

G Protein-Coupled Receptor Signaling in Growth and Inflammation of Vascular Smooth Muscle

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Abstract

Cardiovascular disease (CVD) remains the primary cause of morbidity and mortality in the United States and worldwide. The exaggerated release of serine proteases and reduced blood flow and ensuing development of extracellular acidosis in affected tissues have previously been theorized to contribute to CVD pathophysiology. A broad family of transmembrane receptors known as G protein-coupled receptors (GPCRs) has been implicated in various roles of cardiovascular pathophysiology, yet the roles of several specific GPCR subfamilies in CVD pathology remain unclear. In this study, two understudied GPCR subfamily members of interest are the serine protease-activated receptor PAR2 and the proton sensing receptor GPR68, both identified in vascular smooth muscle cells (VSMCs) and believed to signal through multiple intracellular G proteins. In the stimulatory G_s pathway, activation of cyclic AMP contributes to stimulation of multiple downstream effectors; however, the discrete influence of PAR2 and GPR68 and their precise G signaling pathways in CVD remain unclear. In this study, we hypothesized that PAR stimulation, via serine proteases, and GPR68, through acidosis, promote proliferation and inflammation in VSM and that these operate via stimulatory G_s signaling. Using an arterial distension injury model, whole vessel PAR2 expression and

activity were both increased 30 minutes after injury compared to sham-operated controls. In cultured VSMCs, cumulative data suggest that PAR2 induction is regulated after stimulation through β -arrestin signaling. Interestingly, in the presence of vascular distension injury, we saw decreased arterial wall GPR68 expression compared to controls after 30 minutes. Using a carotid artery ligation injury, which more adequately mimics vascular ischemia/acidosis, in GPR68-deficient (gene knockout (KO)) mice, we saw reduced vascular remodeling and neointimal formation compared to wild type (WT) controls. In vitro, GPR68 KO cells exhibited increased proliferation in both growth stimulated (10-20% serum) and hypoxic (1% oxygen (O₂)) conditions after 48 and 72 hours. Cell cycle data displayed increased proliferation rate of KO cells (~12 hours) compared to WT cells. In WT cells, evidence points towards reduced G_s signaling via the cAMP targets Rap1A/1B and ERK1/2 under acidotic and hypoxic conditions, whereas no changes were observed in GPR68 KO cells in acidic versus normal conditions. Following treatment under ischemia/hypoxia conditions, β -arrestin appears to be upregulated compared to normoxic WT cells, suggesting its involvement in the regulation of GPR68. Further, WT cells treated with acidotic media displayed increased levels of the inflammatory cytokine interleukin-6 (IL-6) compared to WT and KO cells in normal pH media. These cumulative findings suggest that both PAR2 and GPR68 have capacity to regulate VSMC growth and inflammation as foundations of CVD. With this information, insight into these crucial yet understudied GPCR pathways, PAR2 and GPR68, may provide targets of interest in CVD treatment and/or management.

**G Protein-Coupled Receptor Signaling in Growth and Inflammation of Vascular
Smooth Muscle**

A Thesis

Presented to the Faculty of the Department of Physiology
East Carolina University

In Partial Fulfillment of the Requirements for the Degree
Master of Science in Biomedical Science

by

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LIST OF ABBREVIATIONS

VSMCs	Vascular smooth muscle cells
CVD	Cardiovascular disease
GPCRs	G protein-coupled receptors
PARs	Protease activated receptors
GPR68	Proton sensing G protein-coupled receptor 68
ECs	Endothelial cells
ECM	Extracellular matrix
ER	Endoplasmic reticulum
KO	Knockout
WT	Wild type
VVG	Verhoeff-van Gieson
AC	Adenylyl cyclase
CAF	Cancer associated fibroblasts
cAMP	3',5'-cyclic adenosine monophosphate
DMEM	Dulbecco's modified eagle medium
EPAC	Exchange protein activated by cAMP
FBS	Fetal bovine serum
G _i	Inhibitory G alpha subunit
G _s	Stimulatory G alpha subunit
IBMX	3-isobutyl-1-methylxanthine
IL-6	Interleukin 6
KA	Knockout cells treated with acidic media

KN	Knockout cells treated with normal/normoxic media
WA	Wild type cells treated with acidic media
WN	Wild type cells treated with normal/normoxic media
WH	Wild type cells treated with hypoxic media
KH	Knockout cells treated with hypoxic media
LCA	Left carotid artery
MAP	Mean arterial pressure
PDE	Phosphodiesterase
PVDF	Polyvinylidene fluoride
PP	Pulse pressure
Rap1	Ras associated protein 1
RPP	Rate pressure product
DBP	Diastolic blood pressure
SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST	Tris-buffered saline + Tween-20

Introduction

The cardiovascular system consists of the heart connected to an extensive network of arteries and veins. The heart serves as a pump while the blood vessels serve as a systemic delivery system for fluid transport, i.e., blood and organ perfusion. Homeostasis and proper organ function are achieved by transporting nutrients, molecules, and gases in the blood to tissues to meet their metabolic demands. Transportation of blood throughout the system is achieved through forceful contraction of the heart and is modulated by constriction and dilation of vascular smooth muscle cells (VSMCs) in arteries that alter vascular blood flow and moderate tissue blood perfusion. Deviation from normal/homeostatic VSMC behavior contributes to various cardiovascular disease (CVD) pathologies [1, 2, 3].

Broadly, the term CVD applies to various dysfunctions of the heart and/or blood vessels. The development of CVD can result in complications such as hypertension, angina, congestive heart failure, atherosclerosis, and stroke. The vascular system, primarily the arteries and small arterioles, is a tissue of primary concern for many CVD pathologies. A hallmark of CVD is a shift from a normal contractile vascular phenotype to a proliferative and synthetic phenotype; this abnormal growth is a chief concern for the development of vascular occlusion or vascular stenosis. This phenotypic change results in diminished ability for arterial contraction and/or relaxation while simultaneously constricting luminal blood flow from infiltrating VSMCs that narrow the vessel lumen. The American Heart Association has identified abnormal and dysfunctional VSMCs as a significant contributor

to CVD [4]. Thus, components of the vessel wall, specifically VSMCs, are a main focus for investigating the underlying abnormal mechanisms behind CVD [5]. Uncontrolled VSMC growth, apoptosis, and cell migration have all been recognized as contributors in the pathogenesis of CVD [1, 2, 3, 4]. When activated, these cellular mechanisms result in remodeling of the vessel wall and lumen stenosis, with subsequent blood flow changes to downstream tissues. Thus, VSMCs are a primary contributor to the pathogenesis of CVD, and it is essential to understand the pathophysiology of VSMCs as a potential regulator for disease progression, in turn, a central focus for experimental study. Lastly, once CVD becomes fully developed, its treatment varies from lifestyle changes to pharmacological medications to surgical interventions.

