Endothelium in Dyslipidemia

BY

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THESIS

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LIST OF ACRONYMS

CAD - Coronary artery disease
OxLDL - Oxidized low-density lipoprotein
Cu-oxLDL – Copper oxidized LDL
LPO-oxLDL – Lipoxygenase oxidized LDL
HAEC(s) - Human Aortic Endothelial Cell(s)
BrdU - 5-Bromo-2'-deoxy-uridine Labeling Detection Kit III
MβCD - Methyl-β-cyclodextrin
FIV – Flow-induced vasodilation
ET-1 – Endothelin-1
ROS - Reactive Oxygen Species
DKO – ApoE-/- / Kir2.1+/-
ApoE-/- - Apolipoprotein E deficient
ROCK – RhoA Kinase
C3 – RhoA inhibitor, C3 transferase
Y27632 – ROCK inhibitor
MK2206 – AKT inhibitor

Purpose of Study

Atherosclerosis

Heart disease is the leading cause of death in the United States (Xu, Murphy, Kochanek, & Bastian, 2016). Coronary artery disease (CAD) is the most common type of heart disease. CAD is caused by plaque build-up on the arterial wall, narrowing the space for blood flow; this process is formally known as atherosclerosis. Atherosclerosis is the thickening and narrowing of arteries due to plaque formation. Plaque rupture can cause stroke, heart attacks, and possibly death. The oxidation of LDL is a major component to earlier stages of atherosclerosis and endothelial dysfunction. This paper clarifies the impact of oxLDL on endothelial cells.

Dyslipidemia

Dyslipidemia is well known to play role in the initiation of atherosclerosis but mechanisms related to this progression are poorly understood (Dopico). Chronic hyperlipidemia and hypercholesterolemia are known to be risk factors for atherosclerosis. More specifically, hypercholesterolemia may be playing a critical role in endothelial dysfunction by causing Kir channels to be dysfunctional. In this paper, we clarify the role of cholesterol in: suppressing Kir2.1 function in flow-induced vasodilatation; oxLDL-induced proliferation; and oxLDL-induced Akt-phosphorylation.

Review of Literature

Angiogenesis, oxLDL, and RhoA/Rock and Akt pathway

Oxidized low-density lipoprotein (oxLDL) has been known to play an active role in the progression of atherosclerosis and endothelial dysfunction. Higher levels of reactive oxygen species (ROS) is an outcome of various diseases including atherosclerosis, resulting in the oxidation of low-density lipoproteins (Pirillo, Norata, and Catapano). From here, oxLDL activates the recruitment of monocytes to internalize into intima wall and oxLDL activates the conversion from monocytes to macrophages (Pirillo, Norata, and Catapano). Macrophages uptake oxLDL, creating foam cells which ultimately lead to plaque formation and plaque destabilization (Hansson, Robertson, and Söderberg-Nauclér).

More recently, investigators have been trying to gather information on the mechanism behind oxLDL activity. Previously, oxLDL was shown to activate the RhoA and RhoA kinase (ROCK) pathway (Wraith et al.). OxLDL was also shown to increase angiogenesis in mice using matrigel plugs in vivo (Oh et al.). Akt activation by RhoA has been previously shown but not in the context of angiogenesis in atherosclerosis (Del Re, Miyamoto, and Brown; Basile, Gavard, and Gutkind). A detailed hypothetical mechanism is displayed in Figure 1.

Angiogenesis, the formation of new blood vessels, is necessary for oxygen supply and nutrient delivery to help form the atherosclerotic plaque via migration and/or proliferation of cells (Moulton). Many studies have described the progression of angiogenesis in atherosclerotic plaques (Jeziorska and Woolley; Herrmann et al.; Kamat et al.). Other investigators have shown that the formation of new blood vessels in developing atherosclerotic plaques increase the risk for rupture (Sluimer and Daemen), which can lead to stroke, heart attack, and/or death. Because oxLDL is known to induce angiogenesis, an investigation was done on the angiogenic effect of

oxLDL on human aortic endothelial cells (HAECs) by examining proliferation and migration in relation to the Rho/Rock pathway and the Akt pathway.

Conflicting data of OxLDL on Proliferation

The oxidation of LDL can be done by two different methods of modification: copper (cu-oxLDL) or lipoxygenase (LPO-oxLDL), with each able to have varying degrees of oxidation based on the amount of time that is allowed for the LDL to be oxidized. Using lipoxygenase, an enzyme that oxidizes LDL, is a more physiological way of oxidizing LDL than copper because it is expressed in macrophages (Liebert and Al). Also, other labs treat their cells with different concentrations of oxLDL, often without reporting the oxidation of their oxLDL, creating conflicting data on the proliferative effect of oxLDL.

Previous studies have shown that proliferation is increased with exposure to oxLDL (Yu et al.; A. Heinloth et al.; Oh et al.; Byfield et al.) Although there is a plethora of studies that investigated the proliferative effect of oxLDL on endothelial cells, there is a lack of consensus on whether oxLDL increases or decreases proliferation (Chen et al.; Alexandra Heinloth et al.; Eto et al.; Theurl et al.; Ji et al.). The conflicting results are possibly due to the varying degrees of oxidation, dosage, and modification. An investigation was conducted in HAECs to clarify the proliferative effect of oxLDL using minimally, moderately, and strongly oxidized cu-oxLDL and by comparing cu-oxLDL and LPO-oxLDL.

Hypercholesterolemia

Hypercholesterolemia is a well-known risk factor for cardiovascular disease (Steinberg). Oxidized low-density lipoprotein (oxLDL) is well-known to play a role in the progression of endothelial dysfunction by being a factor in cholesterol accumulation (Levitan, Volkov, and Subbaiah). Previously, cholesterol enrichment was shown to prevent the oxLDL-induced

angiogenic response (Oh et al.). Therefore, the effect of cholesterol loading on the oxLDL-proliferative effect and Akt activation of HAECs was also investigated.

The possible effect of cholesterol on Kir2.1-dependent endothelial response

Impaired flow-mediated dilatation (FMD) is known to be long -term predictor of cardiovascular events because it reflects endothelium vasodilator function (Gokce et al.; Huang et al.; Kwon et al.; An, Marc, and Christiaan). Flow-induced vasodilatation (FIV) using a microfluidic chamber is a method to measure endothelial responses to shear stress, rather than brachial artery responses to shear stress. FIV is also a method that allows the manipulation of flow and the addition of pharmacological drugs.

Inwardly rectifying potassium (Kir) channels are known to be involved in endothelial responses to flow and may be involved in endothelial dysfunction under hypercholesterolemic conditions (Dopico; Fang et al.; Romanenko, Rothblat, and Levitan, "Modulation of Endothelial Inward-Rectifier K+ Current by Optical Isomers of Cholesterol"). They are present in murine and human resistance arteries (Olesen, Clapham, and Davies; Ahn et al.). Kir2, a subfamily of Kir (Kubo et al.), current activity can be regulated and suppressed by cholesterol loading and recovered with cholesterol depletion (Romanenko, Rothblat, and Levitan, "Modulation of Endothelial Inward-Rectifier K+current by Optical Isomers of Cholesterol"). Cholesterol can directly bind to Kir2.1 channels (Rosenhouse-Dantsker et al.; Levitan, Singh, and Rosenhouse-Dantsker). It was previously shown that Kir2.1 had higher levels of expression in endothelial cells versus Kir2.2 and Kir2.3 (Ahn et al.). Therefore, a main focus was placed on Kir2.1. Previously, it was also shown that Kir2.1 can be blocked by $BaCl_2$ (Kubo et al.). More recently, $BaCl_2$ was shown to block Kir2.1-dependent FIV endothelial response in mouse mesenteric arteries (Ahn et al.).

In our experiments, we used apolipoprotein E deficient (ApoE-/-) knockout mice, a mouse model used to study atherosclerosis because of their ability to develop spontaneous lesions and hypercholesterolemic state. We also crossed Kir2.1+/- heterozygous mice, which has reduced endothelial Kir2.1 expression and acts as a functional knock-out (Ahn et al.), with ApoE-/- mice to create ApoE-/-/Kir2.1+/-, a double knock-out mouse (DKO). Because cholesterol is known to affect current activity of Kir2 channels, an experiment was done on ApoE -/- knockout mice and DKO mice to investigate the effect of cholesterol depletion on Kir2.1 channels in mesenteric arteries in the context of FIV.

