structure. Three random fields per well were quantified and mean tube formation was expressed as a percentage of control.

2.8 | Measurement of neovascularization—Chorioallantoic membrane (CAM) assay

Fertilized hen eggs were incubated horizontally for 8 days at 40°C under a humidified atmosphere in a bacteriological incubator (KIS-2, Kemi, India) with an intermittent manual rotation at every 8 h. At day 8-post fertilization, CAM assay was carried out according to the assay procedures detailed elsewhere.[40] Briefly, eggs were cleaned with 70% ethanol and a small incision (approximately 0.5 cm^2) was made using pointed, sterile forceps on the eggshell where the air sac is located under sterile conditions. Eggshell fragments were carefully removed to access the CAM beneath. Through the incision, a 20 μL volume of 1 mg/mL sample [PBS, native apo(a), and de-O-glycosylated apo(a)] was carefully injected into the CAM. The incision was

then covered with sterile non-transparent, surgical tape, and eggs were further incubated as described above. A group of 2 eggs was assigned for each sample. Embryos were inspected daily by candling. At day 12-post treatment, eggs were opened, embryos were carefully removed and the CAM was retained on the eggshell. Blood vessels formed on CAM were photographed using a digital camera. Angiogenesis on CAM was evaluated macroscopically by carefully counting the number of new blood vessels formed on CAM after treatment.

2.9 | HUVEC protein extraction

HUVECs treated with and without native, glycosylation-modified apo(a) and lactose were detached from the cell-culture plates by incubating with 0.25% trypsin-EDTA solution at 37°C for 1–2 min. HUVEC pellets collected after gentle centrifugation at 1000× *g* for 5–10 min were lyzed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 8 with 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 140 mM NaCl) with protease inhibitor cocktail (P8340; Sigma-Aldrich, USA) on ice for 30 min. Cell lysates were then centrifuged at 13 000× *g* for 20 min at 4°C and the supernatants were collected. Protein estimation was performed using Bio-Rad protein assay reagent (Bio-Rad, USA) according to manufacture's instruction.

2.10 | Immunoblot analysis

Proteins extracted from the HUVECs treated, non-treated, and the protein molecular weight marker (PM007-0500; GeneDireX, Inc.) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. For galectin-1, ERK1/2 and phospho-ERK1/2, a 12% gel was used while for NRP-1, VEGFR2 and phospho-VEGFR2, a 5% gel was used. Resolved proteins were transferred onto Immobilon-P-Polyvinylidene fluoride (PVDF) membrane for 1 h in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH-8.3) under cold conditions and a constant current of 0.8 mA/cm^2 membrane using a Mini Trans-Blot Module (Bio Rad, USA).[41] Transfer of proteins on the PVDF membrane was verified by Ponceau S staining. The remaining unbound sites on the membrane were blocked by incubating the membrane for 1 h in 5% bovine serum albumin (BSA) in 200 mM Tris-buffered saline, pH 7.6 containing 150 mM NaCl with 0.5% Tween-20 (5% BSA-TBS-T) at room temperature. After that, the membrane was incubated separately overnight at 4°C with specified dilutions of primary antibodies in 3% BSA in TBS-T solution. After washing three times with TBS-T, the membrane was further incubated with 1:10000 dilutions of HRP-labeled secondary antibody for 45 min at 37°C on an orbital shaker (Spinix Orbital Shaker, Tarsons, India). The bound HRP was assayed using a chemiluminescent western blot hyper HRP substrate (T7103A, Takara Bio, USA) and images were taken with VersaDoc MP 4000 Imaging System (Bio-Rad). β-actin was used as an internal control. Protein bands on PVDF membrane were quantified using Quantity One software, BioRad.

List of primary antibodies used; monoclonal mouse anti-human apo(a) antibody (1.5 μg/mL; 5684-9485) from Bio-Rad Laboratories, US; polyclonal rabbit anti-human NRP-1 antibody (1 μg/mL; 10011-RP01-400) and polyclonal rabbit anti-human galectin-1 antibody (1 μg/mL; 10290-RP01-400) from SinoBiologicals, China; polyclonal goat anti-human apoB antibody (1.5 μg/mL; PA1-26901), polyclonal rabbit anti-human VEGFR2 antibody (5 μg/mL; PA5-16487), polyclonal rabbit anti-human phospho-VEGFR2 antibody (1:1000; PA5-12598), polyclonal rabbit anti-human ERK1 antibody (1:1000; PA5-32395), monoclonal mouse anti-human ERK2 antibody (1:1000; MA1-099), and monoclonal rabbit phospho-ERK1/ERK2 antibody (1 μg/mL; 700012) from Thermofischer Scientific, US; monoclonal mouse anti-human β-actin antibody (1:2000; sc-47778) from Santa Cruz Biotechnology, Inc.

List of secondary antibodies used; rabbit anti-goat IgG-HRP (sc-2768), goat anti-mouse IgG-HRP (sc-2005), and polyclonal goat anti-rabbit IgG-HRP (sc-2030) from Santa Cruz Biotechnology, Inc.

2.11 | Far-western blot analysis

Proteins (2 mg/mL) extracted from untreated, HUVECs containing the prey protein, NRP-1 were separated by loading the sample into a single well with 5% SDS-PAGE under reducing conditions. Separated proteins were transferred to a PVDF membrane as described in Section 2.10 and the PVDF membrane was cut vertically into several strips of equal width. The unbound sites on each strip were then blocked with 5% BSA-TBS-T. Each blocked strip was separately incubated for 2 h at 4°C with 2 μg/mL of the bait protein, recombinant human galectin-1 (NBP1-30230; Novus Biologicals, USA), which was pre-incubated for 1 h at 4°C with apo(a) (10 μg/mL), de-O-glycosylated apo(a) (10 μg/mL) and 50 mM lactose, respectively. After washing each strip separately in 5% BSA-TBS-T, they were incubated in 1 μg/mL of goat anti-human galectin-1 antibody (10290-RP01-400; SinoBiologicals, China) in 3% BSA-TBS-T solution overnight at 4°C. After washing with 5% BSA in TBS-T, strips were incubated with

1:10000 dilutions of HRP-labeled anti-goat IgG antibody in 3% BSA-TBS-T solution. Along with this, two blocked strips were separately incubated for 2 h at 4°C with 15 μg/mL HRP-labeled jacalin and 1 μg/mL rabbit anti-human NRP-1 antibody (10011-RP01-400; SinoBiologicals, China) followed by HRP-labeled anti-rabbit IgG antibody (1:10000) in 3% BSA-TBS-T solution. Afterward, the strips were washed thrice with 5% BSA-TBS-T. Membrane-bound HRP was then detected using a chemiluminescent western blot hyper HRP substrate (T7103A, Takara Bio, USA), and the images of the band on the strips that correspond to the prey protein, NRP-1 were taken and quantified as detailed in Section 2.10. Densitometric analysis of bands was performed by Image J Software.

2.12 | Gelatin-zymography

For detecting matrix metalloproteinase (MMP) activity, supernatants from HUVECs (2×10^5 cells/well, six wells per plate) treated with 10 μg/mL each native apo(a), de-O-glycosylated apo(a) and 50 mM lactose in serum-deprived EGM-2 media were collected and subjected to gelatin-zymography. Briefly, gelatin (1 mg/mL) was prepolymerized on a 7.5% SDS-polyacrylamide gel and electrophoresis was carried out under non-reducing conditions. After electrophoresis, the gel was washed twice with renaturation buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 2.5% Triton X-100), followed by incubation in a substrate buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3) at 37°C for 72 h. After that, the gel was stained with Coommassie Brilliant Blue R-250 and subsequently destained. The cleared zone on the gelatin zymogram was visualized and captured using a Gel-Doc System (Bio-Rad, USA).

2.13 | Co-immunoprecipitation

HUVECs treated with and without native apo(a) were treated with 0.25% trypsin-EDTA solution as described in Section 2.9 and the cell pellets collected were lysed at 4°C in 50 mM Tris-HCl, pH 7.4 buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate with protease inhibitor cocktail. Cell lysates were centrifuged for 10 min at 16 000×*g* at 4°C, supernatants were collected and the protein estimation was performed as described in Section 2.9. To prevent non-specific binding, HUVEC proteins (1 mg/mL) were pre-incubated with 50 μL of protein A/G–agarose bead suspension (Roche Diagnostics, Germany) for 3 h at 37°C on a RotoSpin (Tarsons, India). After centrifugation at 12 000×*g* for 2 min at 4°C, the unbound HUVEC proteins collected from the top of the agarose bead suspension were mixed with 10 μg of rabbit polyclonal anti-human NRP-1 antibody and incubated for 2 h at 4°C. To this antigen-antibody mixture, a 50 μL of fresh protein A/G–agarose bead suspension was added and incubated again overnight at 4°C. Agarose beads with the attached immune complexes were pelleted by centrifugation for at 16 000×*g* for 10 min at 4°C. Beads were collected and washed four times in ice-cold lysis buffer. Afterward, beads were subjected to SDS-PAGE (5% gel for NRP-1 and 12% gel for galectin-1) and continued with western blot analyses using rabbit anti-human galectin-1 antibody (1:100) and anti-human NRP-1 antibody (1:100) as described in Section 2.10.

2.14 | Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked lectin assay (ELLA)

A 200 μL of a specified concentration of apo(a) sample, HUVEC lysates, or other protein samples in PBS were coated on polystyrene wells of 96-well ELISA plates (Lockwell C8; Maxisorp, Nunc Thermofischer, USA) by incubating the samples at 37°C for 3 h in a water bath. After decanting the supernatants, wells were washed thrice with PBS containing 0.05% Tween 20 (PBS-T), and the unbound sites on the wells were blocked with PBS containing 0.5% Tween 20 for 30 min at 37°C. Finally, wells were again washed with PBS-T and then incubated with 200 μL of the specified concentrations of HRP-labeled lectins (such as jacalin, con A, PNA, and galectin-1) for ELLA or antibody solution in PBS-T for ELISA at 4°C under dark. After an incubation period of 2 h, supernatants were decanted and unbound proteins were removed by washing the wells three times with the same buffer. Bound HRP was assayed by treating the washed wells with 200 μL (5 mg/mL) of o-phenylenediamine (OPD, P5412; Sigma-Aldrich, USA) in 100 mM citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2 for 30 min at room temperature under dark. The reaction was stopped by the addition of 50 μL of 12.5% H_2SO_4 and absorbance was measured at 490 nm in a microplate reader (Infinite M200, Tecan Inc., USA).

For ELISA to check the effect of apo(a) O-glycans on galectin-1 protein level in HUVEC, polystyrene wells were coated with 5 μg/mL polyclonal rabbit anti-human galectin-1 antibody (10290-RP01-400; SinoBiologicals, China) as described earlier. After blocking the unbound sites on the wells, 5 μg/mL HUVEC cell lysates were added and incubated for 2 h at 4°C. After washing, the wells were further incubated with the same anti-human galectin-1 antibody (5 μg/mL) followed by HRP-labeled polyclonal goat anti-rabbit IgG antibody (1:1000; sc-2030; Santa Cruz Biotechnology, Inc.). The amount of galectin-1 in

cell lysates was assayed by measuring the absorbance of bound HRP as described earlier.

2.15 | Immunofluorescence assay

HUVECs (1×10^4 cells/well) were seeded on 18 mm coverslips coated with 0.1% sterile gelatin solution placed in the wells of a 12-well cell-culture plate and cultured in a CO_2 incubator until the growth reaches 50% confluency. Cells were then treated with native apo(a), de-O-glycosylated apo(a), and lactose as described in Section 2.5. After the treatment, cells were fixed on the coverslips by incubating them in 4% buffered p-formaldehyde fixative solution for 30 min at 37°C. Cells were then washed thrice with 50 mM glycine in PBS and subsequently, incubated in 2% BSA in 0.1% Triton-X-100 solution for 5 min at 37°C. Cells were then blocked with 5% BSA in Hank's balanced salt solution (HBSS) for 1 h at 37°C, and incubated with specified dilutions of primary antibodies prepared in 3% BSA in HBBS overnight at 4°C under dark. Afterward, cells were washed thrice with 0.1% Triton X-100 in PBS and incubated with 1:1000 dilutions of fluorescent-labeled secondary antibody for 45 min at room temperature in dark on an orbital shaker (Spinix Orbital Shaker, Tarsons, India). Cells were then washed with 50 mM glycine in PBS and further incubated with Hoechst 33342 stain (10 μg/mL; Sigma-Aldrich) for 1 min at 37°C. Finally, after washing the cells with PBS, coverslips were gently removed from the wells and mounted in a fluorescent mounting medium (Fluoromount G, Invitrogen) coated on a clean glass slide. Cell-bound fluorescence was visualized and images were captured by inverted confocal fluorescence microscopy.