In addition to the disease process, vascular stenosis can result from medical interventions. Patients with advanced atherosclerosis may undergo percutaneous coronary intervention or coronary artery bypass grafting to clear an occlusion. However, the incidental damage to the endothelium and the excess stretch of medial wall VSMCs during these interventions may inadvertently promote proliferation and migration and inward growth of VSMCs, causing a secondary obstruction in an iatrogenic process known as restenosis. Luminal occlusion contributes to a lack of local blood flow and ultimately ischemia leading to extracellular acidosis [1, 2, 3, 14-18]. Lastly, fully effective treatments for CVD have not been discovered due to the lack of complete understanding of the disease mechanisms, thus helping to develop the focus of this study.

This introduction strives to provide background on vascular anatomy and physiology and how dysfunction within the vasculature contributes to CVD pathogenesis. This section will include an overview of cardiovascular anatomy and physiology, normal and abnormal VSMC pathologies, CVD, and GPCRs, including PARs and acid-sensing GPCRs as potential therapeutic targets against CVD.

Cardiovascular Anatomy and Physiology

The cardiovascular system consists of the heart as a pumping mechanism and an expansive network of vessels as conduits for circulating blood. In brief, blood flows into the right atrium from the vena cava (returning deoxygenated blood from systemic distribution), and after passive filling (i.e., venous return), the right atrium partially contracts (i.e., atrial kick) to help propel blood through the tricuspid valve and into the right ventricle. Right ventricular contraction then propels blood into the pulmonary artery and the lungs, where the blood becomes oxygenated through pulmonary gas exchange. Oxygenated blood returns from the lungs to the left atrium and passively (along with contribution from left atrial kick) flows into the left ventricle. When the left ventricle contracts, blood flows through the aorta and into a systemic vascular network composed of numerous tissues and cell types. Circulating blood flows through arteries, arterioles, and capillaries, in turn perfusing regional tissues before returning as deoxygenated blood through venules and veins for recirculation.

While vessels differ somewhat in their precise functions and anatomical wall layers, the basic components of the vessel wall include the *tunica externa*, *tunica media*, and *tunica intima*. The *tunica externa*, the outermost layer, is primarily formed of collagen and elastic fibers used to maintain the vascular structure. The *tunica media* is composed of mononucleated, elongated VSMCs, elastic fibers, and collagen to support vessel structure and physiological functions. The *tunica intima* lines the lumen and is composed of a single layer of endothelial cells (ECs). The *tunica intima* is a selectively permeable barrier between the blood and the vascular wall (media). The intimal layer plays an essential role as a regulator of vascular physiology to restrict VSMC proliferation and mediate vessel relaxation and constriction.

Vascular smooth muscle cells are found within the *tunica media* and compose a large percentage of the vessel wall. The smooth muscle contracts within the vessel wall, in turn manipulating the vessel's diameter, managing blood flow and volume, and localized pressures to downstream tissues. This ability to manage vessel diameter and blood volume ensures proper oxygen delivery to areas with enhanced metabolic activity. Arteries contain more substantial quantities of VSMCs than veins due to their responsibility for delivering oxygenated blood to downstream tissues. As mentioned, excessive proliferation of VSMCs has been linked to cardiovascular pathologies [1, 2, 3]. Research has shown that VSMCs comprise the majority of the cells within an atherosclerotic plaque [5]. The *tunica media* composition can vary in the quantity of VSMCs, elastin fibers, and collagen, depending on the location and function of the vessel [6]. Figure 1 displays blood vessel anatomy, as described above [6].

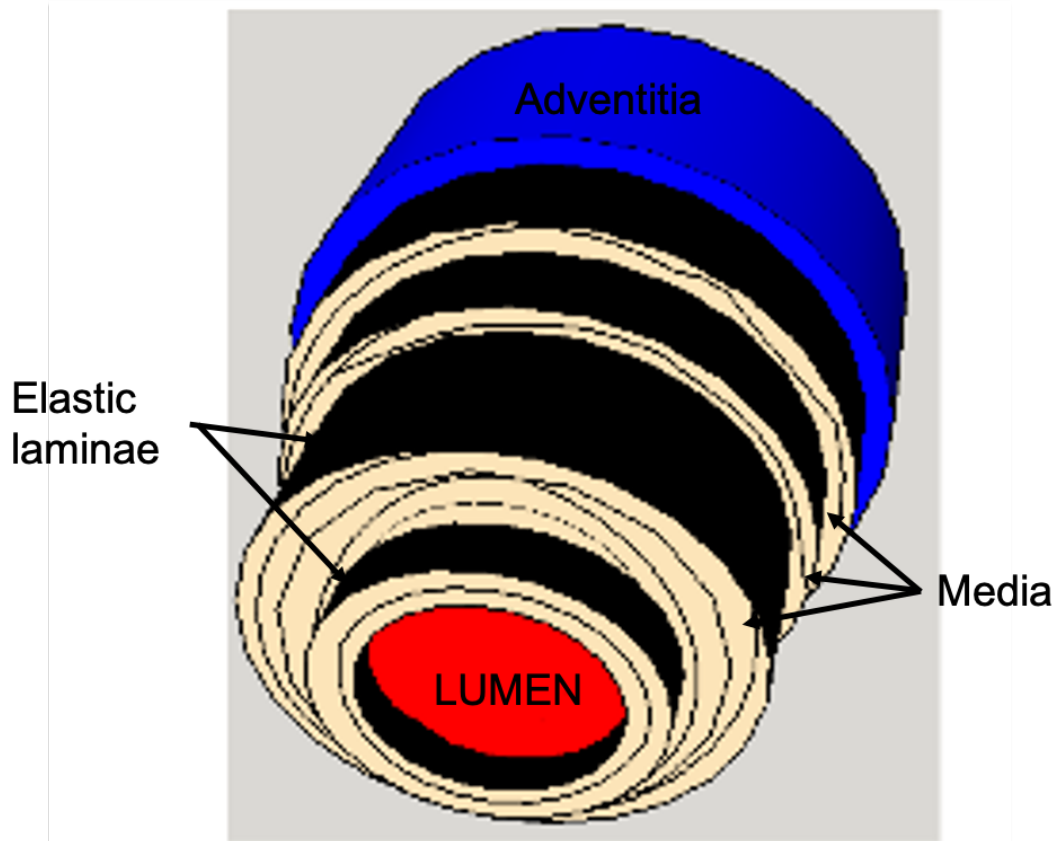


Figure 1. Blood vessel anatomy. The three layers of blood vessels: *tunica intima*, *tunica media*, and *tunica externa*. The *tunica externa*, the outermost layer, is primarily formed of collagen and elastic fibers used to maintain the vascular structure. The *tunica media* is composed of mononucleated, elongated VSMCs, elastic fibers, and collagen to support vessel structure and physiological functions. The *tunica intima* lines the lumen and is composed of a single layer of endothelial cells (ECs) [6].