Methods

Cells and reagents

Human Aortic Endothelial Cells (HAECs; Lonza, Allendale, NJ) were grown and stored in 37°C with 5% CO2, with the EGM-2 Bullet Kit media. The media for the cells was changed every other day. For experiments, cells were treated with bullet kit free media and 2% lipoprotein deficient serum (LDS). Cholesterol and lipoxygenase were purchased from Sigma (St. Louis, MO); Y27632 [((+) -(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl))] from Caymanchem (Michigan, USA); C3 transferase (C3), from Cytoskeleton (Denver, CO).

Preparation methyl-β cyclodextrin loaded with cholesterol

Methyl-β-cyclodextrin (MβCD) saturated with cholesterol was prepared by incubating 5mM of MβCD in serum free media with the cholesterol (50mg/ml) evaporated ethanol/methanol solution. This MβCD cholesterol solution was vortexed, sonicated, and incubated overnight at 37°C. The next morning, the MβCD cholesterol solution was filtered using a 0.45µm filter to remove the cholesterol crystals.

Migration Measurements

HAECs were seeded in a 48-well plate. Cells were grown to confluency, serum starved for 1hr, incubated with inhibitor for 1 hour, and then incubated with oxLDL 1 hr. Post treatment a scratch was made in each well and a picture was taken in the same spot at 0hr and 8hr post treatment.

Proliferation Measurements using BrdU

Proliferation was measured using the 5-Bromo-2'-deoxy-uridine Labeling Detection Kit III (BrdU) purchased from Roche. HAECs were grown to 50% confluency and seeded in a 48-well plate. Cells were treated with cu- oxLDL or LPO (50µg/ml) for 1 or 24hrs in supplement free

media with 2% LDS. Cells were then washed with PBS+. BrdU labeling reagent was added for 6 hours and the cells were washed with PBS+. The cells were then fixed with precooled ethanol fixative for 20 mins in -20° C and washed. The fixed cells were then incubated with nuclease working solution and washed. Cells were then incubated with Anti-BrdU-POD Fab fragments working solution and washed. Cells were then incubated with Anti-mouse-Ig-AP solution [1:100 in PBS+] and washed. Cells were then covered in color substrate solution, NBT/BCIP Solution 1:50 in Substrate Buffer [100mM Tris-HCl-buffer, 100mM NaCl, 50mM $MgCl_2$, pH 9.5] at 15-25°C for 15-30mins and washed. Pictures were taken at 10x with Zeiss microscope, three pictures per well. All incubations after the cells were fixed were done for 30 mins. at 37°C and washed with PBS+ three times.

Proliferation Measurements using MTT

Proliferation methods via MTT kit were previously described (Zhang et al.). The MTT kit was purchased from ATCC (Manassas, VA). HAECs were seeded into 48-well plates at 50% confluency. After cells were adhered, full media was changed with supplement free media with 2% LDS prior to oxLDL treatments. Cells were then treated with oxLDL for 24hr with 0, 10, 50, or 100 μg/ml. After cells were treated with oxLDL, MTT dye was added (0.5mg/ml) to each and incubated for 2hr at 37°C. Cells were then lysed with DMSO. Absorbance at 570nm was measured using a plate reader (Spectra Max M5; Molecular Devices, Sunnyvale, CA).

LDL isolation and oxLDL preparation

LDL isolation and oxidation methods were previously described previously (Oh et al.). Human plasma was purchased from a blood blank. LDL was isolated from the human plasma by centrifugation in KBr with a final density of 1.063g/ml. The preparation was then dialyzed to remove KBr and EDTA. To oxidize the LDL, copper sulfate was added to LDL with a final

concentration of 25 μM for 16hr at 37°C. The reaction was stopped by added 0.5M EDTA to the oxidized mixture. Oxidation of LDL using lipoxygenase was previously described. 1 mg/ml human LDL was incubated with 5,000U/ml soybean lipoxygenase overnight at 37°C.
A thiobarbituric acid-reactive substances (TBARS) assay kit (ZeptoMetrix, Buffalo, NY) was used to determine the level of oxidation of the LDL.

Western Blot Analysis

Western Blot Analysis were previously described (Zhang et al.). HAECs were lysed in lysis buffer (Cell signaling Technology). Lysates were sonicated, and total protein was measured (Bio-Rad Protein Assay). 30μg of total protein samples was suspended in a reduced sample buffer and then electrophoresed on a 10-15% gel with Tris running buffer, blotted to PVDF membrane, and probed with primary antibodies: phosphorylated Akt and total Akt. A horseradish peroxidase-conjugated goat anti-rabbit antibody was added and analyzed through autoradiography using enhanced chemiluminescence (ECL Plus; General Electric Healthcare, Milwaukee, WI). C3 was introduced into HAECs first followed by 50μg/ml oxLDL for 24hr before lysis.

Isolation of mesenteric arteries and vasodilatation measurements

Methods were described previously (Ahn et al.). Mesenteric arteries were isolated and cannulated ex vivo in a flow chamber by securing the arteries with micropipettes filled with KREBs solution. Intraluminal pressure of 60 cmH2O was maintained by elevating two micropipette-attached reservoirs to 60cm above the chamber. Vessels are then pressurized for 30mins. After, vessels are pre-constricted with Endothelin -1 (ET-1) (120pM). Flow-induced vasodilatation (FIV) was determined by changing pressure gradients using the reservoirs to Δ10,

Δ20, Δ40, Δ60, Δ100, inducing flow. Then intraluminal diameter was measured using video microscopy.

Results

Cu-oxLDL-induced migration not facilitated by the RhoA/ROCK pathway

As previously discussed, we questioned whether or not migration and/or proliferation were the main drivers of oxLDL-induced angiogenesis. Migration was measured and analyzed using a scratch assay. Representative pictures display the endothelial cells migrating, moving towards the midline of the scratch (Figure 2A). Pretreatment of cells with cu-oxLDL (50μg/mL) (TBARS:15±3) increased the migration of HAECs after 8 hours (Figure 2B). When HAECs are pretreated for 1 hour with RhoA inhibitor (C3) (1μg/mL) and Rock inhibitor (Y27632) (1μg/mL), migration of cells significantly increased (Figure 2 B).

Cu-oxLDL-induced proliferation

Proliferation was measured using a BrdU assay (Figure 3A). HAECs pretreated with cu-oxLDL (50μg/mL) (TBARS:15±3) for 1 hour increased proliferation in a dose-dependent manner, where 50μg/mL increased proliferation the most (Figure 3B, 9) (Zhang et al.). HAECs treated with LDL for 1hour did not show a significant increase in comparison with control (Figure 3C).

Components that affect the oxLDL induced- proliferation of HAECs

Pretreatment with oxLDL with varying amount of incubation time, oxidation, dosage, and modes affected proliferation (Figure 4). First, pre-incubating HAECs with cu-oxLDL (50μg/mL) for 24hours had a 2-fold increase in proliferation from cells treated for 1hour (Figure 4A). Cells pretreated with LDL for 24hours had increased proliferation significantly from control. LDL treated cells proliferated less than cells treated cu-oxLDL (Figure A). It is important to note that LDL did not have an effect on proliferation at 1hour (Figure 3C) but did at 24hours (Figure 4A).

Lipoxygenase (LPO) oxLDL, a more novel and physiological mode to oxidize LDL, was tested for its proliferative effect at 24 hours (Figure 4B). LPO also had a 2-fold increase on proliferation in comparison to controls when HAECs were pretreated for 24 hours ($p<0.05$) (Figure 4B). Both cu-oxLDL and LPO-oxLDL proliferation mentioned above were done using the BrdU assay.

Proliferation using moderately and highly oxidized cu-oxLDL at different doses was also investigated. As mentioned earlier, there wasn't a consensus in literature on the whether oxLDL had activated or inhibited proliferation. This was probably due to the fact that different labs used different oxidations and doses to investigate proliferation. Using a MTT assay, cu-oxLDL-induced increase in proliferation was shown with both moderately (TBARS: 7.45) and highly (TBARS: 13.62) oxidized LDL in a dose dependent manner using 0, 10, 50, 100 μg/mL (Figure 4C)(Zhang et al.).