List of primary antibodies used; monoclonal mouse anti-human von Willebrand Factor (vWF) antibody (1:100; MA5-14029) from Thermo Fischer Scientific, USA; monoclonal mouse anti-human apo(a) antibody (1:100; 5684-9485) from Bio-Rad Laboratories, US; polyclonal rabbit anti-human NRP-1 antibody (1:300; 10011-RP01-400) and polyclonal rabbit anti-human galectin-1 antibody (1:300; 10290-RP01-400) from Sino Biologicals, China.

List of secondary antibodies used; polyclonal goat anti-rabbit IgG (H&L)-Alexa Fluor 488 (ab15077) and polyclonal donkey anti-mouse IgG (H&L)-Alexa Fluor 647 (ab150111) from Abcam, US.

2.16 | RNA isolation and real-time polymerase chain reaction

HUVECs after treatment were lysed with Trizol reagent (Thermo Fischer, USA) and RNA was isolated as per the manufacturer's instruction. The purity and quantity of RNA were evaluated by NanoDrop 1000 (Thermo Scientific, USA). cDNA synthesis was done with GoScript™ Reverse Transcription System (Promega Corporation, USA) in C1000 Touch Thermal Cycler (BioRad, USA). Sybr green-based qPCR analysis was performed with Kapa Sybr Fast qPCR kit (Kapa Biosystems, USA) in ABI 7500 Fast system (Applied Biosystems Inc., USA). Change in gene expression of galectin-1 and NRP-1 was compared with that of β-actin. The following primer sequences were used:

Galectin-1 Forward: 5′-TCGCCAGCAACCTGAATC TC-3′
Galectin-1 Reverse: 5′-GCACGAAGCTCTTAGCGT CA-3′
NRP-1 Forward: 5′-GGCGCTTTTCGCAACGATAA A-3′
NRP-1 Reverse: 5′-TCGCATTTTTCACTTGGGTGAT-3′
β-actin Forward: 5′-CCGTGAAAAGATGACCCAGA TC-3′
β-actin Reverse: 5′-CACAGCCTGGATGGCTACGT-3′

2.17 | Statistical analysis

All data are presented as mean ± standard deviation. The means and standard deviations were calculated from three independent experiments. All treated groups were compared to the relevant control group. The statistical significance was determined by Student's *t* test and ANOVA using Graph pad Prism Version 9.1.2 (226). $p<.05$ was considered as a significant difference.

3 | RESULTS

3.1 | Affinity of N-and O-glycan specific lectins toward human apo(a) before and after glycosylation modification

To demonstrate the anti-angiogenic potential of apo(a)-linked O-glycans of Lp(a), Lp(a) was isolated from outdated human plasma and its apo(a) subunit was separated by chemical reduction[39] (Figures S1 and S2). The released apo(a) subunit was collected and its O-glycan structures were removed chemically.[39] Apo(a) with intact O-glycan structures was named "native apo(a)", whereas apo(a) without any O-glycan structures as 'de-O-glycosylated apo(a) [DOA]'. HRP-labeled plant lectins such as jacalin, galectin-1, peanut agglutinin (PNA), and concanavalin A (con A) were used to check the presence of O-glycans, Galβ1→3GalNAc residue in O-glycans, de-sialo (asialo) O-glycans (O-glycans devoid of a terminal sialic acid group)

and N-glycans respectively on both native and de-O-glycosylated apo(a) as described previously.[3]

Results show that jacalin, an O-glycan-specific plant lectin failed to bind de-O-glycosylated apo(a) prepared both chemically and enzymatically in contrast to its high affinity towards native apo(a) suggesting the complete removal of all O-glycan structures of apo(a) (Figures 1 and S5A). The comparatively low Con A response in both native and de-O-glycosylated apo(a) indicates the low abundance of N-glycans in apo(a). Our data also shows that after the removal of O-glycan structures from apo(a), the resulting de-O-glycosylated apo(a) became a poor ligand for the binding of galectin-1. Moreover, the absence or very low response of HRP-labeled PNA shows that O-glycan structures of native apo(a) are protected with intact terminal sialic acid groups. In addition, as expected, no PNA binding was observed in de-O-glycosylated apo(a) due to the absence or removal of sialo O-glycan structures.

3.2 | O-Glycans in apo(a) subunit of human Lp(a) suppress the proliferative, migratory, and tube-forming ability of HUVECs

A 75%–80% confluent HUVEC was used for our in vitro studies and an immunofluorescence analysis for vWF, an endothelial cell-specific marker on HUVEC was performed (Figure S3) to confirm the authenticity of HUVEC. Using confocal imaging, we have also verified

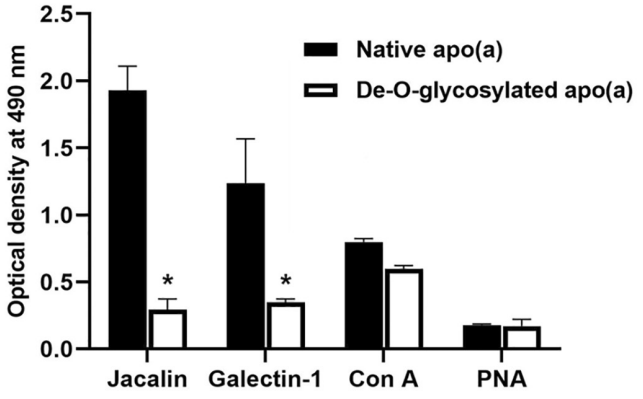

FIGURE 1 Glycan pattern in native and de-O-glycosylated apo(a): Native apo(a) and de-O-glycosylated apo(a) [DOA] were coated (5 μg protein/mL) on polystyrene wells. After blocking the unbound sites, wells were incubated with HRP-labeled jacalin (1.5 μg/mL), galectin-1 (10 μg/mL), con A (10 μg/mL), and PNA (5 μg/mL). Bound HRP was assayed as optical density at 490 nm using an ELISA. Values are mean ± SD of three independent trials. *Different ($p < .05$) from native apo(a).

the binding of apo(a) on HUVECs. From Figure S4, it is clear that after 6 h incubation, apo(a) binds to HUVECs and starts to enter into the cells. Using native and de-O-glycosylated apo(a), we demonstrated the anti-angiogenic potential of apo(a) in terms of its ability to modulate proliferative, migratory, and tube-forming capacity of HUVECs in endothelial cell growth medium supplemented with a pro-angiogenic factor, vascular endothelial growth factor (VEGF). The potential of apo(a)-linked O-glycans to modulate endothelial cell tube formation was investigated by observing the formation of capillary-like structures (CLS) on completely confluent HUVECs as well as the formation of tubular structures on the Matrigel matrix. Both CLS and Matrigel tubes are in vitro representations of the ability of endothelial cells to form blood vessel lumen.[42] Figure 2 shows that HUVECs upon incubation with native apo(a), lost their ability to proliferate and to migrate into a wounded area created on the HUVEC monolayer. Our result (Figure 2) also shows that incubation with native apo(a) significantly inhibits ($p < .05$) CLS formation and tube formation, while the proliferative, migratory, and tube forming ability of untreated and de-O-glycosylated apo(a)-treated HUVECs remained less or unchanged.

In addition, the de-O-glycosylated apo(a) prepared by the enzymatic degradation of native apo(a) by O-glycosidase (Cat.No:11347101001, Sigma-Aldrich, USA)[6] also failed to make any response on angiogenic processes (Figure S5B). To avoid any interference of enzyme remnants in the cellular assays, we preferred a chemical method for the removal of apo(a) O-glycans. Moreover, an earlier report showed the absence of any apo(a) fragmentation by the chemical methods employed for apo(a) isolation and removal of its O-glycan structures.[6,39] The smallest glycan unit recognized by galectin-1 is the β-galactoside residue.[9] Lactose (Gal β 1→4 Glu), a β-galactoside containing disaccharide is a simple carbohydrate ligand necessary for binding to galectin-1.[9] So, in all our in vitro experiments, we used lactose as a positive control. However, being a low-affinity ligand of galectin-1, high concentrations of lactose (50-150 mM) are needed to inhibit galectin-1.[43] Here, we have used 50 mM lactose to ensure maximum binding and inhibition of galectin-1. A significant inhibition ($p < .05$) on the proliferation, migration, and tube formation of HUVECs similar to that with native apo(a) was observed when HUVECs were incubated with 50 mM lactose (Figure 2). In addition, HUVECs were also treated with de-sialylated apo(a) [DSA], in which the terminal sialic acid group of apo(a) was removed, and we found inhibition in the proliferation of cells (Figure S6). All these results confirm the potential of apo(a) O-glycans

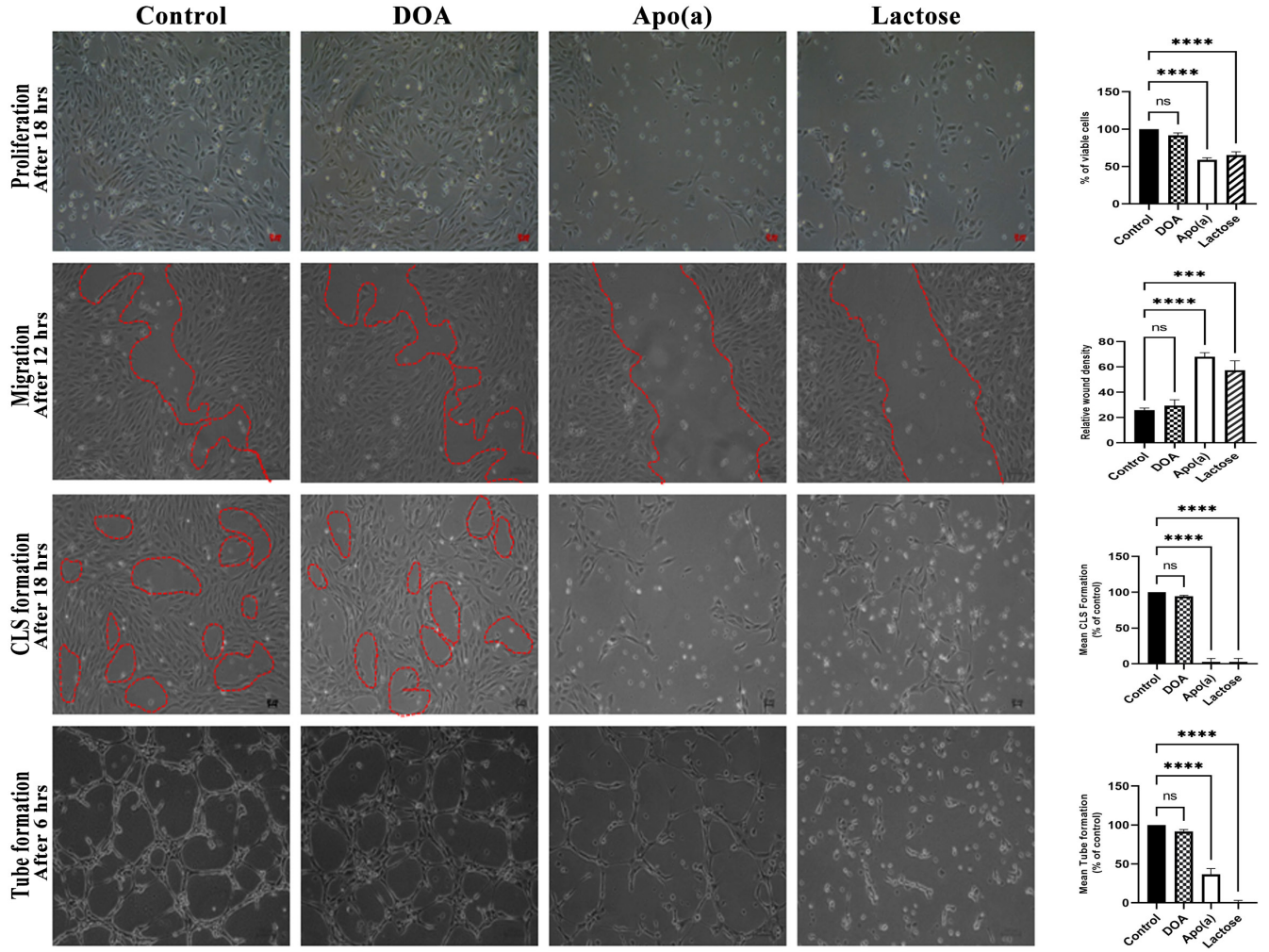

FIGURE 2 Effect of apo(a)-linked O-glycans on proliferation, migration, and tube formation of HUVECs: Cells were incubated with native, de-O-glycosylated apo(a) [DOA] and 50 mM lactose in EGM-2 at 37°C under 5% CO_2 and images were taken by an inverted microscope (Nikon Eclipse Ti, USA, magnification, ×10). The effect of apo(a) O-glycans on HUVEC proliferation, migration, and tube formation was demonstrated using MTT assay, wound-scratch assay, and CLS formation assay or Matrigel-tube formation assay respectively. The rate of cell migration was quantified after the indicated time intervals using image J software as relative wound density and expressed as a percentage. The number of CLS formed and the number of tubes formed were calculated by manual counting. Tube formation was scored as follows: A three-branch point event was defined as one tubular structure. Three random fields per well were quantified by manual counting and mean tube formation was expressed as a percentage of control. The control cultures were incubated in EGM-2 with PBS 7.4. Experiments were repeated in three different trials with duplicates. Values are mean ± SD of three independent trials. *Different ($p < .05$) from control.

to inhibit the proliferative, migratory, and tube-forming capacity of HUVECs.