Cardiovascular Disease and G Protein-Coupled Receptors

Cardiovascular disease remains the number one cause of morbidity and mortality in the United States and worldwide, resulting in approximately 31% of global deaths [7, 8]. The loss of life and decreased quality of life of individuals with CVD is also accompanied with a significant economic burden [4]. The direct and indirect costs of CVD are expected to rise above 700 billion annually by 2035 [4]. As morbidity, mortality, and the economic

consequences of CVD mount, the importance of developing targeted and effective treatments for CVD manifestation is paramount.

G protein-coupled receptors have been identified in numerous cardiovascular settings and are the target of approximately 34% of FDA-approved drugs [9,10]. G protein-coupled receptors are integral membrane proteins that are anchored via seven-transmembrane alpha-helical segments. Intracellular and extracellular loops connect these transmembrane segments [11]. The receptors, comprised of amino acids, begin at an extracellular N-terminus and end at an intracellular C-terminus. The overall receptor structure arranges the transmembrane segments in the shape of a barrel to form a central cavity for ligand binding. The N-terminus also serves as a ligand-binding site in specific GPCRs for protons and other activators. The C-terminus is often phosphorylated (i.e., serine and threonine residues) to increase the binding of scaffolding proteins like beta-arrestin. Phosphorylation of the C-terminus often occurs as a result of receptor activation and generally initiates a cascade of intracellular G protein-coupled signaling events.

These receptors are characterized by their ability to act as second messengers by detecting outside molecules and activating intracellular pathways without diffusion of molecules. The specific intracellular subunits represent select activities of the receptors. The receptor is activated by an external signal, either a ligand or other signaling molecule, leading to a change in the receptor's conformation and activating intracellular G proteins. While inactive, the receptor remains bound to a heterotrimeric G protein with alpha, beta, and gamma subunits. Binding of an agonist results in a conformational change that

proceeds to the alpha subunit that exchanges GDP for GTP. The alpha subunit then dissociates from the beta and gamma subunits and interacts with other intracellular proteins to carry out receptor activity [12]. Beta-arrestin may be involved in signaling pathways, including the MAPK/PI3K system as well as receptor endocytosis. When GPCRs become stimulated, activation of the heterotrimeric subunits occurs. GPCR activity is managed through two beta-arrestin pathways. One pathway that may occur is binding the arrestin to the internal portion of the receptor to inhibit activation. The second pathway occurs by linking the receptor to internalization proteins that will move the proteins from the membrane and into cytosolic endosomes to either be destroyed or recycled later. Figure 2 displays differential GPCR signaling [13].

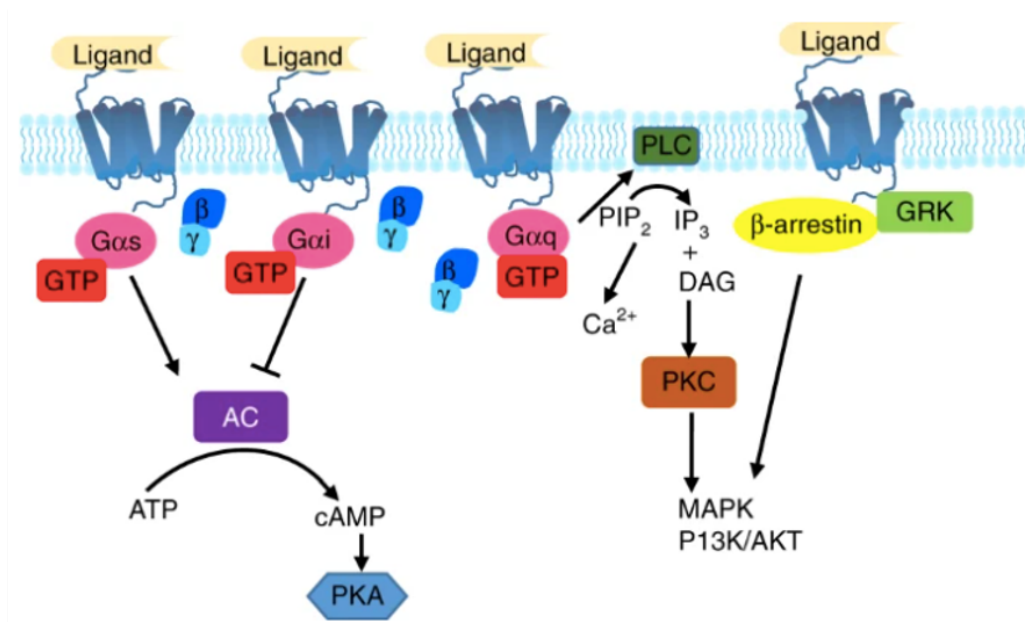


Figure 2. GPCR Signaling. Signaling of G protein coupled receptors (GPCRs) occurs through multiple pathways which all act as second messengers. The Gs and Gi pathways regulate cAMP through interactions with adenylate cyclase (AC). Through stimulation of PLC, Gq pathway results in increases of intracellular Ca²⁺, MAPK, and PI3K/Akt. Stimulation of the β-arrestin/GRK pathway converge on downstream MAPK and PI3K/Akt signals [13].

Protease Activated Receptors

Serine proteases have been identified as important contributors to vascular physiology and pathology. Proteases have been noted to contribute to cellular proliferation, migration, extracellular matrix (ECM) remodeling, and/or inflammation in response to injury [44]. During vascular distress, proteases may activate a particular subfamily of GPCRs known as serine protease-activated receptors (PARs). The unique mechanism of activation for PARs occurs through cleavage of the external amino terminus and exposure to peptide ligands, in turn leading to intracellular signaling [44-46]. Four members of the PAR family have been identified to date: PAR1, PAR2, PAR3, and PAR4. Specifically, PAR1, PAR3, and PAR4 are activated primarily by thrombin, whereas PAR2 is activated primarily by trypsin and mast cell tryptase [47].