OxLDL- induced proliferation is facilitated through the RhoA/ROCK/Akt pathway

Both cu-oxLDL and LPO oxLDL-induced proliferation was blocked using inhibitors of RhoA (Figure 5A) and ROCK (Figure 5B). These data suggest that, oxLDL-induced proliferation is activated through the RhoA/ROCK pathway (Zhang et al.).

In an attempt to the connect the RhoA/ROCK pathway to Akt, using western blot analysis, we investigated the effect of oxLDL and RhoA/ROCK inhibitors on Akt phosphorylation (Figure 5 C, D) (Zhang et al.). Both cu-oxLDL and LPO-oxLDL increased Akt-phosphorylation significantly, 2.9-fold and 4.7-fold, respectfully (Figure 5 C, D) (Zhang et al.). Both cu-oxLDL and LPO-oxLDL treated HAECs inhibited Akt-phosphorylation when pre-incubated with RhoA inhibitor (C3) (Figure 5C) and ROCK inhibitor (Y27632) (Figure 5D). In

addition, when Akt is blocked with an Akt inhibitor, MK2206, Akt phosphorylation is blocked (Figure 6B) and proliferation is reduced (Figure 6A)(Zhang et al.).

OxLDL-induced Akt Phosphorylation and Proliferation is inhibited with Cholesterol Loading

Previously, cholesterol enrichment was shown to prevent the angiogenic response to oxLDL (Oh et al.). Because we wanted to investigate the proliferative ability of HAECs in a cholesterol rich environment in vitro, HAECs were preloaded with MβCD-cholesterol prior to oxLDL exposure. Both cu-oxLDL and LPO oxLDL induced Akt-phosphorylation was reduced when HAECs were preloaded with MβCD-cholesterol (Figure 7A). Both cu-oxLDL and LPO oxLDL-induced proliferation is also reduced with cholesterol loading in HAECs. (Figure 7B).

Cholesterol depletion recovers Kir2.1-mediated FIV in ApoE-/- mice but not in DKO mice

Apolipoprotein E deficient (ApoE-/-) mice are a well-known atherosclerotic model. Because ApoE-/- mice have high cholesterol, we wanted to investigate the effect of cholesterol depletion on flow-induced vasodilatation (FIV), previously explained in the methods. ApoE-/- mesenteric arteries were pre-incubated with unloaded MβCD (5mM), causing cholesterol depletion. Arteriole endothelial response to flow was then measured with FIV. Normal endothelial response to flow is 80-100% of baseline flow (Ahn et al.). ApoE-/- mesenteric arteries had reduced dilatations to flow (48.14% at Δ100), with no further block with $BaCl_2$ (Figure 8A, C). When ApoE-/- arteries were pre-incubated with MβCD, dilations increased and were able to be blocked with $BaCl_2$.

ApoE-/- mice were then crossed with Kir2.1 deficient mice (Kir2.1 +/-) to create a DKO. DKO mesenteric arteries had reduced dilatations to flow (57.02% at Δ100) and did not recover when arteries were depleted of cholesterol (Figure 8 B, C). No further block was seen with

$BaCl_2$. Kir2.1 +/- mesenteric arteries (not crossed with ApoE-/-) also resulted in reduced dilations to flow (Ahn et al.).