3.3 | Apo(a)-linked O-glycans also have the potential to inhibit the in vivo neovascularization in chick chorioallantoic membrane

To demonstrate the in vivo anti-angiogenic potential of O-glycans of apo(a), we examined the effect of native apo(a) and de-O-glycosylated apo(a) on new blood vessel formation (neovascularization) in chick chorioallantoic membrane (CAM) as described previously.[40] The assays were conducted between the 8th day and 12th day of egg fertilization which is the period of maximum angiogenesis in CAM.[40] Figure 3 shows that the number of newly formed blood vessels (shown in the insert) in embryonic eggs treated with native apo(a) is significantly ($p < .05$) lesser than that of untreated or de-O-glycosylated apo(a)-treated eggs. This result suggests that apo(a) via its heavily O-glycosylated structures, significantly suppresses the formation and growth of new embryonic blood vessels.

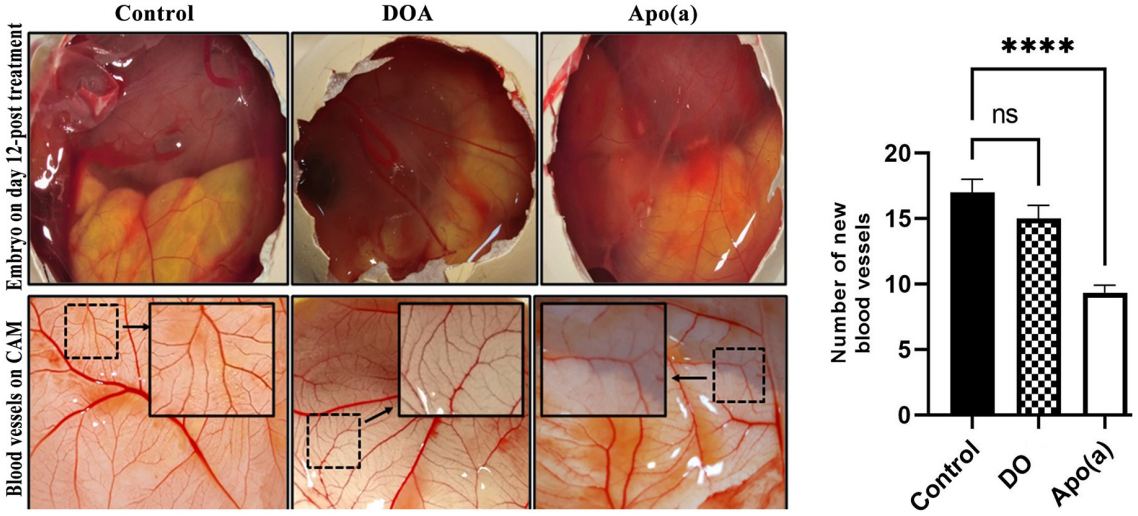

FIGURE 3 Effect of Apo(a)-linked O-glycans on new blood vessel formation on chick chorioallantoic membrane (CAM): A 20 μL volume (1 mg/mL) of native apo(a), de-O-glycosylated apo(a) [DOA] and PBS 7.4 was carefully injected separately on the CAM of eight days old fertilized hen eggs through a small incision made on the eggshell near the air sac and incubated at 40°C under humidified atmosphere. On day 12-post treatment, eggs were opened and images were taken using a digital camera. The anti-angiogenic effect of apo(a)-linked O-glycans was evaluated macroscopically by observing the number of new blood vessels formed in the CAM. Experiments were repeated in five different trials with duplicates. Values are mean ± SD of three independent trials. *Different ($p < .05$) from control.

3.4 | Apo(a)-linked O-Glycans reduce the pro-angiogenic activity of galectin-1 in HUVECs

Results so far revealed that apo(a)-linked O-glycans significantly suppress the angiogenic processes both in vitro and in vivo. Next, we investigated whether the O-glycan-mediated inhibitory potential of apo(a) in angiogenesis is mediated by inhibiting the pro-angiogenic effect of galectin-1. Confluent, serum-deprived, scratch-wounded HUVEC monolayer was treated with recombinant human galectin-1 (2 μg/mL) pre-incubated with native and de-O-glycosylated apo(a). From Figure 4Aa, it is clear that in response to galectin-1 stimulation, HUVECs migrated and covered up to ~80% of the wounded area. Similarly, galectin-1-mediated migration of HUVECs was less affected in the presence of de-O-glycosylated apo(a) resulting in ~80% closure of wounded HUVEC monolayer (Figure 4Ab). To rule out the possibility of galectin-1-mediated wound closure due to endothelial cell proliferation rather than migration, wound-scratch experiments were performed in the presence of mitomycin (5 μg/mL). Moreover, the anti-human galectin-1 antibody was used to neutralize the pro-angiogenic effect of galectin-1 prior to the addition of galectin-1 on the wounded HUVEC monolayer.

Figure 4Af shows that HUVECs failed to migrate and close the wounded area in response to galectin-1 pre-incubated with anti-human galectin-1 antibody ($p < .05$). This result indicates the complete neutralization of galectin-1 by anti-human galectin-1 antibody and thus verifies the pro-angiogenic effect of galectin-1 on HUVECs. Similar to the effect of galectin-1 neutralizing antibody, the wounded area remained unaffected by galectin-1 pre-incubated with native apo(a) (Figure 4Ac), suggesting the potential of apo(a)-linked O-glycans to inhibit the galectin-1-induced HUVEC migration. To further confirm the role of apo(a) O-glycans, especially Gal β 1→3 GalNAc structure in inhibiting galectin-1-stimulated HUVEC migration and wound closure, we have tested the migratory potential of HUVEC in the presence of galectin-1 pre-incubated with 50 mM lactose and also with heat-inactivated apo(a) [+H apo(a)]. Boiling of apo(a) in 150 mM PBS (pH 7.4) buffer at 90°C for 5 min denatures the protein structure of apo(a) with minimal or no effect on its O-glycosylated groups.[43] Hence, we found a significant ($p < .05$) inhibitory effect with lactose (Figure 4Ad) and heat-inactivated apo(a) (Figure 4Ae) on HUVEC migration similar to that observed with native apo(a). These results confirm that apo(a) O-glycans inhibit galectin-1-induced migration of HUVEC.

3.5 | O-glycans of apo(a) neither modulates MMP production nor its activity in HUVECs

The well-established regulatory function of matrix metalloproteinases (such as MMP-2 and MMP-9) in early phases of angiogenesis such as cell migration[44] prompted us to investigate whether the inhibitory potential of apo(a)-linked O-glycans on galectin-1-induced angiogenesis is

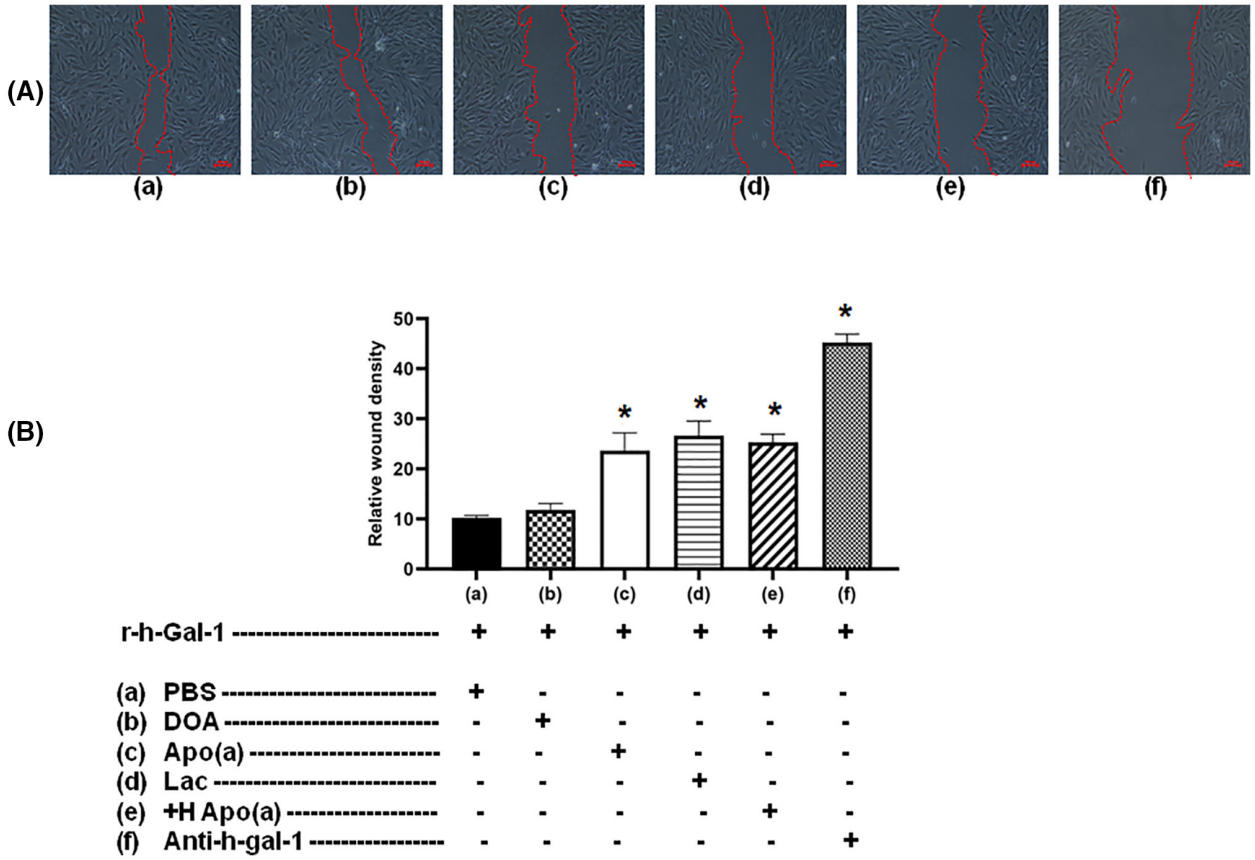

FIGURE 4 (A, B) Effect of apo(a)-linked O-glycans on the pro-angiogenic activity of galectin-1 in HUVECs: Fully confluent, serum-deprived, scratch-wounded HUVEC monolayer was treated with 2 μg/mL recombinant human galectin-1 protein pre-incubated with (a) PBS 7.4, (b) 10 μg/mL de-O-glycosylated apo(a) [DOA], (c) 10 μg/mL native apo(a), (d) 50 mM lactose, (e) 10 μg/mL heat-inactivated apo(a) [+H apo(a)], and (f) 1 μg/mL anti-human galectin-1 antibody. After an incubation period of 12 h at 37°C under 5% CO_2, images of the cells that migrated into the wounded area were photographed. Using image J software, the migration rate was quantified as relative wound density and expressed as a percentage. Experiments were repeated in three different trials with duplicates. Values are mean ± SD of three independent trials. *Different ($p < .05$) from PBS 7.4.

modulated by the production or activity of MMPs (MMP-2 and MMP-9) in HUVECs. Gelatin zymography was performed to determine the activity of gelatinase (MMP-2 and MMP-9) released into the HUVEC supernatant after treatment with apo(a), de-O-glycosylated apo(a), and 50 mM lactose. Our result shows that treatment of HUVEC monolayer with native apo(a) or de-O-glycosylated apo(a) made no significant difference in the release of MMP-2 and MMP-9 proteins into the medium (Figure 5A). Moreover, the gelatin-zymography image of HUVEC-supernatant collected after treatment with native and de-O-glycosylated apo(a) (Figure 5B) shows the inability of apo(a) to alter the matrix (gelatin)-degrading activity of MMPs. Similarly, the failure of lactose to make any change in the production or activity of MMPs in HUVECs (Figure 5A,B) confirms that not only the O-glycan structures but also the intact apo(a) protein subunit lack any significant role in modulating MMP activity and its production by endothelial cells. These results suggest that the inhibitory effect of apo(a)-linked O-glycans on angiogenesis is not due to a decrease in the production, release, or activation of MMPs. This observation is consistent with an earlier report[37] and suggests the less or no effect of apo(a) with or without O-glycans in inhibiting extracellular matrix remodeling during angiogenesis.