Thrombin and trypsin are contributors to the coagulation cascade and serve as physiologically relevant activators of PARs [48, 49]. PAR1 has been studied widely and has provoked interest in assessing the remaining PARs as possible contributors of CVD [50-52]. PAR2 and PAR4 have been limited in their study but are theorized to be involved in VSMC disorders but remain unclear in their roles [50, 53-56]. Expression of PARs under normal physiological conditions may be limited, but upregulation of PARs and their activation under inimical conditions may be crucial to vascular pathophysiology, but this remains to be discerned.

Acidosis and GPR68

Extracellular acidosis is a direct result of reduced blood flow and subsequently reduced oxygen supply that results in localized hypoxia, and that creates a metabolic shift leading to proton (H^+) accumulation and membrane localization of carbonic anhydrase [19-21]. While oxygenated, vessel metabolism occurs predominantly through aerobic glycolytic mechanisms. During episodes of hypoxia, reduced oxygen leads to a shift from aerobic to anaerobic metabolism, resulting in the accumulation of intracellular lactic acid. As lactic acid increases, CO_2 levels rise, which stimulates carbonic anhydrase and generates H^+ and HCO_3^- [22]. As these levels escalate, the transport of H^+ to the extracellular space increases extracellular acidity and decreases extracellular pH [22-24]. The role of pH in cardiovascular function has been well known for over a century [25], although its regulatory influence on the pathophysiology of CVD remains unclear. Research into cellular pH has failed to consider the role of acidosis in an ischemic or hypoxic state and the importance of varying levels of acidosis precisely at the cellular membrane [26-28].

The theory of acidosis as a possible contributor to the pathogenesis and/or progression of CVD, as discussed, and perhaps other diseases (i.e., tumors) too has led to the identification of a subfamily of acid-sensing GPCRs [29-32]. Within this subfamily, GPR68 has been identified to be localized primarily in VSMCs and cardiomyocytes [33-35] and modestly in other cell types [33]. GPR68 operates through multiple signaling pathways and likely activates these pathways depending on receptor activity and ligand specificity in a highly context-specific and often biased fashion [36]. Maximal activation of acid-

sensing GPCRs occurs when pH levels fall to a range of 6.4-6.9 (normal range is 7.35-7.45) due largely to the protonation of extracellular histidine residues [18, 30, 37, 38]. Research results suggest the receptor involvement potentially regulates cellular proliferation in airway endothelial cells [22]. The heterodimerization of GPR68 with other acid-sensing GPCRs has also been noted [38]. While GPR68 has been studied in other environments outside of the cardiovascular system, the receptor has been reported to be primarily active within VSMCs [30, 34, 39, 40], yet its precise (patho)physiological functions have yet to be fully determined.

Multiple intracellular G protein signaling pathways have been identified for GPCRs. Pathways of interest include the signaling cascades of stimulatory G_s (adenylate cyclase/cAMP activation), inhibitory G_i (adenylate cyclase/cAMP inhibition), G_q (PLC/DAG/IP₃; MAPK/PI3K; Ca²⁺ stimulation), and $G_{12/13}$ (RhoA/Ras activation). Information on GPR68 is rapidly emerging but remains limited, and previous studies have given us a basic understanding of its role in vascular physiology; however, precise mechanisms for GPR68 in VSMCs remain unclear. Previous studies have suggested that GPR68 activates the cytokine interleukin-6 (IL-6) in the brain, increases bone density, regulates endoplasmic reticulum (ER) stress, and reduces cancer cell proliferation [38, 39, 41, 42]. One study in human VSMCs found that activation of GPR68 at acidic levels (6.3-6.8) increased levels of cAMP, calcium, and prostacyclin (PGI₂); however, in that study, precise cellular mechanisms were not identified [43]. A follow-up study on GPR68 in VSMCs suggested that the cellular response to extracellular acidosis might occur via

two separate pathways, a GPR68-dependent pathway and a GPR68-independent pathway [34].

This study aims to understand the potential role of the GPCRs PAR2 and acid-sensing GPR68 in regulating VSMC proliferation and their underlying mechanisms using in vivo surgical methods and in vitro models. We hypothesize that PAR stimulation, via serine proteases, and GPR68, through acidosis, via the G_s pathway promote proliferation and inflammation in VSM. With this information, insight into these crucial yet understudied GPCR pathways may provide targets of interest in CVD treatment and/or management.

Materials and Methods

Animals

Rats

All animal care and animal-based experimental procedures in this study strictly adhered to the guidelines established by the Guide for Care and Use of Laboratory Animals (National Research Council, revised 2011), the Public Health Service Policy on Humane Care and Use of Laboratory Animals (revised 2015), and East Carolina Institutional Animal Care and Use Committee. These studies utilized male juvenile (aged 3-5 months) Sprague-Dawley rats (Charles River Laboratories) within 400-450 grams body weight.

Mice

All animal care and animal-based experimental procedures in this study strictly adhered to the guidelines established by the Guide for Care and Use of Laboratory Animals (National Research Council, revised 2011), the Public Health Service Policy on Humane Care and Use of Laboratory Animals (revised 2015), and East Carolina Institutional Animal Care and Use Committee. These studies utilized female and male juvenile (aged 3-5 months) C57BL6/J mice (Jackson Laboratories) within a weight range of 20-40 grams body weight as wild type (WT) control mice. Knockout (KO) GPR68 colonies were established from cryoarchive from the Mutant Mouse Resource and Research Center,

initially established by Dr. B. Koller, UNC-Chapel Hill [57]. GPR68 KO mice were generated employing homologous recombination of the targeting construct of the GPR68 gene. GPR68 KO and WT homogenates from male and female mouse tail snips were evaluated through automated RT-PCR genotyping by Transnetyx, Inc. using GPR68 custom primers or neomycin primers (as a positive control in KOs).

Blood Pressure Measurement

Measurement of systolic and diastolic pressures and heart rates were measured using a noninvasive tail-cuff system (SC1000, Hatteras Instruments). Conscious male WT and KO animals of similar age and weight were placed on a warm platform at approximately 37°C within a metal holding container to restrict movement. A tail-cuff was placed on the tail, which was immobilized with tape. The minimum pulse amplitude was set to 20%, systolic threshold to 5%, and maximum pressure to 200 mmHg to ensure proper pulse detection. Animals received five acclimatization cycles before data were recorded for ten measurement cycles. Measurements occurred at approximately the same time every day for four consecutive days.