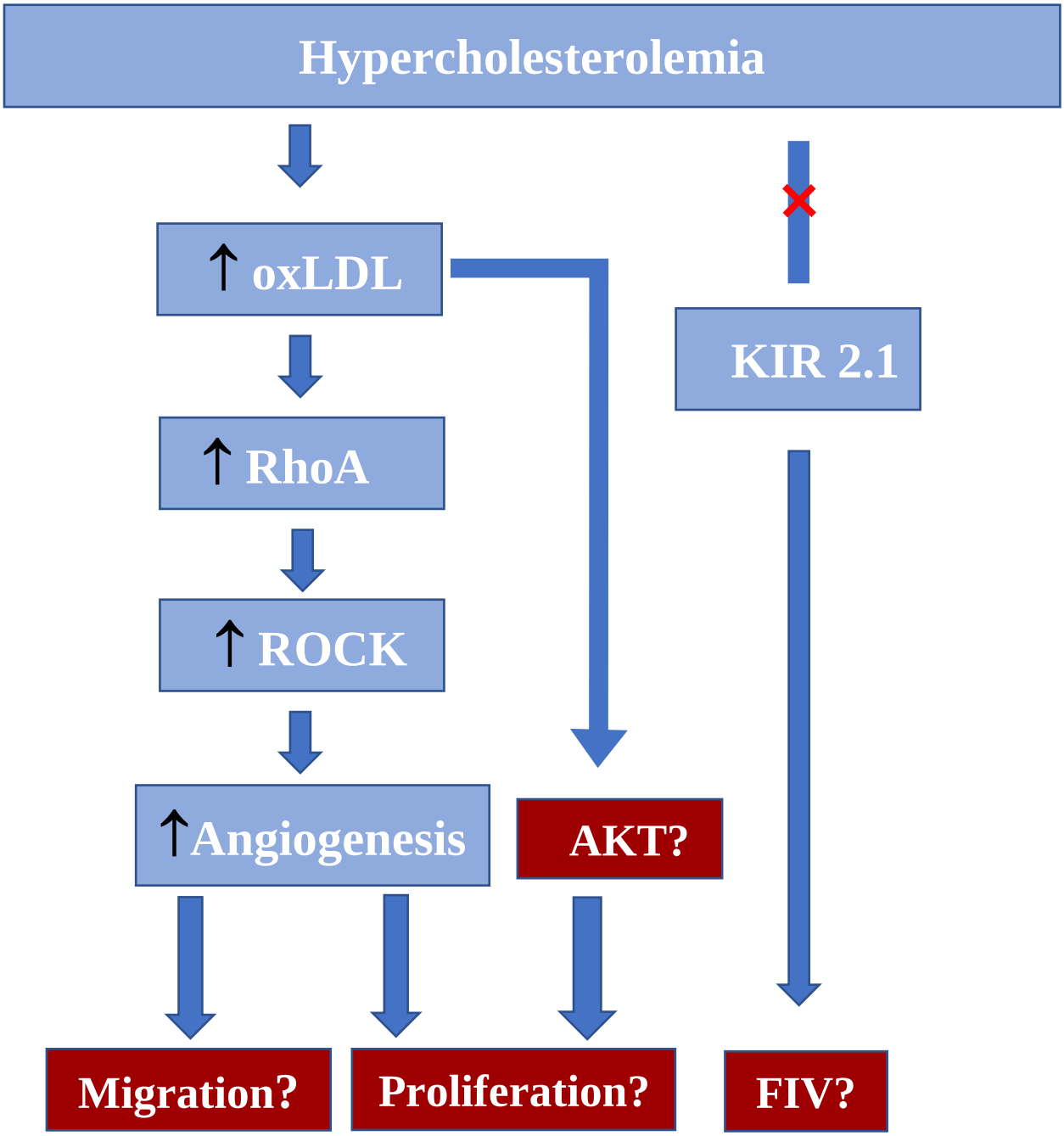

Figure 1. Hypothetical Schematic

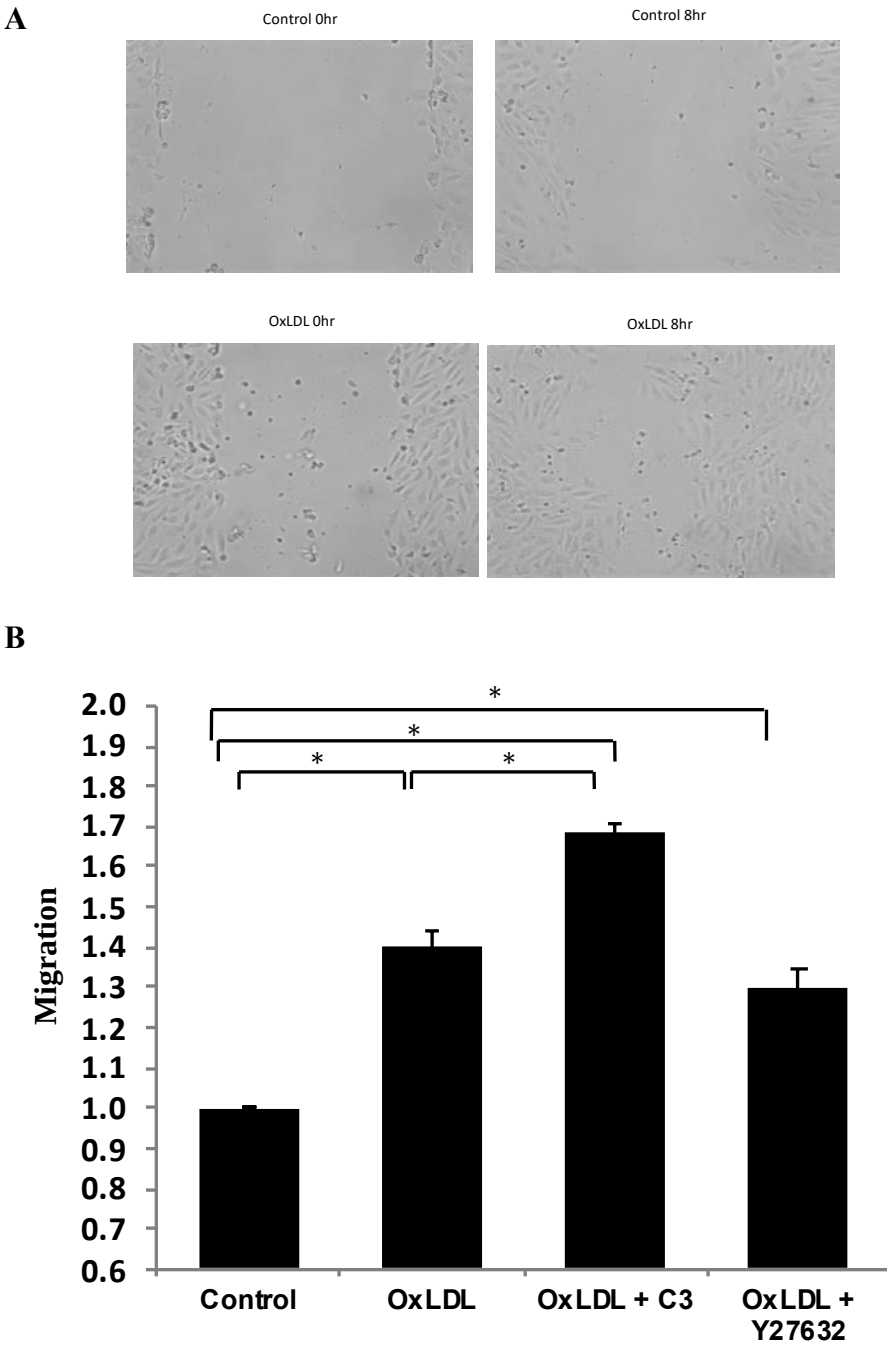

Figure 2. The effect of oxLDL and Rho/Rock Inhibitors on Migration in HAECs
A. Representative pictures of the migration scratch assay with cells treated with and without oxLDL. Pictures were taken at 0hr and 8hr.
B. OxLDL (50μg/mL) induces migration of HAECs. Inhibition of Rho increases oxLDL induced migration. Inhibition of ROCK increases oxLDL induced migration (*, $p < 0.05$) (n=3-6).
Source: Oh, Myung-jin et al. "Oxidized-LDL Signals through Rho-GTPase to Induce Endothelial Cell Stiffening and Promote Capillary Formation." *Journal of Lipid Research*. (2016): 1-33. Print.

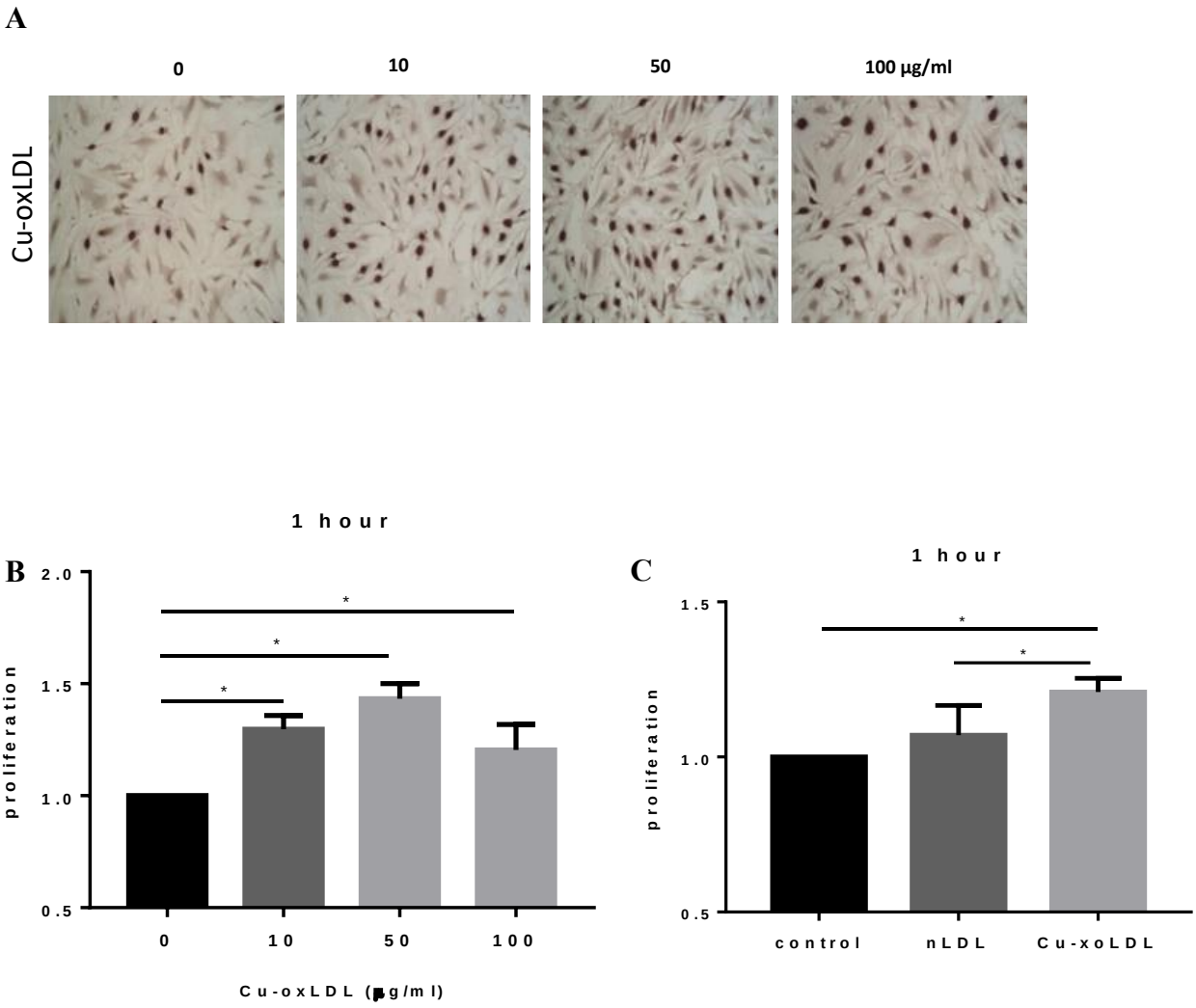

Figure 3. OxLDL increases proliferation dose-dependently after 1hr of treatment
A. Representative pictures of proliferation with HAECs stained with BrdU. HAECs were treated cu-oxLDL for 1hr.
B. Cu-oxLDL induces proliferation in a dose dependent manner. Cells were treated with 0, 10, 50, and 100µg/mL cu-oxLDL (*, $p < 0.05$, $n \geq 3$)
C. HAECs treated with LDL for 1hr did not induce proliferation. HAECs treated with 50µg/mL cu-oxLDL for 1hr did increase proliferation. (*, $p < 0.05$, $n \geq 3$)
Source: Zhang, Chongxu et al. "OxLDL-Induced Endothelial Proliferation via Rho/ROCK/Akt/$p27^{kip1}$ Signaling: Prevention by Cholesterol Loading." *American Journal of Physiology - Cell Physiology* (2017): C340-C351. Web.