3.6 | Apo(a)-linked O-Glycan structures reduce the expression of galectin-1 protein with no effect on its mRNA

As apo(a) O-glycans could inhibit galectin-1-stimulated angiogenesis in HUVEC (Figure 4), our next objective was to investigate the underlying molecular mechanism of the inhibitory potential of apo(a) O-glycans. Interestingly, a recent study reported down-regulation of galectin-1 protein expression in pre-eclamptic placenta.[29] Moreover, Hirashima et al.[45] have demonstrated a lower serum concentration of galectin-1 in the second trimester as an independent risk factor for both gestational hypertension and

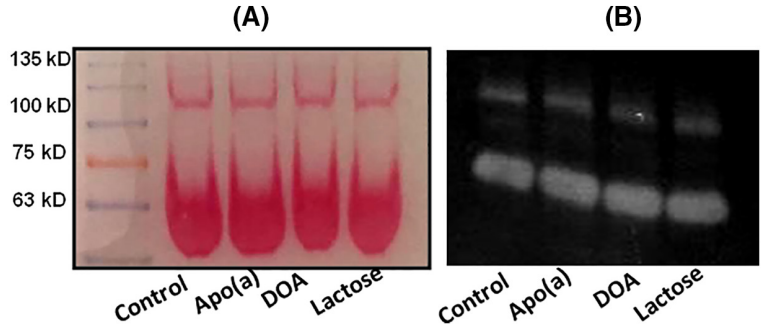

FIGURE 5 Effect of apo(a) O-glycans on MMP production and its activity: (A) Ponceau S staining of proteins transferred on the PVDF membrane from 7.5% SDS-PAGE of HUVEC-culture supernatants treated with or without apo(a). The image was taken using a digital camera; (B) Gelatin zymogram of 7.5% SDS-PAGE of HUVEC-supernatant performed under non-reducing conditions. After incubation in a substrate buffer at 37°C for 72 h, the gel was stained with Coommassie Brilliant Blue R-250 and subsequently destained. The cleared zone on the gelatin zymogram was visualized and captured by a Gel-Doc System (Bio-Rad, USA). Lane (a): HUVEC culture supernatant from non-treated cells, lane (b): HUVEC culture supernatant from native apo(a)-treated cells, lane (c): HUVEC culture supernatant from de-O-glycosylated apo(a) [DOA]-treated cells, and lane (d): HUVEC culture supernatant from cells treated with 50 mM lactose. The experiment was repeated in three different trials.

pre-eclampsia. But the underlying molecular mechanism in the reduced level of galectin-1 during pre-eclampsia is not yet elucidated. Many studies have reported that pre-eclamptic women possess a higher plasma Lp(a) level and placental Lp(a) deposits.[30–33] So, we propose that apo(a) O-glycans may possess some modulatory effect on the expression of galectin-1 protein and/or mRNA, which may further affect the galectin-1-mediated angiogenesis in HUVECs. Both immunoblotting and ELISA of HUVEC lysates collected after treatment with native apo(a) and lactose showed a decrease in galectin-1 protein level (Figure 6A–C). Immunofluorescence of HUVECs confirms that cellular expression of the galectin-1 protein is diminished upon treatment with native apo(a) and lactose (Figure 7). At the same time, galectin-1 mRNA level in HUVECs remained unchanged (Figure 6D). These results confirm an apo(a)-O-glycan-mediated inhibition of galectin-1 protein expression in HUVEC and suggest that apo(a) reduce the galectin-1 protein level.

3.7 | Apo(a) via its O-glycan structures competitively inhibit the carbohydrate-mediated binding of galectin-1 to its ligand neuropilin-1, expressed on HUVEC

Galectin-1-mediated angiogenic processes were initiated by the binding of its carbohydrate-recognition domain to neuropilin-1 (NRP-1), an O-glycosylated transmembrane protein expressed on endothelial cells.[20,25] Therefore, we hypothesize that being a heavily O-glycosylated protein and a potent ligand of galectin-1, apo(a) can competitively inhibit the carbohydrate-mediated binding between galectin-1 and NRP-1. We performed a far-western blot analysis for NRP-1 after immobilizing HUVEC-lysate proteins on the PVDF membrane (Figure 8A). Using anti-human NRP-1 antibody and HRP-labeled jacalin we confirmed that the antigenicity, as well as O-glycan structures of NRP-1 in the immobilized HUVEC lysate, is preserved (Figures 8Aa and S7). From Figure 8Bb, it is clear that after an incubation period of 2 h at 4°C, recombinant human galectin-1 binds to membrane-immobilized NRP-1. The significant decrease ($p < .05$) in the intensity of the galectin-1 band in the presence of native apo(a) (Figure 8Bd) and lactose (Figure 8Be) compared to that in the presence of de-O-glycosylated apo(a) (Figure 8Bc) indicates an apo(a)-O-glycan-mediated inhibition in the binding of the carbohydrate-binding domain of galectin-1 to immobilized neuropilin-1. To further prove the apo(a)-O-glycan-mediated interference in the galectin-1:NRP-1 binding, we performed immunoprecipitation of proteins collected from native apo(a)-treated and non-treated HUVEC cell lysates. Using rabbit anti-human NRP-1 antibody, the galectin-1:NRP-1 complex was precipitated from HUVEC lysates. The resulting immune complex [anti-human NRP-1-(galectin-1:NRP-1) complex] was then captured by protein A/G-agarose beads and subjected to immunoblotting (Figure 8C). Presence of both NRP-1 and galectin-1 bands in non-treated HUVEC lysate indicates the formation of galectin-1:NRP-1 complex, while the absence of a galectin-1 band in native apo(a)-treated HUVEC indicates the failure of galectin-1:NRP-1 complex formation in the presence of apo(a) (Figure 8C).

This result confirms that interaction between galectin-1 and NRP-1 is mediated by apo(a) through its O-glycan structures. Similar to the apo(a)-O-glycan induced reduction in the level of galectin-1 (Figure 6), levels in NRP-1 also reduced when cells were treated with native

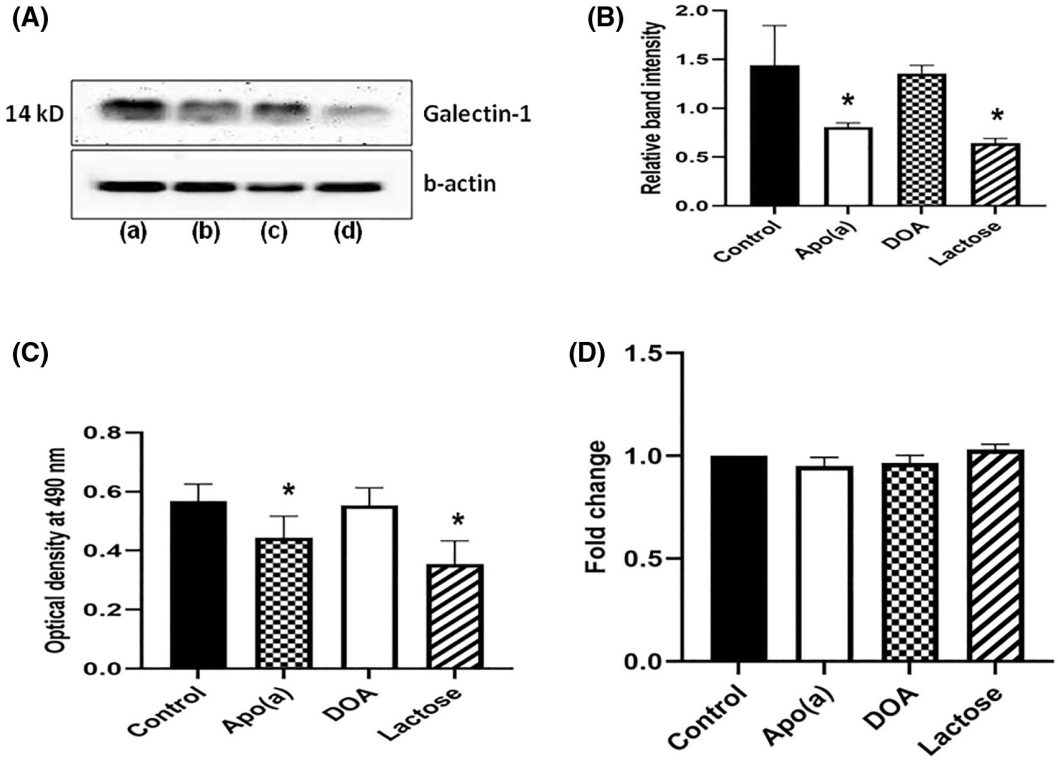

FIGURE 6 Effect of apo(a) O-glycans on galectin-1 protein and mRNA level: (A) In immunoblotting, HUVEC lysates (30 μg protein/well) were subjected to 12% SDS-PAGE. Afterward, western-blotting analyses were performed by, probing with rabbit anti-human galectin-1 antibody (1 μg/mL) followed by HRP-labelled anti-rabbit IgG antibody (1:10000). β-actin was used as an internal control. The bound HRP was detected using a chemiluminescent substrate. Images were taken with VersaDoc MP 4000 Molecular Imager. Lane (a): HUVEC lysate from non-treated cells, lane (b): HUVEC lysate from native apo(a)-treated cells, lane (c): HUVEC lysate from de-O-glycosylated apo(a) [DOA]-treated cells, and lane (d): HUVEC lysate from cells treated with 50 mM lactose. (B) Intensity of each band was quantitated using Quantity One software and expressed as relative band intensity. Values are mean ± SD of three independent trials. (C) For ELISA, after blocking the unbound sites, rabbit anti-human galectin-1 antibody-coated (5 μg/mL) polystyrene wells were incubated with 5 μg/mL HUVEC cell lysates for 2 h at 4°C. After washing the wells, the bound galectin-1 was detected by incubating the wells with rabbit anti-human galectin-1 antibody (5 μg/mL) followed by HRP-labelled anti-rabbit IgG antibody (1:1000). The amount of galectin-1 in cell-lysates was assayed by measuring the absorbance of bound HRP at 490 nm using an ELISA reader. Values are mean ± SD of six independent trials. (D) Effect of native, de-O-glycosylated apo(a) [DOA], and lactose on the galectin-1 mRNA level was detected by an RT-PCR. β-actin was used as an internal control. The experiment was repeated in three different trials. *Different ($p < .05$) from control.

apo(a) compared to that of non-treated cells (Figure 8C). Immunofluorescence analysis of HUVEC after treatment with native apo(a) and lactose further confirms a reduced expression of NRP-1 compared to that in non-treated and de-O-glycosylated apo(a)-treated HUVEC (Figure 9). Moreover, our RT-PCR result indicates that similar to galectin-1, apo(a) O-glycans have no effect on the mRNA level of NRP-1 (Figure S8).