Mouse Left Carotid Artery Ligation

Male mice (3-5 months) were given buprenorphine (0.05-0.1 mg/kg; SC) as an analgesic 10 minutes before being anesthetized with isoflurane (2-5%). The animal's legs were retracted, and the neck was shaved and sterilized using surgical iodine and 70% alcohol.

Using a sterile scalpel, a midline incision was made below the chin and down to the sternum. Using forceps, the fascia and glandular tissue were gently dissected away to expose the underlying muscular layer. The muscular layer was blunt dissected, separated, and retracted to expose the left common carotid artery. The artery was further dissected to expose more of the left common carotid artery down to the internal and external bifurcation and was isolated from connective tissue and nerves, including the vagus nerve. Once isolated, a sterile 6-0 Prolene suture was tied around the common carotid artery just proximal to the bifurcation using a surgeon's knot to result in complete vessel ligation and lumen obstruction. The muscular and glandular tissues were closed in layers, and the skin was sealed using Vetbond tissue adhesive. Animals were provided supplemental fluids and kept on a heated surface until complete recovery. Animals were returned to individual cages and were regularly monitored for the first 24 hours post-surgery. After 24 hours, animals were observed twice a day and given analgesic every day for up to three days. After twenty-four hours to four weeks, animals were euthanized, and tissues were harvested and analyzed for histology and protein analysis [58, 59].

Rat Carotid Artery Balloon Injury

Male Sprague-Dawley rats (400-450 grams) were given buprenorphine (0.05-0.1 mg/kg; SC) as an analgesic 10 minutes before being anesthetized using ketamine/xylazine cocktail (1 ml/kg). Similar to carotid surgery performed in the mouse, exposure of the left common carotid artery was achieved surgically, and a Fogarty 2 French embolectomy catheter (Baxter Healthcare Corp., Irvine, CA) was inserted into the carotid branch

through an incision. Once the catheter was inserted, it was advanced to the aortic arch and inflated. After inflation, the balloon was withdrawn three times to injure the vessel mechanically. After the mechanical injury was achieved, the catheter was removed, and the vessel was ligated. Animals were then euthanized, and carotid arteries were extracted after thirty minutes and processed for subsequent analyses [63].

Histological Analysis

Once tissues were collected, they were processed and stained for histomorphometry. Processing involves the tissue passing through a series of graded alcohols (70-100%) to ensure dehydration, and then tissues were cleared with xylene. The tissues were then infiltrated by liquid paraffin under vacuum. The tissues were placed in a plastic cassette labeled with their proper ID and were incubated overnight in liquid paraffin to be prepared for embedding the following morning. The cassette was removed from the liquid paraffin and placed on a heating block to avoid wax hardening. The tissue was placed in a stainless-steel base mold and filled with liquified wax. The tissue was positioned upright with forceps, with the prelabeled cassette positioned on top of the mold. The mold was placed on a cold plate to allow the wax to harden around the tissue. The mold was later removed once the wax had completely hardened around the tissue (≤ 5 minutes on a cold plate).

After tissues sat overnight, the wax was cut into a trapezoid shape around the tissue for orientation once placed on a glass microscope slide. The wax in the cassette was placed

on a rotary microtome and sliced to 5 μ m thickness. A ribbon was created with approximately 10-12 slices per sample and placed in a heated water bath, and then carefully placed on a glass microscope slide. Slides were placed on a slide warmer at 37°C overnight, followed by desiccation in a drying oven to ensure tissue adherence to the slide.

Tissues were processed using the Verhoeff-van Gieson (VVG) staining method of elastic tissue and were deparaffinized with xylene and rehydrated with graded alcohols ending in distilled water. Tissues were stained for 10-20 minutes until elastic fibers turn black, which was achieved using elastic staining solution containing alcoholic hematoxylin, ferric chloride, and Weigert's iodine. Tissues were then washed with running tap water for 1 minute and differentiated in a ferric chloride solution. Slides were rinsed again in running tap water for 1 minute and transitioned to Van Gieson staining solution for 3-5 minutes. Tissues were then dehydrated through graded alcohols and cleared in changes of xylene, air dried, and coverslipped in Permount. After staining, tissues were color photographed, and photomicrographs were electronically saved. Once saved, photos were transferred to ImageJ software and analyzed for histomorphometry [60].

Tissues were processed using Masson's trichrome staining for collagen and were deparaffinized with xylene and rehydrated with graded alcohols ending in distilled water. Following deparaffinization and rehydration, tissues were placed in a Celestin blue-hematoxylin to stain nuclei. Staining was differentiated in a 1% acid alcohol, and tissues were rinsed in running tap water. Slides were placed in Solution A (acid fuchsin (0.5 g.),

glacial acetic acid (0.5 ml), distilled water (100 ml)) for 5 minutes, and rinsed with distilled water. Following incubation in Solution A, slides were placed in Solution B (phosphomolybdic acid (1.0 g.), distilled water (100 ml)), drained, and put into Solution C (methyl blue (2.0 g.), glacial acetic acid (2.5 ml), distilled water (100 ml)) for 3-5 minutes. Tissues were then rinsed with distilled water and treated with 1% acetic acid for 2 minutes. Tissues were dehydrated through graded alcohols and cleared in changes of xylene, air dried, and coverslipped in Permount [60].

Isolation of Mouse and Rat VSMCs

Male WT and KO mice and male Sprague-Dawley rats were given buprenorphine (0.05-0.1 mg/kg; SC) as an analgesic 10 minutes before being anesthetized using isoflurane (2-5%), supplied by the Department of Comparative Medicine, Brody School of Medicine, East Carolina University. Animals were placed in a supine position on a sterile surface and euthanized via pneumothorax and exsanguination. The thoracic aorta was surgically removed and mechanically digested. Tissue fragments were transferred to a digesting solution containing collagenase and elastase. The solution was incubated in a bead bath at 37°C for 2-4 hrs until the tissue fragments broke down into cellular components. The cell-containing solutions were then centrifuged at 400 rcf for 5 minutes at room temperature. After a cellular pellet had formed, the pellet was resuspended in 5 mL of Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher) containing 10% fetal bovine serum (FBS) and 0.2% Primocin (InvivoGen) and seeded into a T12 vented flask. The

primary VSMCs were incubated in 5% CO₂ at 37° untouched for 72 hrs to allow for adherence and spreading [61].