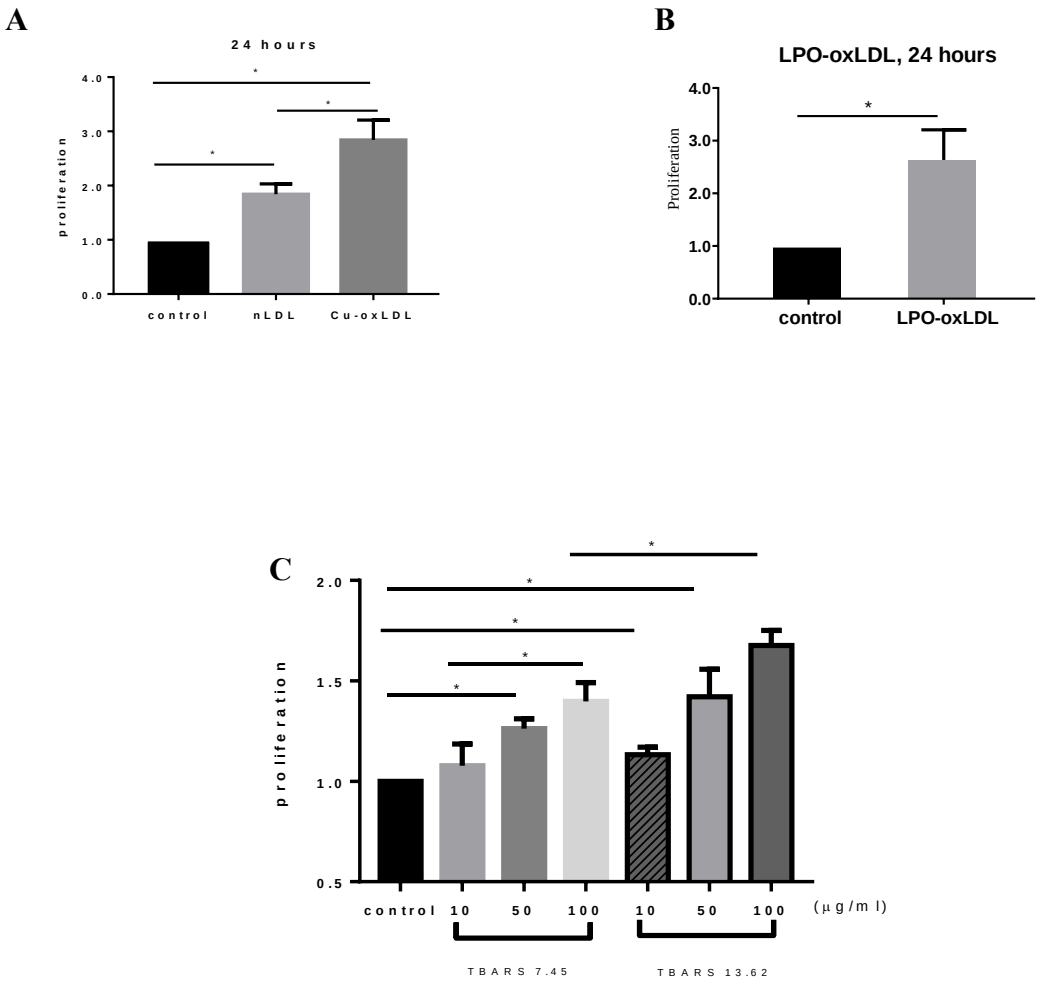

Figure 4. The effect of oxLDL on Proliferation with varying degrees of oxidation, dosage, and modes with 24-hour treatments

A. HAECs treated with LDL for 24hr increased proliferation. HAECs treated with cu-oxLDL (50μg/mL) for 24hr increased proliferation significantly more than LDL (50μg/mL) (*, $p < 0.05$, $n \geq 3$).

B. HAECs treated with LPO for 24hrs significantly increased proliferation. (50μg/mL) (*, $p < 0.05$, $n \geq 4$.)

C. HAECs were treated with cu-oxLDL for 24hrs using minimally and moderately oxidized-LDL (TBARS = minimally, 7.45 and moderately, 13.62) at doses: 0, 10, 50, 100 μg/ml. (*, $p < 0.05$, n=4)

Source: Zhang, Chongxu et al. "OxLDL-Induced Endothelial Proliferation via Rho/ROCK/Akt/$p27^{kip1}$ Signaling: Prevention by Cholesterol Loading." *American Journal of Physiology - Cell Physiology* (2017): C340-C351. Web.

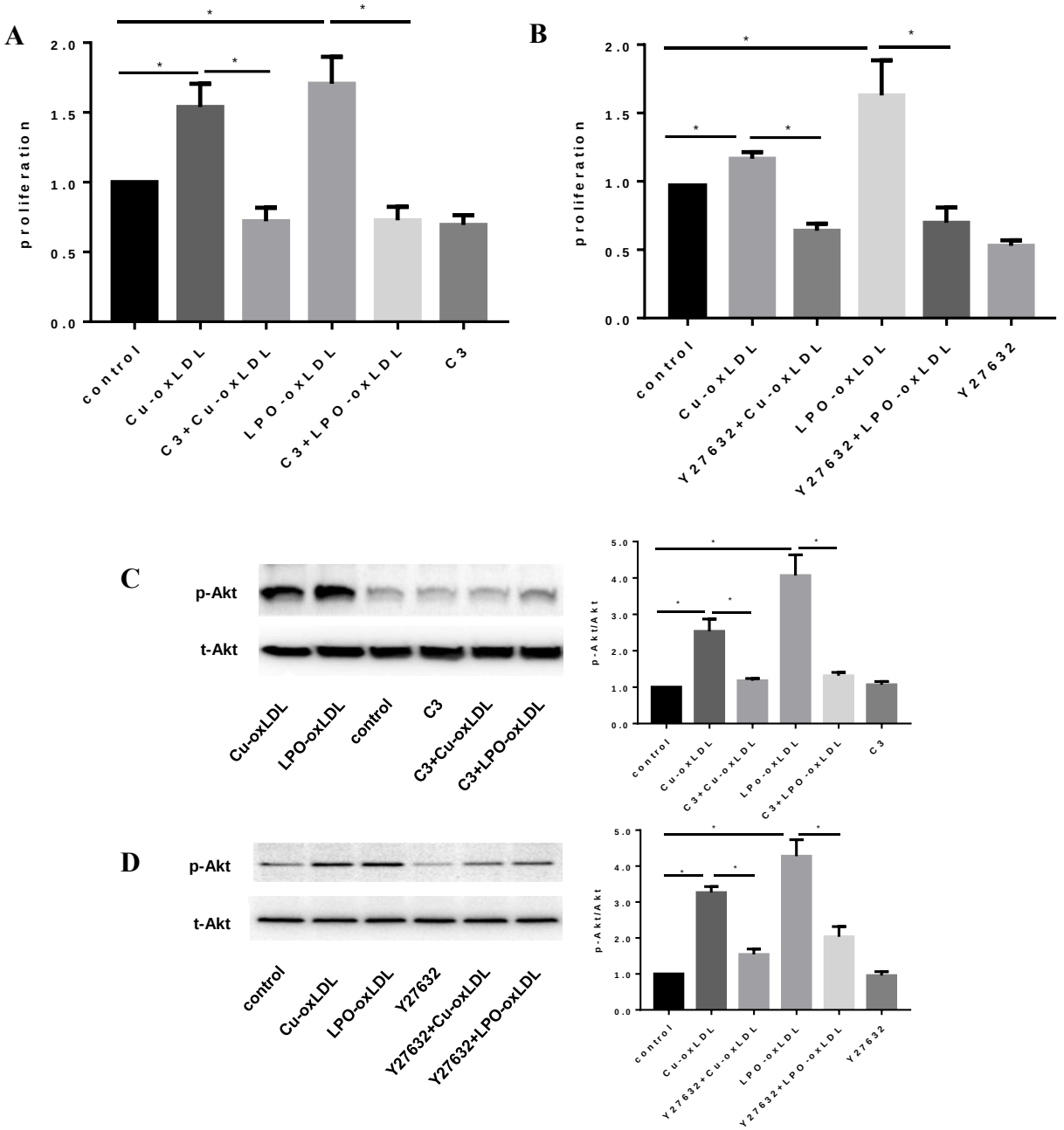

Figure 5. OxLDL- induced proliferation is through the RhoA/ROCK/Akt pathway

A. Cu and LPO OxLDL induced proliferation is reduced by Rho Inhibitor (C3) (1μg/mL). (*, $p < 0.05$, $n \geq 4$).

B. Cu and LPO OxLDL induced proliferation is reduced by Rock Inhibitor (Y27632) (1μg/mL). (*, $p < 0.05$, $n \geq 4$).

C. Cu and LPO OxLDL induced Akt phosphorylation is reduced by Rho Inhibitor. Akt phosphorylation was normalized to Total Akt.