3.8 | Apo(a)-linked O-Glycans inhibit galectin-1/neuropilin-1/VEGFR-2/MAPK-mediated angiogenic signaling pathway in HUVEC

Previous studies have demonstrated that the interaction of the carbohydrate-binding domain of galectin-1 with NRP-1 on endothelial cells promotes the VEGFR-2/MAPK-mediated angiogenic signaling.[20] VEGFR-2 is another O-glycoprotein on endothelial cells, which is essential for pregnancy-associated angiogenesis.[14,20] It gets activated after the carbohydrate-mediated binding of galectin-1 with NRP-1 and promotes the downstream MAPK pathway,[20] thereby promoting the adhesion and migration of endothelial cells.[14,20] Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are important downstream proteins in MAPK signaling, which regulates both endothelial cell migration and proliferation through the phosphorylation/de-phosphorylation mechanisms.[23] In this study, we tested whether the apo(a)-O-glycan-mediated inhibition of the binding of galectin-1 to NRP-1 diminishes the expression or the phosphorylation of VEGFR-2 and ERK1/2 in HUVEC. Immunoblotting of HUVEC lysates collected after treatment (Figure 10A) shows that both

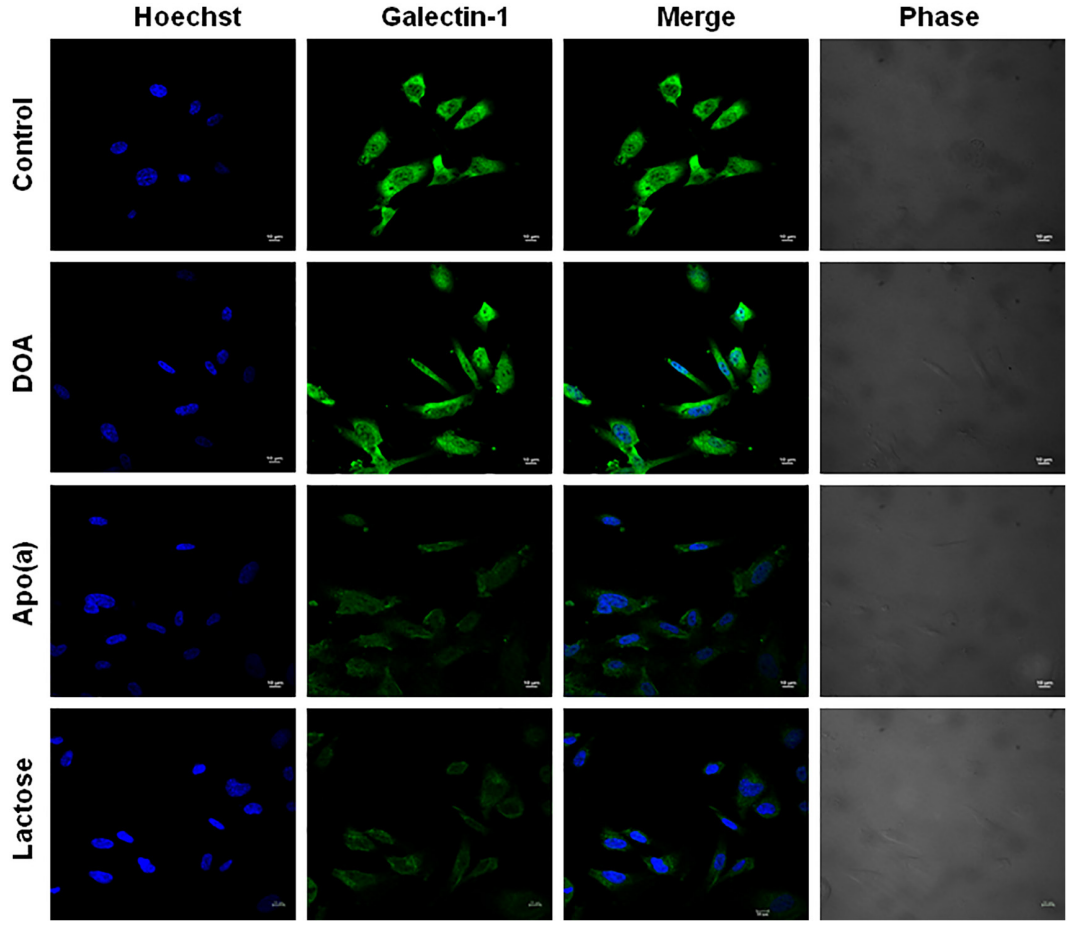

FIGURE 7 Immunofluorescence analysis of galectin-1 expression on HUVECs treated with and without apo(a): Starved HUVECs grown on sterile coverslips were treated with native apo(a), de-O-glycosylated apo(a) [DOA] and lactose. After 12 h incubation at 37°C, cells were fixed with p-formaldehyde and stained with rabbit anti-human galectin-1 antibody (diluted 1:100 in 3% BSA in HBBS), followed by fluorescence-labeled anti-rabbit IgG antibody (diluted 1:1000 in 3% BSA in HBBS). Fluorescence was captured using an inverted confocal fluorescence microscopy. The blue color indicates Hoechst staining of the nucleus and the green color indicates Alexa 488 staining of galectin-1. Experiments were repeated in three different trials with duplicates.

native apo(a) and lactose hinder the expression as well as the phosphorylation of VEGFR-2 and ERK2 (Figure 10B–E). Even though the protein expression of ERK1 is not affected much, its phosphorylation is significantly reduced ($p < .05$) in the presence of native apo(a) and 50 mM lactose. To verify the inhibitory action of apo(a) O-glycan structures on the MAPK pathway, we went on to look at the expression of galectin-1, NRP-1, VEGFR-2, ERK1, and ERK2 as well as the phosphorylation status of VEGFR-2, ERK1, and ERK2 in HUVEC lysates collected after treatment of cells with heat-inactivated apo(a) [+H apo(a)] and de-sialylated apo(a) [DSA] (Figure 10A).

Figure 10 shows that similar to the effect of native apo(a), both heat-inactivated and de-sialylated apo(a) significantly downregulates ($p < .05$) the expression of galectin-1, ERK2, and VEGFR-2, without affecting the expression of ERK1. Moreover, the phosphorylation of ERK1, ERK2, and VEGFR-2 is also significantly decreased ($p < .05$) by heat-inactivated and de-sialylated apo(a). But de-sialylated apo(a) failed to make any change in the expression of NRP-1 (Figure 10C,E). Our data confirm that apo(a) via its O-glycan structures inhibits the expression and activation of downstream proteins in the MAPK signaling pathway.

4 | DISCUSSION

Galectin-1 is an O-glycan-binding animal lectin ubiquitously expressed in human reproductive tracts, placenta, and developing embryo,[14–18] where it interacts with the specific cell-surface glycoconjugates via its specific evolutionarily conserved carbohydrate-binding domain[14] and regulates each and every stages of pregnancy such as implantation, fetomaternal tolerance, spiral artery remodeling, angiogenesis, and placentation.[17,18,25] In vivo administration of agents which mimic its glycan ligand or its carbohydrate-binding domain blocks galectin-1-mediated

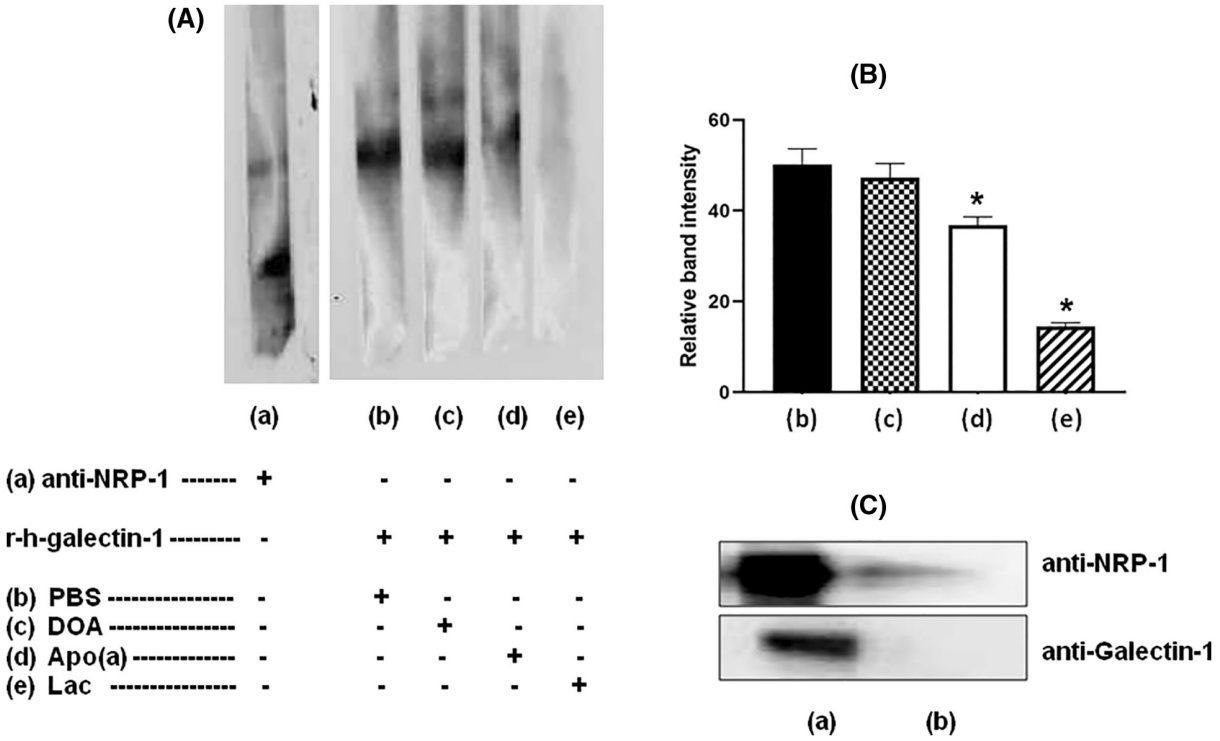

FIGURE 8 Apo(a) via its O-glycan structure competitively inhibits the interaction between galectin-1 and neuropilin-1 (A) Far-Western blotting: Proteins collected from untreated HUVEC lysates (2 mg/mL) were subjected to 5% SDS-PAGE and resolved proteins were transferred on PVDF membrane. The membrane was cut into five small strips. (a) One strip was incubated with rabbit anti-human NRP-1 antibody (1 μg/mL) followed by HRP-labeled anti-rabbit IgG antibody (1:10000). The remaining four PVDF strips were separately incubated in a solution of 2 μg/mL of recombinant human galectin-1 pre-treated with (b) PBS 7.4, (c) de-O-glycosylated apo(a) [DOA] (10 μg/mL), (d) native apo(a) (10 μg/mL), and (e) 50 mM lactose. Galectin-1 bound on PVDF-immobilized NRP-1 was detected by incubating each strip separately in rabbit anti-human galectin-1 antibody (1 μg/mL) followed by HRP-labeled anti-rabbit IgG antibody (1:10000). The bound HRP on PVDF was detected using a chemiluminescent western blot hyper HRP substrate and images were taken with VersaDoc MP 4000 Molecular Imager. (B) Intensity of each band was quantitated using Quantity One software and expressed as relative band intensity. Values are mean ± SD of three independent trials. (C) Immunoprecipitation: Proteins collected from HUVEC lysates after treatment with and without native apo(a) were incubated with rabbit anti-human NRP-1 antibody. The resulting immune complexes formed (NRP-1:anti-NRP-1 complex) were captured on protein A/G-Agarose beads. After washing, the beads were subjected to 5% SDS-PAGE and separated proteins were transferred on PVDF membrane and probed separately with rabbit-anti-human NRP-1 antibody (1 μg/mL) and rabbit-anti-human galectin-1 antibody (1 μg/mL) respectively. This is followed by the incubation with HRP-labeled anti-rabbit IgG antibody (1:10000). The bound HRP was detected as described above. Lane (a): HUVEC supernatant from non-treated cells, lane (b): HUVEC supernatant from native apo(a)-treated cells. Experiments were repeated in three different trials. *Different ($p < .05$) from control.

placental angiogenesis and leads to pre-eclampsia in mice.[25] The core-1 type O-glycan containing Gal β 1→3 GalNAc (T antigen) structure with or without sialic acid substitution is one of the most efficient ligands for galectin-1.[6] Interestingly, the Gal β 1→3 GalNAc structure is a hidden oncofetal antigen, ie, proteins that are typically present only during fetal development and certain kinds of cancer.[46] In the placenta, the interaction between the disaccharide structure, Gal β 1→3 GalNAc, and galectin-1 plays a vital regulatory role.[47] Hence, the observation of increased deposits of an O-glycan-rich Lp(a), a strong affinity ligand of placental galectin-1,[6] in the pre-eclamptic placenta needs special attention.