Cell Culture

Following the 72 hr incubation, cell confluence was evaluated using a Leica inverted microscope (DMI 4000B). Once 70 - 80% confluent, the primary VSMCs were passaged by aspirating media from the cells and washing twice with room temperature Dulbecco's Phosphate-Buffered Saline (PBS). Trypsin (0.25%) was added to the cells and allowed to incubate (5% CO₂ at 37°C) for 5 minutes. Following incubation, complete media (CM; DMEM containing 10% FBS and 0.2% Primocin) was added to the cells to halt trypsin activity resulting in a cellular suspension solution. The suspended cells were transferred to a T25 treated vented flask and left undisturbed for 48 hrs (considered passage 1 (p1)). This process was repeated up to p8. Cells were maintained and managed in a sterile environment to prevent contamination.

Hypoxia Chamber

For hypoxic exposure, cells were treated with CM for 24 hours at 10% CO₂, 1% O₂, and 89% N₂, then media was aspirated, and cells treated with ice-cold RIPA buffer (ThermoFisher) supplemented (1:100) with Halt protease and phosphatase inhibitor cocktail (ThermoFisher). Cells were treated with RIPA for 5 minutes at 37°C and removed from the plate using a cell scraper to release proteins into suspension. Protein

suspensions were collected and analyzed via gel electrophoresis (see protein analysis method below).

Cell Cycle Analysis

Cells were treated with complete media (CM; DMEM with 10% FBS and 0.2% Primocin) for 24, 48, or 72 hours, then media was aspirated, and cells were trypsinized. Cells were treated with trypsin (0.25%) for 5 minutes in 5% CO₂ at 37°C and cleaved from the plate, and released into suspension. Trypsin reactions were halted using CM, and cells were collected into Eppendorf tubes. Suspended cells were centrifuged at 500 rcf for 5 minutes, and pelleted cells were fixed in a solution of ice-cold 70% EtOH solution containing 30% PBS and incubated at 4°C for 10 minutes. Fixed cells were centrifuged at 1,000 rcf for 5 min and treated with propidium iodide (PI)/RNase Staining Solution. Cell cycle data were obtained using a Beckton Dickson FACScan flow cytometer and analyzed with ModFit LT software [50].

Cell Counting

Cells were treated with complete media (CM; DMEM with 10% FBS and 0.2% Primocin) for 24, 48, or 72 hours, then media was aspirated, and cells were trypsinized. Cells were treated with trypsin (0.25%) for 5 minutes in 5% CO₂ at 37°C and cleaved from the plate, and released into suspension. Trypsin reactions were halted using CM, and cells were collected into Eppendorf tubes. The cells were then diluted in a 1:10 concentration of cell

suspension to CM for cell number (viable cells) and viability percentages obtained for each treatment group using ViCell automated cell counting (BD) and software.

Protein Isolation and Analysis

Vascular smooth muscle cells were washed with PBS and trypsinized, centrifuged at 500 rcf, and cell pellets were washed twice with ice-cold PBS. Proteins were extracted from cells using ice-cold RIPA buffer (ThermoFisher) supplemented (1:100) with Halt protease and phosphatase inhibitor cocktail (ThermoFisher). Cell lysates were gathered and transferred to an Eppendorf tube and centrifuged at 14,000 rcf for 15 min at 4°C. Concentrations of protein were determined using the Bradford Protein Assay Kit (ThermoFisher).

Cell lysates with appropriate protein concentrations were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with stain-free blots. During gel electrophoresis, proteins migrated from large to small based on molecular weight and then were transferred to low fluorescence polyvinylidene fluoride (LF-PVDF) membranes with Trans-Blot Turbo RTA Transfer Kit system (Bio-Rad). Using the mixed molecular weight setting, proteins were transferred to PVDF for 7 minutes at 2.5 amps and 25 volts. Following the transfer, PVDF membranes were analyzed for total protein using the ChemiDoc MP Imaging System (Bio-Rad). To prepare for primary antibody treatment, membranes were blocked for 1 hour at room temperature in 5% dry milk solution mixed with Tris-buffered saline with 0.1% Tween-20 (TBST). After 1 hour, membranes were

washed three times using TBTS for 10 minutes at room temperature. Membranes were incubated overnight at 4°C with primary antibodies prepared within 5% bovine serum albumin (BSA) in 0.1% TBST using 1:500-1:1000 concentrations (per antibody) recommended by the manufacturer.

After the incubation (approximately 16-18 hours), membranes were washed three times with TBST for 10 minutes each. Following washes, membranes were incubated at room temperature for 1 hour using 1:10,000 horseradish peroxidase (HRP)-conjugated secondary antibodies. Once more, membranes were washed three times for 10 minutes each with TBST following secondary antibody incubation and were developed using West Pico substrates (ThermoFisher). Chemiluminescence was utilized for protein detection with the ChemiDoc MP Imaging System. Detected protein was quantified using Bio-Rad ImageLab software and normalized to total protein [62].

Cellular Fractionation

Cells were trypsinized and centrifuged at 500g for 5 minutes. Cell pellets were washed with ice-cold PBS and re-centrifuged twice. Cells were then treated with recommended reagents at appropriate ratios and times per cell fractionation protocol (Subcellular Protein Fractionation Kit; Thermo Scientific). Cytoplasmic fractions were first extracted after centrifugation at 500g for 5 minutes using a cytoplasmic extraction buffer (CEB). Second, the membrane extraction was obtained using membrane extraction buffer (MEB) and

gently mixing for 10 mins at 4°C, followed by a 3000g centrifugation for 5 minutes. The remaining nuclear and cytoskeletal fractionations were discarded.

Milliplex Assay

Vascular smooth muscle cells (in normal pH or acidic conditions) and whole tissue homogenates (injured and uninjured left carotid arteries (LCAs)) from WT and KO samples were analyzed for protein target interleukin-6 (IL-6). Using a Milliplex MAP Cytokine/Chemokine Panel (EMD Millipore, Billerica, MA), 25µl of protein extract was loaded into the assay plate to measure the concentration of IL-6. Once loaded onto the Milliplex assay plate, samples were run on a MagPix system (Luminex, Austin, TX). All results were analyzed using the Milliplex Analyst software (Version 5.1, EMD Millipore, Billerica, MA).