D. Cu and LPO OxLDL induced Akt phosphorylation is reduced by Rock Inhibitor. Akt phosphorylation was normalized to Total Akt.

Source: Zhang, Chongxu et al. "OxLDL-Induced Endothelial Proliferation via Rho/ROCK/Akt/$p27^{kip1}$ Signaling: Prevention by Cholesterol Loading." *American Journal of Physiology - Cell Physiology* (2017): C340-C351. Web.

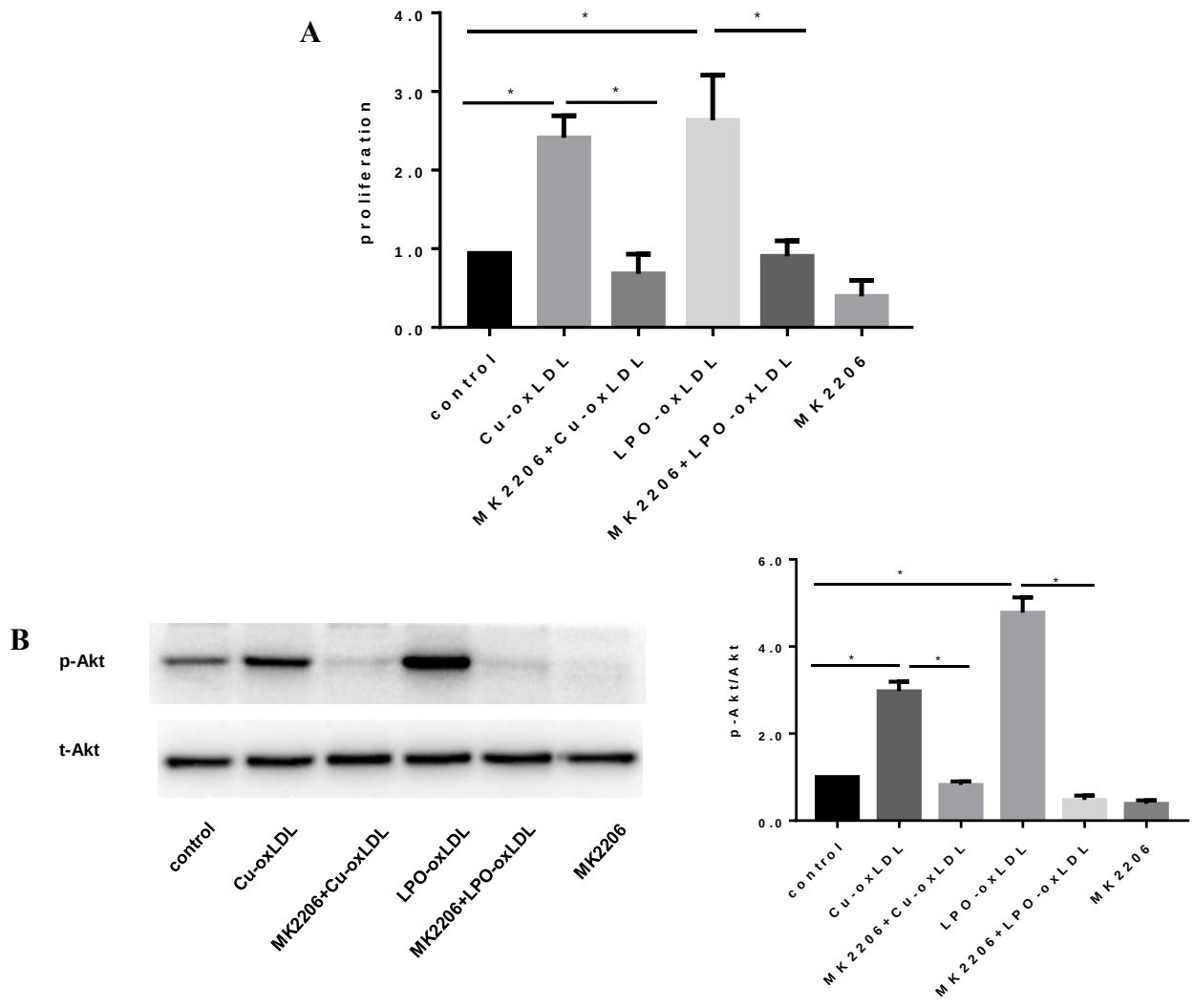

Figure 6. Akt inhibitor blocks proliferation and Akt phosphorylation

A. OxLDL-induced (50μg/mL) proliferation is blocked by Akt inhibitor, MK2206 (100nM). (*, $p < 0.05$, $n \geq 4$)

B. OxLDL-induced Akt activation is blocked by Akt inhibitor, MK2206 (100nM). (*, $p < 0.05$, $n \geq 4$)

Source: Zhang, Chongxu et al. "OxLDL-Induced Endothelial Proliferation via Rho/ROCK/Akt/$p27^{kip1}$ Signaling: Prevention by Cholesterol Loading." *American Journal of Physiology - Cell Physiology* (2017): C340-C351. Web.