In this study, our first aim was to demonstrate the O-glycan-mediated anti-angiogenic potential of apo(a) subunit of Lp(a). For this purpose, native apo(a) was isolated from human plasma and its O-glycan structures were removed chemically without affecting its protein structure.[6,39] Similar to the previous reports,[3,6,8] our study also revealed that apo(a) possesses both O-and N-glycan structures in sialylated form. (Figure 1). Using native and de-O-glycosylated apo(a), we demonstrated the O-glycan-mediated inhibitory effect of apo(a) on proliferation, migration, and tube formation in HUVECs (Figure 2). In addition, HUVEC proliferation was also inhibited in the presence of de-sialylated apo(a) (Figure S6). Conversely, an earlier study found that de-sialylated recombinant apo(a) enhances the angiogenic response of endothelial cells.[37] This discrepancy in the result may be due to the variation in the glycan structure of apo(a) used or the

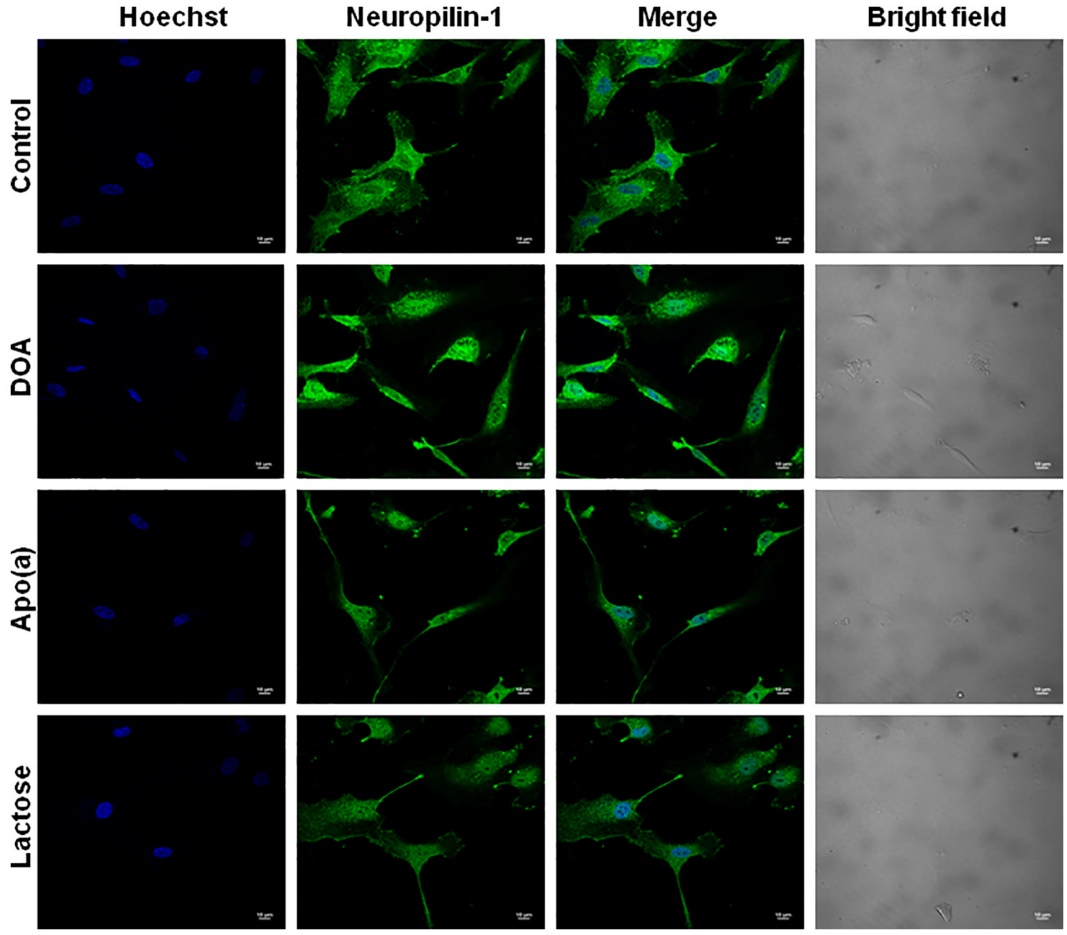

FIGURE 9 Confocal imaging to confirm the apo(a)-mediated reduction in the expression of neuropilin-1: Starved HUVECs grown on sterile coverslips were treated with native apo(a), de-O-glycosylated apo(a) [DOA] and lactose. After incubation for 12 h at 37°C, cells were fixed with p-formaldehyde and stained with rabbit anti-human NRP-1 antibody (diluted 1:100 in 3% BSA in HBBS), followed by fluorescence-labeled anti-rabbit IgG antibody (diluted 1:1000 in 3% BSA in HBBS). Fluorescence was captured using inverted confocal fluorescence microscopy. The blue color indicates Hoechst staining of the nucleus and the green color indicates Alexa 488 staining of NRP-1. The experiment was repeated in three different trials with duplicates.

method employed to remove the sialic acid group from apo(a). Lactose is one of the most used and relatively small β-galactoside-containing disaccharides to inhibit the lectin activity of galectin-1.[14,48] In this study, we have used 50 mM lactose to inhibit the glycan-mediated binding of galectin-1. Furthermore, a reduced count and intensity of newly formed blood vessels in chick chorioallantoic membrane was observed after the treatment of eight days old fertilized hen eggs with apo(a) and its subsequent incubation till the 12th day (Figure 3). In contrast, a much lesser effect was found in CAM treated with de-O-glycosylated apo(a) and PBS 7.4. This result clearly shows the involvement of apo(a)-linked O-glycans in neovascularization and so in angiogenesis. Altogether, our results suggest a glycan-mediated inhibitory potential of apo(a) in various stages of angiogenic processes.

Previous studies have demonstrated a dose-dependent, glycan-mediated stimulatory effect of galectin-1 on HUVEC proliferation and migration.[20] As apo(a)-linked O-glycans in Lp(a) are experimentally proven as strong ligands of galectin-1,[6–8] we investigated its ability to abolish the pro-angiogenic potential of exogenously added galectin-1. In Figure 4, a significant inhibitory effect of native apo(a), lactose, and heat-inactivated apo(a) on galectin-1-mediated HUVEC migration, and tube formation was observed. The endothelial cell culture media (EGM-2, Lonza) used in our study is supplemented with another pro-angiogenic protein, vascular endothelial growth factor (VEGF). The presence of VEGF in cell culture medium stimulates the angiogenic processes of endothelial cells in 2-dimensional culture dishes as well as in a 3-dimensional Matrigel matrix as previously described.[20] Interestingly, the presence of both galectin-1 and VEGF in cell-culture media enhances cell migration in the wounded area as well as the formation of capillary-like tubular structures (Figure 4). This observation is

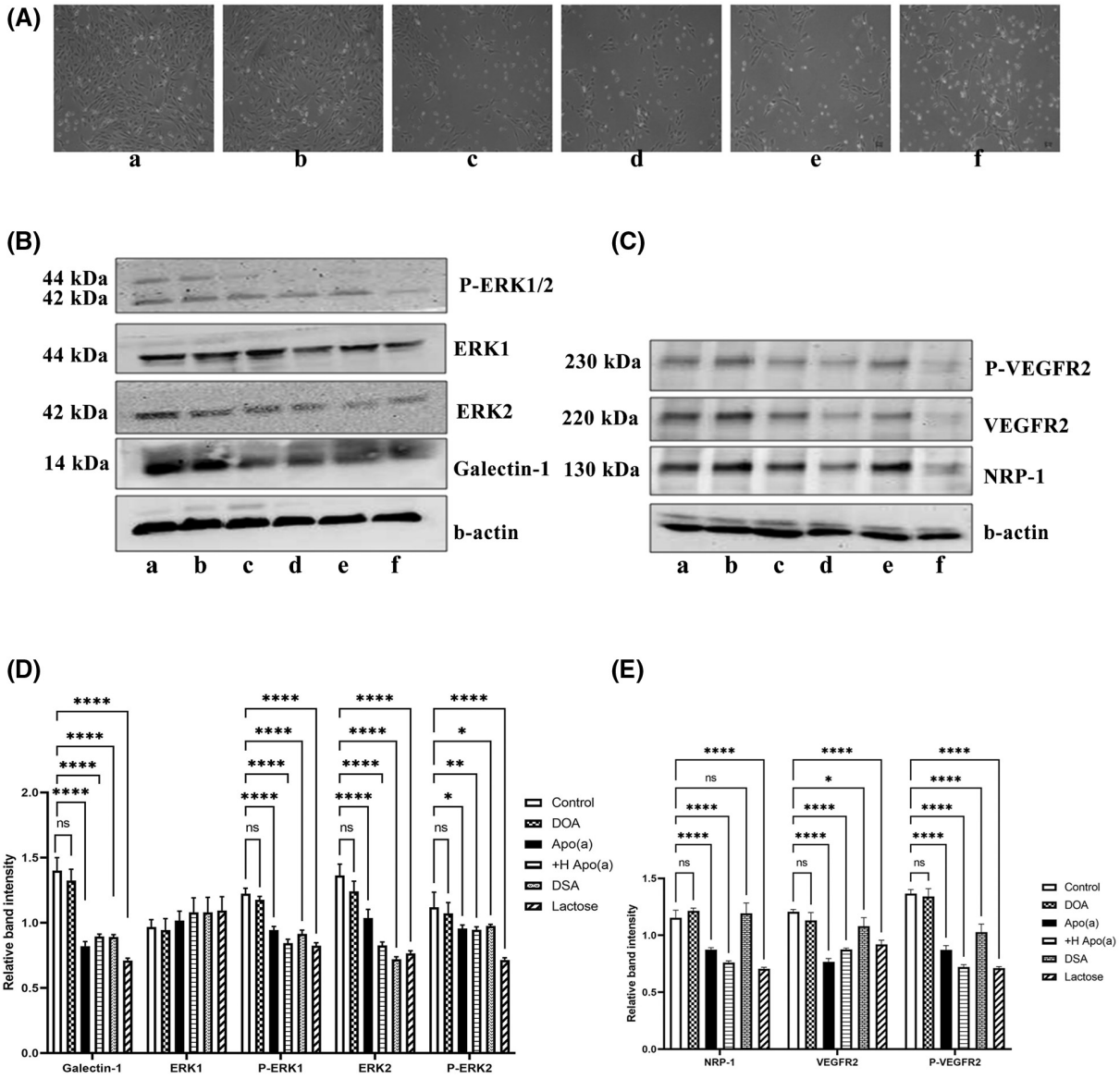

FIGURE 10 Effect of apo(a) O-glycans on the expression of downstream proteins in MAPK pathway (A) HUVECs were treated with (a) PBS 7.4, (b) de-O-glycosylated apo(a) [DOA], (c) native apo(a), (d) heat-inactivated apo(a) [+H apo(a)], (e) de-sialylated apo(a) [DSA], and (f) 50 mM lactose and images were taken as described above. HUVEC lysates (30 μg protein/well) collected after treatment were subjected to SDS-PAGE. (B) 12% gel for galectin-1, ERK1/2 and phospho-ERK1/2, and (C) 5% gel for NRP-1, VEGFR2 and phospho-VEGFR2. After western-blotting of proteins from the PVDF membrane were blocked and incubated separately with rabbit anti-human galectin-1, ERK1/2, phospho-ERK1/2, NRP-1, VEGFR2, and phospho-VEGFR2, followed by HRP-labeled anti-rabbit IgG antibody. The bound HRP was detected using a chemiluminescent substrate. Images were taken with VersaDoc MP 4000 Molecular Imager. β-actin was used as an internal control. Lane (a): HUVEC lysate from PBS-treated cells, lane (b): HUVEC lysate from de-O-glycosylated apo(a) [DOA]-treated cells, lane (c): HUVEC lysate from apo(a)-treated cells, lane (d): HUVEC lysate from heat-inactivated apo(a) [+H apo(a)]-treated cells, lane (e): HUVEC lysate from de-sialylated apo(a) [DSA]-treated cells, and lane (f): HUVEC lysate from cells treated with 50 mM lactose. (D, E) Intensity of each band was quantitated using Quantity One software and expressed as relative band intensity. The experiment was repeated in three different trials. Values are mean ± SD of three independent trials. *Different ($p < .05$) from control.

consistent with an earlier study, which described the resultant angiogenic effect of both galectin-1 and VEGF in endothelial cells as an additive one and not due to a synergistic interaction.[20]

We also evaluated the underlying molecular mechanism of the inhibitory potential of apo(a)-linked O-glycans on galectin-1-induced angiogenesis. First, we analyzed whether the anti-angiogenic activity of apo(a)-linked O-glycans is modulated by MMPs. MMPs are a group of specialized proteinases involved in the regulation of extracellular matrix (ECM) remodeling, an early phase of angiogenesis.[44] Similar to a previous study,[37] we found

that apo(a) via its O-glycan structures failed to make any change in MMP release or its activity (Figure 5).