Statistics

Using GraphPad Prism, statistical analysis was performed with data displayed as a mean +/- standard error of the mean (SEM). Statistical analyses were performed with one-way ANOVA with Tukey's post-hoc tests to compare three or more groups. T-tests were performed for unpaired groups. A p-value < 0.05 was considered statistically significant for all comparisons.

Results

PARs in vascular injury

After mechanical balloon distension injury of the left carotid artery in male Sprague-Dawley rats, Western blot analysis was performed for PAR2 and PAR4 expression on arterial homogenates of sham-operated (naïve) or injured vessels after 30 minutes. A significant increase in PAR2 expression in injured vessels was shown compared to uninjured (naïve) vessels (Figure 3A). A noticeable (not significant; $p=0.16$) increase in PAR4 expression was observed in injured vessels compared to sham controls as well (Figure 3B). In figure 3B, it also appears that absolute expression levels of PAR4 in both control ($p=.03$) and injured ($p=.01$) vessels was significantly higher compared to respective absolute PAR2 expression levels. Using the expression of phosphorylated Erk1/2 (p-Erk1/2) normalized to total Erk1/2 as an indirect readout of PAR2/4 activity, Western blot analysis of arterial homogenates 30 minutes post-injury showed increases in PAR activity compared to uninjured vessels (data not shown).

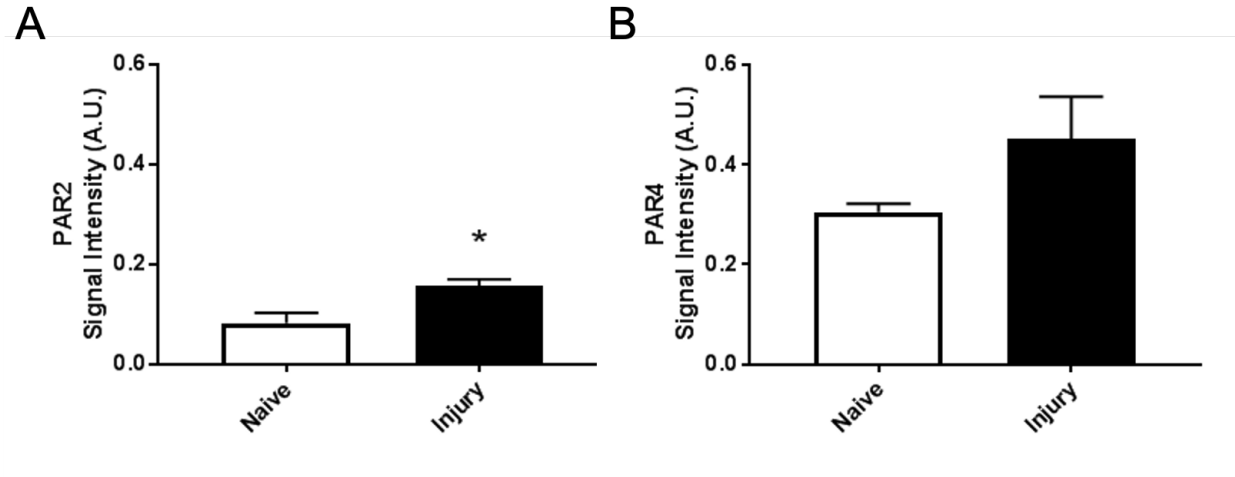


Figure 3: PAR 2 expression increases in the presence of vascular balloon injury. PAR2 and PAR4 Western blot protein expression following 30 minute balloon injury of the carotid artery. **A:** a significant increase in PAR2 expression in injured vessels compared to naïve (uninjured) vessels. **B:** a non-significant increase in PAR4 expression in injured vessels compared to naïve vessels. N=3/group., *p<05.

PAR2 expression in VSMC growth

Given that PAR2 expression was significantly upregulated following rat vascular injury (Figure 3A), PAR2 signaling under stimulated growth conditions was further analyzed in vitro using rat primary VSMCs. Cells were quiesced for 24 hours and then treated with growth media (10-20% serum) for 10, 30, or 60 minutes, after which cell fractions were prepared. PAR2 showed a significant ($p=.01$) increase in membrane expression after 10 minutes, with a return to baseline levels after 30 and 60 minutes (Figure 4A & B). No significant changes were observed in PAR2 cytosolic expression despite noticeable (~50%; $p=.09$) decreases at 60 mins (Figure 4C & D).

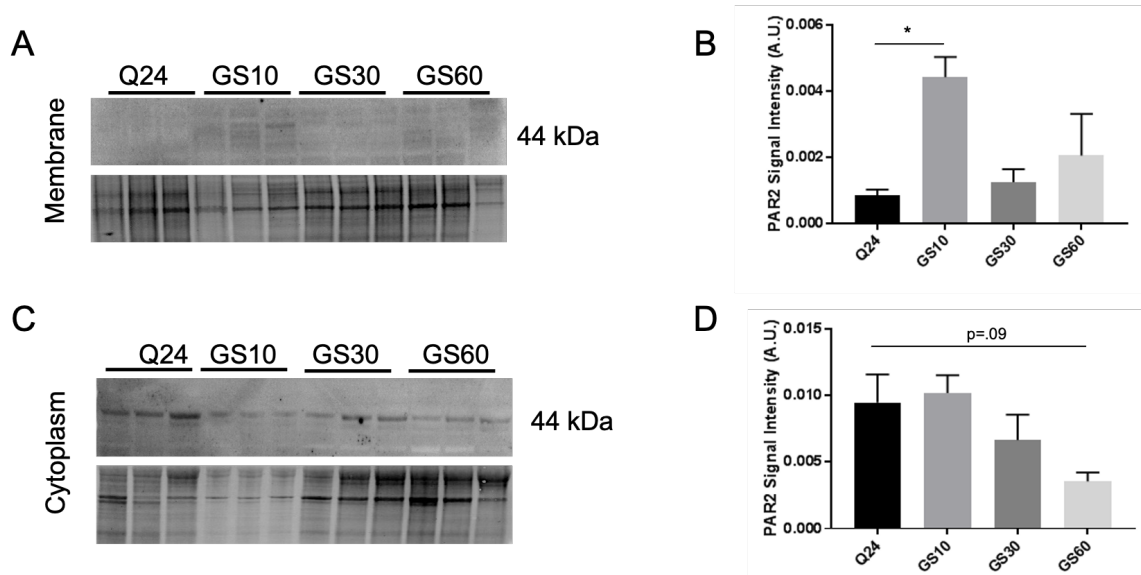


Figure 4: PAR2 expression increases at the plasma membrane in the presence of growth stimulation. Cells were quiesced for 24 hours (Q24) and treated with growth stimulation (GS) for 10, 30, or 60 minutes and cellular fractionation lysates were collected. **A:** PAR2 Membrane fractionation and total protein **B:** PAR2 is significantly increased at the plasma membrane after 10 minutes of growth stimulation with levels returning to baseline at 30 and 60 minutes. **C:** PAR2 cytosolic fractionation and total protein **D:** Cytoplasmic PAR2 expression exhibits no changes through 10 minutes with a decline through 60 minutes. Protein expression normalized to total protein. N=3-6/group, *p<.05.