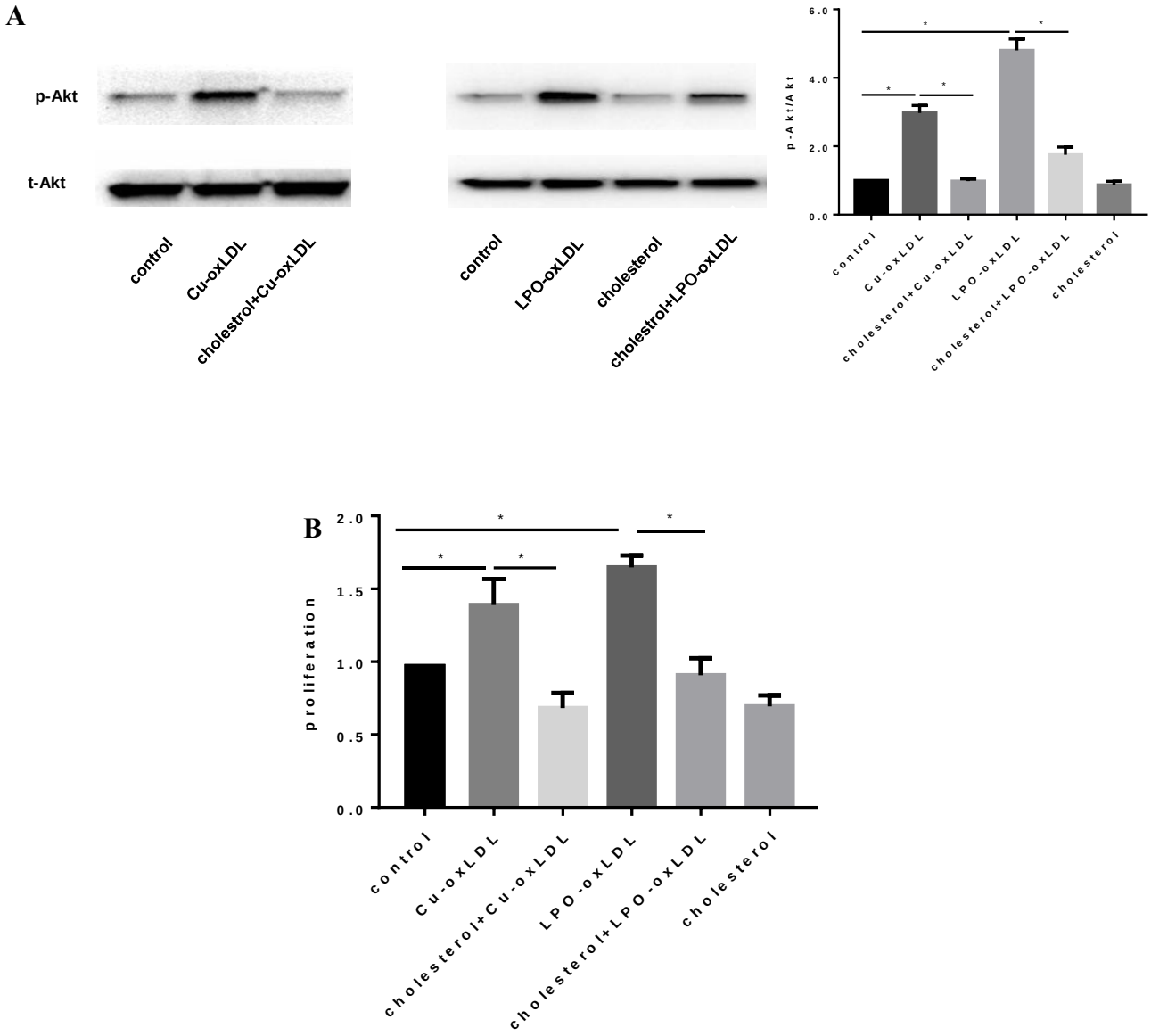

Figure 7. Cholesterol loading on oxLDL-induced Akt Activation and Proliferation
A. Cu-oxLDL and LPO-oxLDL (50μg/mL) activates phosphorylation of Akt (p-Akt). Pretreatment with MβCD-cholesterol reduces oxLDL-induced Akt phosphorylation. (*, $p < 0.05$, $n \geq 4$).
B. Pretreatment with MβCD-cholesterol reduces oxLDL-induced proliferation. (*, $p < 0.05$, $n \geq 4$).
Source: Zhang, Chongxu et al. "OxLDL-Induced Endothelial Proliferation via Rho/ROCK/Akt/$p27^{kip1}$ Signaling: Prevention by Cholesterol Loading." *American Journal of Physiology - Cell Physiology* (2017): C340-C351. Web.

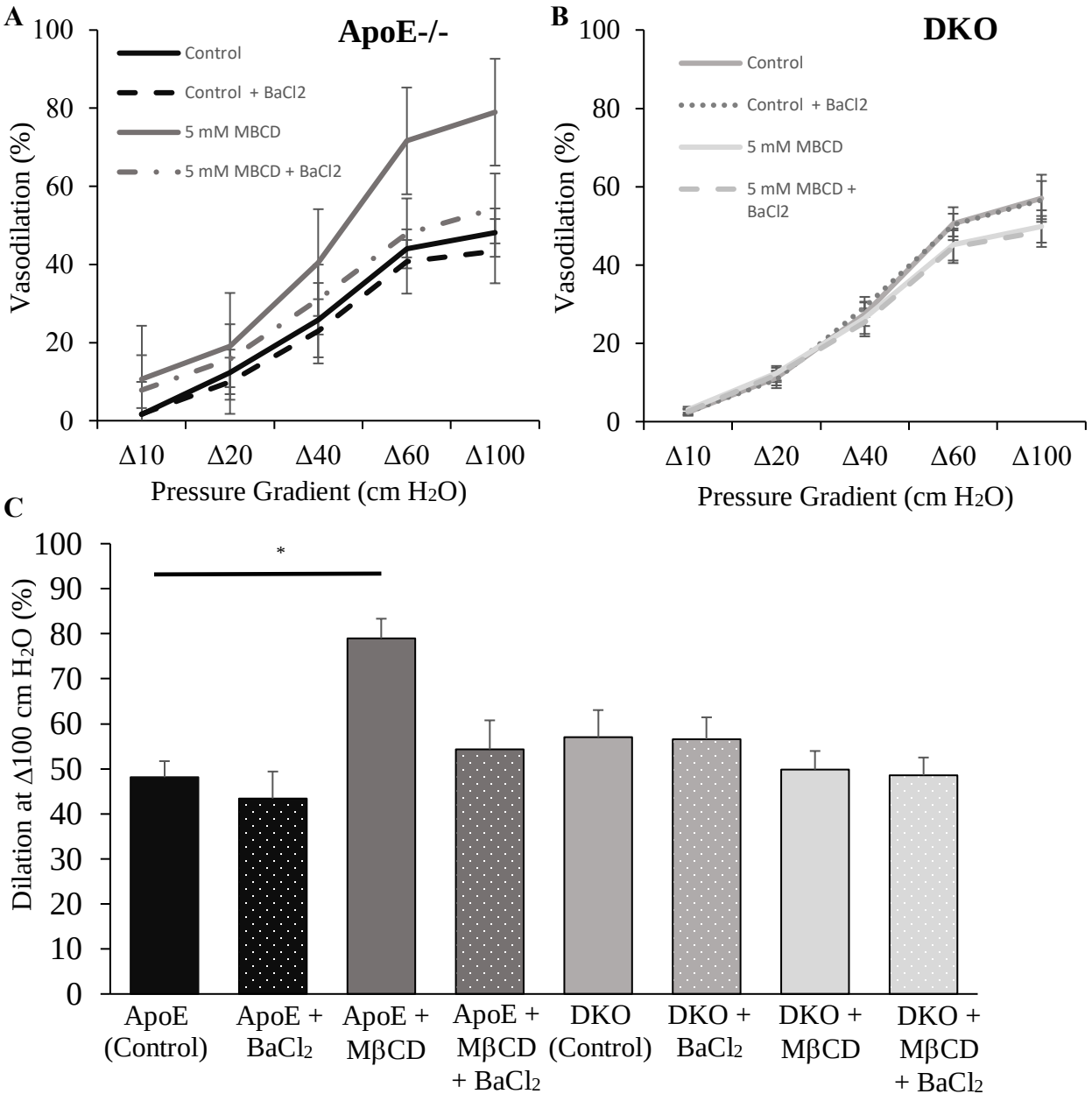

Figure 8. Cholesterol depletion on ApoE-/- and ApoE-/- /Kir2.1+/- (DKO) FVB mice

A. Cholesterol depletion using MβCD (5nM) of ApoE-/- mice arterioles recovered FIV.

B. Cholesterol depletion of DKO mice arterioles did not recover FIV.

C. The graph displays dilatation values at Δ100 from graphs **A** and **B**. (*, $p < 0.05$, $n \geq 4$).

Source**:** Fancher, I., Ahn, S., Adamos, C., Osborn, C., Oh, M., Fang, Y., Reardon, C., Getz, G., Phillips, S., Levitan, I. (2018) Hypercholesterolemia-induced loss of flow-induced vasodilation and lesion formation in ApoE-/- mice critically depend on Kir channels. *JAHA*