Our immunoblotting and immunofluorescence results indicate that apo(a)-linked O-glycans of Lp(a) reduce the protein levels of galectin-1, but not the mRNA levels in apo(a)-treated HUVECs (Figures 6 and 7). Therefore, we propose that the mechanism of apo(a)-mediated reduction in the galectin-1 protein levels occurred may either by inhibiting the galectin-1 translation machinery or by inducing the proteasome machinery leading to the degradation of the galectin-1 protein released into the extracellular matrix medium. The possibility of an apo(a)-mediated induction of proteasome machinery in the reduction of galectin-1 protein levels sounds plausible because an earlier report demonstrates the binding of Lp(a) to the proteasome machinery following an endocytosis and the comparatively protease-resistant apo(a) subunit of Lp(a) recycled back to the extracellular medium.[49] However, more studies are required to confirm our proposition.

Next, our far-western blotting and immunoprecipitation experiments (Figure 8) clearly show that native apo(a) via its O-glycan structures prevents galectin-1 from interacting with NRP-1, an O-glycosylated transmembrane protein involved in a number of signaling pathways mediated by galectin-1.[20,50] We preferred far-western blot analysis to show apo(a) as a competitive inhibitor of NRP-1 in galectin-1 binding because it is a rapid and highly reproducible experimental approach for identifying and understanding the interaction that occurs between natively, structured proteins.[51] Moreover, compared with other biochemical binding assays, far-western blotting does not need any purification or modification of proteins.[51] Other biochemical approaches such as Surface Plasmon Resonance (SPR) and phase solid assays are expensive, time-consuming, and require a good quantity of purified proteins (apo(a), neuropilin-1, and galectin-1). In addition, the commercially available purified (mostly recombinant) proteins lack intact O-glycan structures. Here, we have immobilized the native neuropilin-1 expressed in HUVEC on the PVDF membrane by performing a western blot of HUVEC-lysate. Then, we checked the inhibitory potential of apo(a) in galectin-1-binding toward immobilized neuropilin-1. Jacalin binding toward immobilized NRP-1 confirms the presence of O-glycan structures in immobilized NRP-1 (Figure S7). NRP-1 contains 24 monosaccharide units in the form of sialylated core 1 and core 2 O-glycan structures,[52] while each interkringle region of apo(a) contains 5 O-glycan structures.[3] A single apo(a) subunit possess 10 different kringle IV types and so 9 interkringle regions. Moreover, according to the molecular and peptide analysis studies, in the human population, the number of kringle IV type 2 domains of apo(a) varies from 2 to 42, and two different apo(a) isoforms are present in a single individual.[2,4,5] Hence, the smallest apo(a) possesses 50 O-glycan structures with a predominance of core 1 O-glycan structures. Further, as the number of apo(a) kringle IV type 2 domain increases, the number of O-glycan structures also increases,[4,5] thereby making apo(a) superior to NRP-1 for galectin-1 binding. So, it is obvious that apo(a) could competitively inhibit the carbohydrate-mediated binding between galectin-1 and NRP-1 via its heavily enriched O-glycan structures and our results confirm this notion (Figure 8). Furthermore, just like in galectin-1, the protein level of NRP-1 is also reduced in HUVECs treated with native apo(a) (Figures 9 and S7). Here also, we propose the involvement of an apo(a)-mediated proteasome machinery in the degradation of NRP-1 protein which should also be verified in the future.

Finally, our result also indicates that native apo(a) and lactose hinder VEGFR-2/MAPK-mediated signaling by inhibiting the expression as well as the phosphorylation of VEGFR-2 and ERK1/2 (Figure 10). Collectively, all these results confirm that via the heavily O-glycosylated apo(a) subunit, Lp(a) inhibit galectin-1/NRP-1/VEGR2/MAPK-mediated angiogenic processes in endothelial cells such as proliferation, migration, and tube formation as well as neovascularization in chick chorioallantoic membrane with no or minimal effect on MMPs. The anti-angiogenic effect of Lp(a) without affecting MMPs and so on ECM remodeling may be the possible reason for the impaired angiogenesis in lipid [Lp(a)]-laden vascular tissues but requires more studies for confirmation.

5 | CONCLUSION

Previous studies show that the anti-angiogenic potential of apo(a) lies in its kringle domains, which are structurally similar to that of plasminogen and angiostatin.[2,22–24] A significant role of heavily O-glycosylated interkringle regions of apo(a) in angiogenesis remained unnoticed in those studies because of the absence or variation in the intact glycan structure in the apo(a)/Lp(a) sample they have used.[35–37] However, a couple of studies have found that a large number of kringle IV type 2 repeats (which are enriched with O-glycans) are necessary for the anti-angiogenic activity of apo(a).[53,54] An in vitro study found that after an enzymatic glycosylation modification by sialidase, recombinant apo(a) was less effective in exerting an anti-angiogenic effect on HUVEC.[37] In addition, a few studies have exploited the anti-angiogenic potential of apo(a) kringle domains and demonstrated it as a novel therapeutic agent to inhibit angiogenesis-dependent cancer progression.[55] In our study, we have demonstrated for the first time an inhibitory potential of O-glycan structures of apo(a) subunit of Lp(a) in galectin-1-stimulated angiogenesis. From our results, we conclude

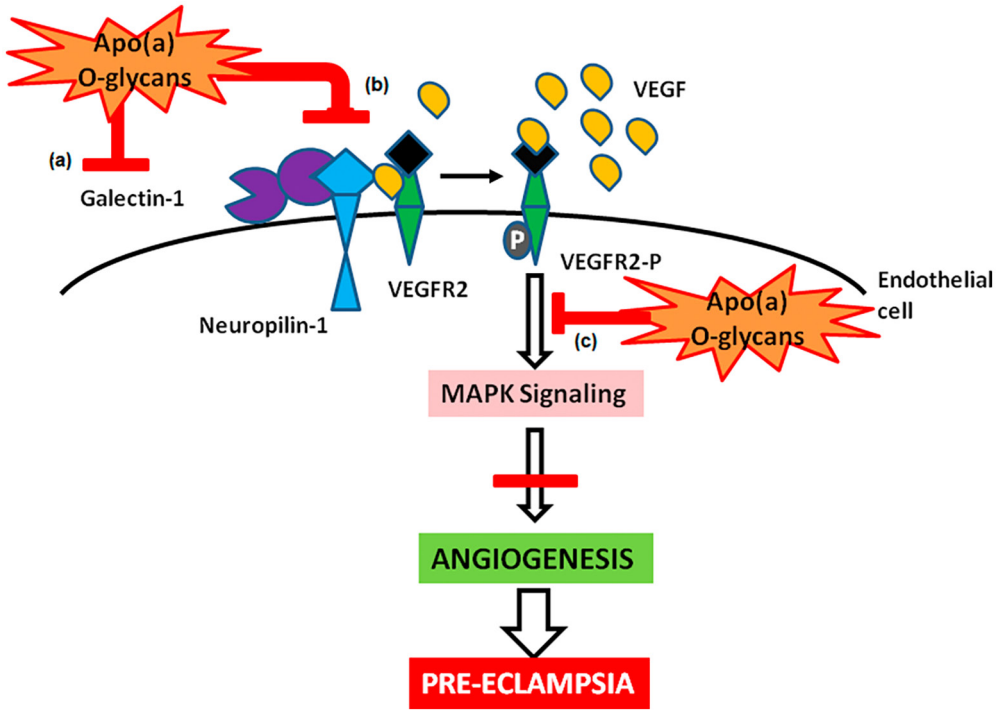

FIGURE 11 Schematic representation of the inhibitory potential of apo(a) O-glycans in galectin-1-mediated angiogenesis: O-glycan structures of apo(a) subunit of Lp(a) inhibit galectin-1/NRP-1/VEGFR-2/MAPK-mediated angiogenic signaling pathway by (a) inhibiting the expression of galectin-1, (b) competitively inhibiting the interaction between galectin-1 and neuropilin-1, and (c) inhibiting the phosphorylation of down-stream signaling proteins in MAPK pathway.

that apo(a)-linked O-glycan-mediated inhibition of (a) galectin-1 and NRP-1 protein expression, (b) the interaction of galectin-1 with NRP-1, and (c) the expression and phosphorylation of MAPK proteins are the underlying molecular mechanisms of the anti-angiogenic potential of Lp(a) in HUVECs (Figure 11). Our observations point toward the possibility that in women with higher plasma Lp(a) level, circulating Lp(a) can bind, and immobilized on vascular tissues enriched with galectin-1 such as placenta and interfere galectin-1-mediated angiogenesis via its heavily O-glycosylated apo(a) protein subunit.

AUTHOR CONTRIBUTIONS

Vasantha Kalaivani and Abdul Jaleel designed the experiments; Vasantha Kalaivani, Mahesh S. Krishna, Asokan Aneesh Kumar, and Gopika Satheesh performed the experiments; Vasantha Kalaivani and Abdul Jaleel wrote the manuscript.

ACKNOWLEDGMENTS

We would like to thank Dr. T. R. Santhosh Kumar and the facility staff, Central Cell and Tissue Repository of Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India for providing HUVECs. We also thank Dr. P.S. Appukuttan, Senior Grade Professor & Former Head of the Department of Biochemistry, Sree Chitra Tirunal Institute for Science and Technology (SCTIMST), Thiruvananthapuram, Kerala, India for providing lectins such as jacalin, peanut agglutinin (PNA), and concanavalin A (Con A). We thank Mr. Rajasekharan for his help in performing ultracentrifugation. We also thank Mrs. Bindu Asokan, Mrs. Jiji, and Mr. Gopikrishnan for their help in doing confocal microscopy.

FUNDING INFORMATION

We thank Science & Engineering Research Board (SERB) National Post-Doctoral Fellowship (NPDF) Scheme, (Fellowship No: PDF/2016/002807), New Delhi, India, and the Intramural Financial Support of Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India.

DISCLOSURES

The authors declare that they have no conflict of interest/competing interests.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Out-dated human plasma was collected from the Blood bank, Ananthapuri Hospitals and Research Institute,

Thiruvananthapuram, Kerala, India after getting approval (IHEC/1/2019/02) from Institute Human Ethical Committee.