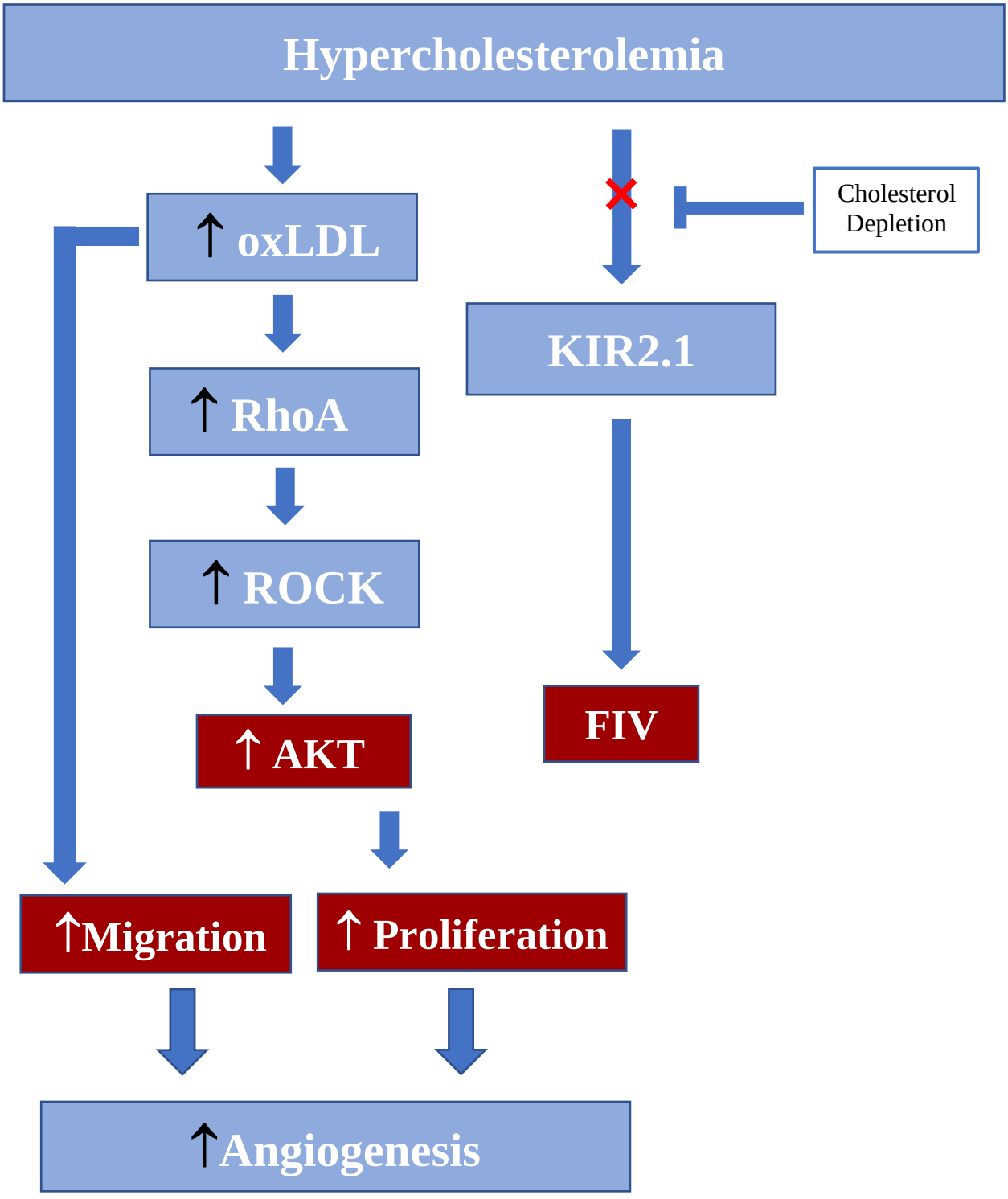

Figure 9. Proposed Schematic

Discussion

The Angiogenic Effect of OxLDL on Human Aortic Endothelial Cells

Previously, it was shown that oxLDL can induce angiogenesis of endothelial cells through the RhoA/Rock pathway (Oh et al.). My lab took a further look into whether this angiogenic effect was due to proliferation and/or migration of endothelial cells and the mechanism behind it.

Although oxLDL induced migration of endothelial cells, RhoA/ROCK inhibitors further increased migration (Figure 2). Therefore, oxLDL-induced migration may not be activated through the RhoA/ ROCK pathway (Figure 9) (Oh et al.). It is possible that RhoA and ROCK inhibition may be activating migration via another pathway.

Proliferation also was induced by both cu-oxLDL and LPO-oxLDL (Figure 3, 5). In contrast to migration, proliferation was inhibited by RhoA/ROCK inhibitors (Figure 5). Also, RhoA/ROCK inhibitors caused inhibition of Akt-phosphorylation. Therefore, since both RhoA and ROCK inhibitors blocked Akt-phosphorylation, Akt is downstream from RhoA and ROCK in the pathway (Figure 9). In addition, proliferation and Akt phosphorylation were blocked with Akt inhibitor (MK2206) (Figure 6). In summary, proliferation is activated through the RhoA/ROCK/AKT pathway (Figure 9) and the angiogenic effect of oxLDL is facilitated through proliferation.

Another major component of this paper was to clarify the effect of the amount of incubation, oxidation, dosage, and modification of oxLDL on proliferation. Both cu-oxLDL and LPO-oxLDL have positive proliferative effects. The proliferative effect also increased when cells were incubated for longer periods of time (1hr vs. 24hrs) (Figure 3,4A, 4B); the oxidation of LDL was increased (TBARS: 7.45 vs.13.62) (Figure 4C); and the concentration of oxLDL was

increased (0, 10, 50, 100μg/mL) (Figure 3B, 4C). Because all these factors can alter the proliferative effect of oxLDL, it is necessary to report these variables specifically in the methods. Different cells types may not be able to survive or have effects at certain oxidations or dosages, making it even more crucial to report and justify oxidations and concentrations. Otherwise, it is difficult to consolidate, replicate, and understand data.

The Effect of Cholesterol on Proliferation, Akt-phosphorylation, and FIV

Cholesterol is known to play a role in atherosclerosis. In light of the oxLDL-induced angiogenic effect, an investigation was done on the effect of cholesterol on proliferation, Akt-phosphorylation, and FIV.

When HAECs are loaded with cholesterol, proliferation and Akt-phosphorylation is inhibited (Figure 7). This is interesting because instead of further activation and exacerbation, cholesterol is inhibitory in this case, leading to the conclusion that too much cholesterol can have inhibitory effects.

When hypercholesterolemic mouse (ApoE-/-) arterioles were depleted of cholesterol, FIV recovered, indicating that endothelial response to flow is not completely lost. And recovered cholesterol-depleted ApoE-/- arteries were able to be blocked with $BaCl_2$, indicating that recovery may be due to the recovery of Kir2.1 channels. (Figure 8A, C) (Fancher et al.). When DKO (a hypercholesterolemic, Kir2.1 deficient mouse) arterioles were depleted of cholesterol, FIV did not recover and did not respond to $BaCl_2$, leading to the conclusion that endothelial recovery response to cholesterol depletion is indeed Kir2.1-dependent (Figure 8B, C).

Clinical Relevance

Because cholesterol has been shown to inhibit and suppress Kir2.1 function, targeting Kir2.1 may be a way to prevent coronary events (Le and Abe) . By activating Kir2.1 function, it may prevent dysfunctional systems related to flow and possibly prevent atherosclerosis. Unfortunately, systemic activation of Kir2.1 may not be a good method of approach because of its relation to atrial fibrillation (Xia et al.). An approach to target endothelial-specific Kir2.1 would have to be pursued instead (Le and Abe).

Future Directions

Questions that still need to be answered

Because the mechanism behind migration is still unclear, a further investigation into the mechanism behind oxLDL-induced migration is an avenue of interest. It's possible that certain conditions required for migration weren't met.

Now that we've been able to show that cholesterol can inhibit proliferation in HAECs in vitro, a further investigation on the effect of cholesterol enrichment or depletion on endothelial proliferation ex vivo or in vivo would be interesting.

From our data we show that cholesterol depletion via MβCD led to recovery in hypercholesterolemic mouse arteries. But it is still unclear whether or not MβCD is removing intercellular cholesterol or if it is able to remove cholesterol from Kir2.1. It would also be helpful to know the amount of cholesterol being removed in order to optimize this process. And if we can show that MβCD can remove cholesterol from Kir2.1, then we would be able to determine whether or not cholesterol is a necessary factor into the function of Kir2.1.

Human studies using FIV on hypercholesterolemic human arterioles are currently being done. We have not yet tested cholesterol depletion in human arterioles which would provide

insight on the effect of cholesterol on Kir2.1 in humans. A limitation to this approach, however, is the number of vessels collected per biopsy is often very low.

Exercise?

It would also be interesting if we looked to see if exercise could improve Kir2.1 function in hypercholesterolemic people. Exercise has been shown to reduce cholesterol in hypercholesterolemic patients and reduce arterial wall thickness (Thijssen, Cable, and Green; Okada et al.). If we could show that exercise was improving Kir2.1 function by lowering cholesterol, then that could be proof of an alternative way to combating coronary diseases. In the future, we could even supplement some of the exercise group with a Kir-activating drug to see if the improvements on vascular function are even greater.

An alternative approach to statins

It has been previously shown that statins that block the Rho/Rock pathway have improved FMD (Rawlings et al.) by increasing endothelial nitric oxide synthesis (Laufs and Liao). A next step would be to further investigate the effect of statins on Kir2.1 in ApoE-/-mice or in people with hypercholesterolemia using FIV. Due to possible adverse effects of statins, such as myopathy or rhabdomyolysis, alternative approaches to reduce cholesterol are necessary (Maji et al.). Using FIV would be an interesting approach because we would be able to use statins and other pharmacological variables together.

Limitations

A limitation for proliferation was that it was done in vitro and only gives partial insight to what is happening in vivo. Also, cu-oxLDL was oxidized in a manner that wouldn't naturally happen in the body. This is another reason for using LPO-oxLDL.

A limitation previously mentioned was the effect of MβCD on cholesterol. It was unclear how much or if MβCD was causing cholesterol to unbind from Kir2.1. Cholesterol is needed for variety of functions and if MβCD is removing all of the cholesterol than that might be skewing the results.

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