ORCID

Vasantha Kalaivani https://orcid.org/0000-0002-5578-5744

REFERENCES

1. Lawn RM, Schwartz K, Patthy L. Convergent evolution of apolipoprotein(a) in primates and hedgehog. *Proc Natl Acad Sci U S A*. 1997;94:11992-11997. doi:10.1073/pnas.94.22.11992
2. McLean JW, Tomlinson JE, Kuang WJ, et al. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature*. 1987;330:132-137. doi:10.1038/330132a0
3. Garner B, Merry AH, Royle L, Harvey DJ, Rudd PM, Thillet J. Structural elucidation of the N- and O-glycans of human apolipoprotein(a): role of o-glycans in conferring protease resistance. *J Biol Chem*. 2001;276:22200-22208. doi:10.1074/jbc.M102150200
4. Marcovina S, Zhang Z, Gaur V. Identification of 34 apolipoprotein(a) isoforms: differential expression of apolipoprotein (a) alleles between American blacks and whites. *Biochem Biophys Res Commun*. 1993;191:1192-1196. doi:10.1006/bbrc.1993.1343
5. Utermann G, Menze HJ, Kraft HG, Duba HC, Kemmler HG, Seitz C. Lp(a) glycoprotein phenotypes-inheritance and relation to Lp(a)-lipoprotein concentrations in plasma. *J Clin Invest*. 1987;80(2):458-465. doi: 10.1172/JCI113093
6. Chellan B, Narayani J, Appukuttan PS. Galectin-1, an endogenous lectin produced by arterial cells, binds lipoprotein(a) [Lp(a)] in situ: relevance to atherogenesis. *Exp Mol Pathol*. 2007;83:399-404. doi:10.1016/j.yexmp.2007.04.004
7. Kalaivani V, Appukuttan PS. Circulating Lp(a):LDL complexes contain LDL molecules proportionate to Lp(a) size and bind to galectin-1: a possible route for LDL entry into cells. *Lipids*. 2014;49:1101-1113. doi:10.1007/s11745-014-3941-4
8. Kalaivani V, Appukuttan PS. Plasma lipoprotein(a) size polymorphism and function: apo(a) subunit size determines galectin-1 recognition and apoB subunit epitope masking. *Open J Biochem*. 2015;2:1-12. doi:10.15764/BIOC.2015.01003
9. Dings RPM, Miller MC, Griffin RJ, Mayo KH. Galectins as molecular targets for therapeutic intervention. *Int J Mol Sci*. 2018;19:905. doi:10.3390/ijms19030905
10. Isabelle C, Marie LM, Florence L, Robert K. Galectin-1: a small protein with major functions. *Glycobiology*. 2006;16(11):137R-157R. doi:10.1093/glycob/cwl025
11. Baum LG, Seilhamer JJ, Pang M, Levine WB, Beynon D, Berliner JA. Synthesis of an endogeneous lectin, galectin-1, by human endothelial cells is up-regulated by endothelial cell activation. *Glycoconj J*. 1995;12:63-68. doi: 10.1007/BF00731870
12. Thijssen VL, Hulsmans S, Griffioen AW. The galectin profile of the endothelium: altered expression and localization in activated and tumor endothelial cells. *Am J Pathol*. 2008;172(2):545-553. doi:10.2353/ajpath.2008.070938
13. Sangeetha SR, Appukuttan PS. IgA1 is the premier serum glycoprotein recognized by human galectin-1 since T antigen (Gal1→3GalNAc-) is far superior to non-repeating N-acetyl lactosamine as ligand. *Int J Biol Macromol*. 2005;35:269-276. doi:10.1016/j.ijbiomac.2005.03.004
14. Barrientos G, Freitag N, Tirado-Gonzalez I, et al. Involvement of galectin-1 in reproduction: past, present and future. *Hum Repro Update*. 2014;20(2):175-193. doi:10.1093/humupd/dmt040
15. Fischer I, Weber M, Kuhn C, et al. Is galectin-1 a trigger for trophoblast cell fusion?: the MAP-kinase pathway and syncytium formation in trophoblast tumour cells BeWo. *Mol Hum Repro*. 2011;17(12):747-757. doi:10.1093/molehr/gar053
16. Kolundzic N, Bojić-Trbojević Z, Kovačević T, Stefanoska I, Kadoya T, Vićovac L. Galectin-1 is part of human trophoblast invasion machinery—a functional study in vitro. *PloS One*. 2011;6(12):e28514. doi:10.1371/journal.pone.0028514
17. Tirado-Gonzalez I, Freitag N, Barrientos G, et al. Galectin-1 influences trophoblast immune evasion and emerges as a predictive factor for the outcome of pregnancy. *Mol Hum Reprod*. 2013;19(1):43-53. doi:10.1093/molehr/gas043
18. Blois SM, Ilarregui JM, Tometten M, et al. A pivotal role for galectin-1 in fetomaternal tolerance. *Nat Med*. 2007;13(12):1450-1457. doi:10.1038/nm1680
19. Than NG, Romero R, Kim CJ, McGowen MR, Papp Z, Wildman DE. Galectins: guardians of eutherian pregnancy at the maternalfetal interface. *Trends Endocrinol Metab*. 2012;23:23-31. doi:10.1016/j.tem.2011.09.003
20. Hsieh SH, Ying NW, Wu MH, et al. Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene*. 2008;27:3746-3753. doi:10.1038/sj.onc.1211029
21. Masahiro S, Kazuhisa H, Junko N, Kiyoshi O. The luteotrophic function of galectin-1 by binding to the glycans on vascular endothelial growth factor receptor-2 in bovine luteal cells. *J Reprod Dev*. 2015;61(5):439-448. doi:10.1262/jrd.2015-056
22. Baston-Buest DM, Porn AC, Schanz A, Kruessel JS, Janni W, Hess AP. Expression of the vascular endothelial growth factor receptor neuropilin-1 at the human embryo-maternal interface. *Eur J Obstet Gynecol Reprod Biol*. 2011;154:151-156. doi:10.1016/j.ejogrb.2010.10.018
23. Sun Y, Liu WZ, Liu T, Feng X, Yang N, Zhou HF. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J Recept Signal Transduct Res*. 2015;35:600-604. doi:10.3109/10799893.2015.1030412
24. Griffioen AW, van der Schaft DW, Barendsz-Janson AF, et al. Anginex, a designed peptide that inhibits angiogenesis. *Biochem J*. 2001;354(Pt 2):233-242. doi:10.1042/0264-6021:3540233
25. Freitag N, Tirado-González I, Barrientos G, et al. Interfering with Gal-1–mediated angiogenesis contributes to the pathogenesis of preeclampsia. *Proc Natl Acad Sci U S A*. 2013;110(28):11451-11456. doi:10.1073/pnas.1303707110
26. Molvarec A, Blois SM, Stenczer B, et al. Peripheral blood galectin-1-expressing T and natural killer cells in normal pregnancy and preeclampsia. *Clin Immunol*. 2011;139:48-56. doi:10.1016/j.clim.2010.12.018
27. Agarwal I, Karumanchi SA. Preeclampsia and the antiangiogenic state. *Pregnancy Hypertens*. 2011;1:17-21. doi:10.1016/j.preghy.2010.10.007
28. Nelson DB, Ziadie MS, McIntire DD, Rogers BB, Leveno KJ. Placental pathology suggesting that preeclampsia is more than one disease. *Am J Obstet Gynecol*. 2014;210:66.e1-66.e7. doi:10.1016/j.ajog.2013.09.010

29. Alese OM, Moodley J, Naicker T. The role of galectin-1 in HIV associated preeclampsia. *Eur J Obstet Gynecol Reprod Biol*. 2020;246:138-144. doi:10.1016/j.ejogrb.2020.01.024
30. Manten GTR, Van Der Hoek YY, Marko Sikkema J, et al. The role of lipoprotein(a) in pregnancies complicated by pre-eclampsia. *Med Hypotheses*. 2005;64:162-169. doi:10.1016/j.mehy.2004.04.026
31. Parvin S, Samsuddin L, Ali A, Chowdhury SA, Siddique I. Lipoprotein(a) level in pre-eclampsia patients. *Bangladesh Med Res Counc Bull*. 2010;36:97-99. doi:10.3329/bmrcb.v36i3.7289
32. Konrad E, Güralp O, Shaalan W, et al. Correlation of elevated levels of lipoprotein(a), high-density lipoprotein and low-density lipoprotein with severity of preeclampsia: a prospective longitudinal study. *J Obstet Gynaecol*. 2020;40(1):53-58. doi:10.1080/01443615.2019.1603214
33. Meekins JW, Pijnenborg R, Hanssens M, van Assche A, McFadyen IR. Immunohistochemical detection of lipoprotein(a) in the wall of placental bed spiral arteries in normal and severe preeclamptic pregnancies. *Placenta*. 1994;15:511-524. doi:10.1016/S0143-4004(05)80420-5
34. Salafia CM, Kathryn A, Starzyk KA, et al. Lipoprotein(a) deposition in the uteroplacental bed and in basal plate uteroplacental arteries in normal and complicated pregnancies. *Placenta*. 1998;11:377-387. doi:10.1016/S0143-4004(98)80026-X
35. Kim JS, Chang JH, Yu HK, et al. Inhibition of angiogenesis and angiogenesis-dependent tumor growth by the cryptic kringle fragments of human apolipoprotein(a). *J Biol Chem*. 2003;278:29000-29008. doi:10.1074/jbc.M301042200
36. Kim JS, Yu HK, Ahn JH, et al. Human apolipoprotein(a) kringle V inhibits angiogenesis in vitro and in vivo by interfering with the activation of focal adhesion kinases. Biochem. *Biophys Res Commun*. 2004;313:534-540. doi:10.1016/j.bbrc.2003.11.148
37. Liu L, Boffa MB, Koschinsky ML. Apolipoprotein(a) inhibits in vitro tube formation in endothelial cells: identification of roles for kringle V and the plasminogen activation system. *PloS One*. 2013;8:e52287. doi:10.1371/journal.pone.0052287
38. Heyderman E, Strudley K, Richardson TC. In: Weir DM, Herzenberg LA, Blackwell C, Herzenberg A, eds. *Handbook of Experimental Immunology*. Vol 129. Blackwell; 1989:1.
39. Geetha M, Kalaivani V, Sabarinath PS, Appukuttan PS. Plasma anti-α-galactoside antibody binds to serine- and threonine-rich peptide sequence of apo(a) subunit in Lp(a). *Glycoconj J*. 2014;31:289-298. doi:10.1007/s10719-014-9521-2
40. Ribatti D, Vacca A, Roncali L, Dammacco F. The chick embryo chorioallantoic membrane as a model for in vivo research on angiogenesis. *Int J Dev Biol*. 1996;40(6):1189-1197.
41. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979;76(9):4350-4354. doi:10.1073/pnas.76.9.4350
42. Ahmad M, Khurana NR, Jaberi JE. Ionizing radiation decreases capillary-like structure formation by endothelial cells in vitro. *Microvasc Res*. 2007;73:14-19. doi:10.1016/j.mvr.2006.08.005
43. Shental-Bechor D, Levy Y. Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proc Natl Acad Sci U S A*. 2008;105(24):8256-8261. doi:10.1073/pnas.0801340105
44. Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol*. 2001;21:1104-1117. doi:10.1161/hq0701.093685
45. Hirashima C, Ohkuchi A, Nagayama S, et al. Galectin-1 as a novel risk factor for both gestational hypertension and preeclampsia, specifically its expression at a low level in the second trimester and a high level after onset. *Hypertens Res*. 2018;41(1):45-52. doi:10.1038/hr.2017.85
46. Sindrewicz P, Lian L-Y, Yu L-G. Interaction of the oncofetal Thomsen–Friedenreich antigen with galectins in cancer progression and metastasis. *Front Oncol*. 2016;6:79. doi:10.3389/fonc.2016.00079
47. Richter DC, Jeschke U, Bergemann C, et al. Expression of the Thomsen-Friedenreich (TF) tumor antigen in human abort placentas. *Anticancer Res*. 2005;25:1675-1678.
48. St-Pierre C, Ouellet M, Tremblay MJ, Sato S. Galectin-1 and HIV-1 infection. *Method Enzymol*. 2010;480:267-294. doi:10.1016/S0076-6879(10)80013-8
49. Sharma M, Redpath GM, Williams MJA, McCormick SPA. Recycling of apolipoprotein(a) after PlgRKT-mediated endocytosis of lipoprotein(a). *Circ Res*. 2017;120:1091-1102. doi:10.1161/CIRCRESAHA.116.310272
50. Wu MH, Ying NW, Hong TM, Chiang WF, Lin YT, Chen YL. Galectin-1 induces vascular permeability through the neuropilin-1/vascular endothelial growth factor receptor-1 complex. *Angiogenesis*. 2014;17:839-849. doi:10.1007/s10456-014-9431-8
51. Jadwin JA, Mayer BJ, Machida K. Detection and quantification of protein–protein interactions by far-western blotting. In: Kurien B, Scofield R, eds. *Western Blotting. Methods in Molecular Biology. 1312*. Humana Press; 2015. doi:10.1007/978-1-4939-2694-7_38
52. Windwarder M, Yelland T, Djordjevic S, Altmann F. Detailed characterization of the O-linked glycosylation of the neuropilin-1 c/MAM-domain. *Glycoconj J*. 2016;33:387-397. doi:10.1007/s10719-015-9602-x
53. Trieu VN, Uckun FM. Apolipoprotein(a), a link between atherosclerosis and tumor angiogenesis. *Biochem Biophys Res Commun*. 1999;257:714-718. doi:10.1006/bbrc.1999.0519
54. Schulter V, Koolwijk P, Peters E, et al. Impact of apolipoprotein(a) on in vitro angiogenesis. *Arterioscler Thromb Vasc Biol*. 2001;21:433-438. doi:10.1161/01.ATV.21.3.433
55. Kim GM, Reid T, Shin SJ, et al. A phase 1, open label, dose escalation study to investigate the safety, tolerability, and pharmacokinetics of MG1102 (apolipoprotein(a) Kringle V) in patients with solid tumors. *Invest New Drugs*. 2017;35:773-781. doi:10.1007/s10637-017-0460-1

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kalaivani V, Krishna MS, Kumar AA, Satheesh G, Jaleel A. O-glycan structures in apo(a) subunit of human lipoprotein(a) suppresses the pro-angiogenic activity of galectin-1 on human umbilical vein endothelial cells. *The FASEB Journal*. 2023;37:e22813. doi:10.1096/fj.202201001RR