Changes in PAR2 expression with pharmacological stimulation

To further examine the expression and activity of PAR2, a selective PAR2 agonist (SLIGRL) and PAR2 antagonist (FSLRY) were used in rat VSMCs. As previously described, cells were quiesced for 24 hours and then treated with media containing either agonist or antagonist for 10, 30, or 60 minutes. After treatment, whole cell homogenates and fractionated cell lysates were obtained. Using p-Erk1/2 normalized to total Erk1/2 as an indirect readout of PAR2/4 activity, preliminary Western blot analysis indicated that cells treated with SLIGRL at 1, 10, and 100 uM showed increases in PAR2 activity after 10 minutes with reduced activities after 30 and 60 minutes (Figure 5A). Cells treated with SLIGRL or FSLRY showed no significant changes in membrane fractions compared to nontreated cells (Figure 5B & C). Cytosolic fractions displayed a significant increase in

PAR2 expression in cells treated with SLIGRL compared to nontreated or FSLTRY treated cells (Figure 5D & E).

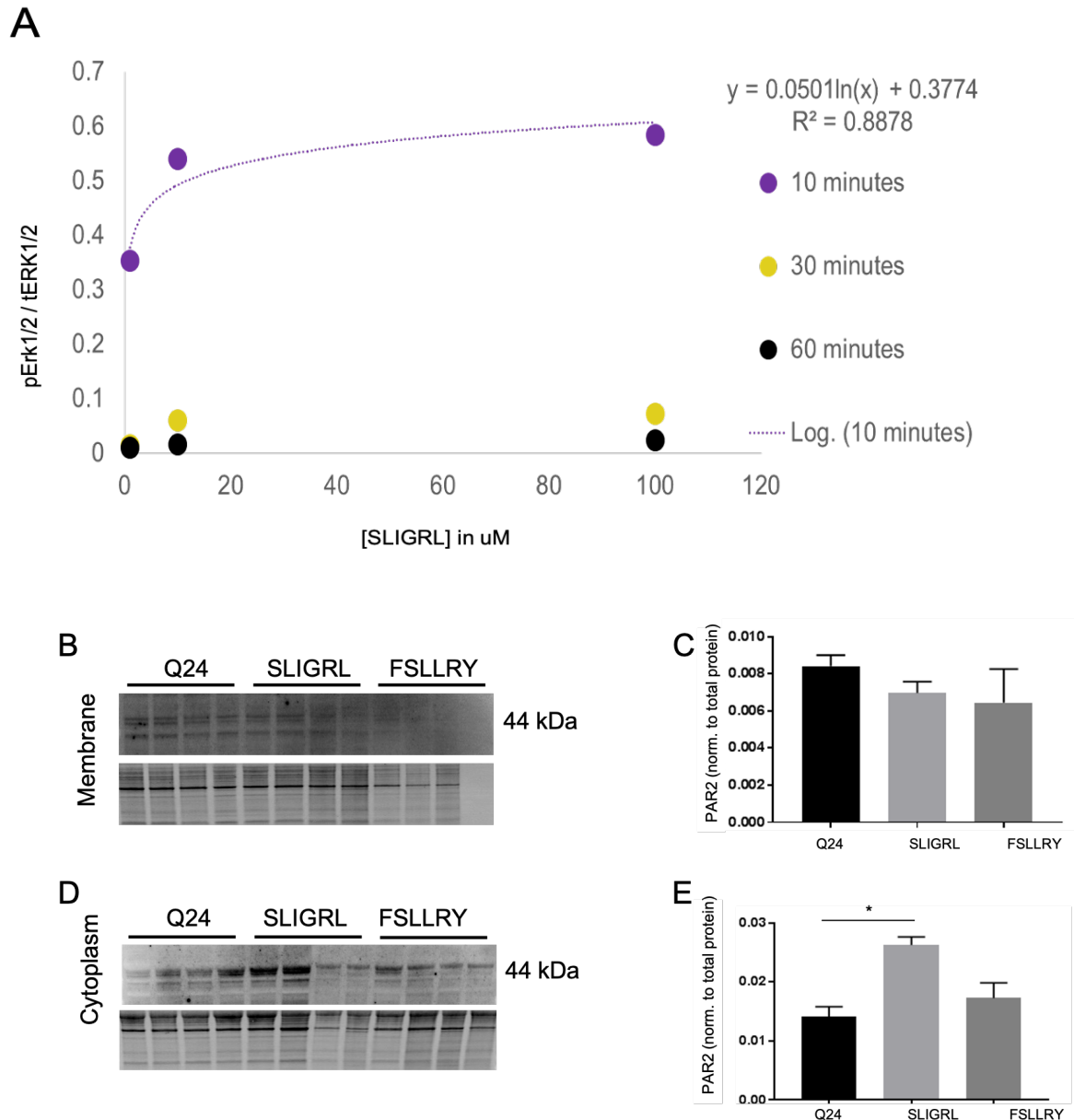


Figure 5: PAR2 expression increases in cytoplasm after pharmacological stimulation. Cells were quiesced for 24 hours (Q24) and treated with PAR2 agonist (SLIGRL) and antagonist (FSLTRY) for 30 minutes and cellular fractionation lysates were collected. **A:** VSMCs were treated with varying concentrations of PAR2 agonist (1-100 uM) for 0 – 60 minutes and probed for pERK/tERK expression for analysis of PAR2 activity. **B:** PAR2 Membrane fractionation and total protein **C:** No changes in PAR2 expression at the plasma membrane in the presence of agonist or antagonist. **D:** PAR2 cytosolic fractionation and total protein **E:** Cytoplasmic PAR2 expression exhibits a significant increase after treatment with agonist with no changes in cell treated with antagonist. Protein expression normalized to total protein. N=3-6/group, *p<.05.

β -arrestin and regulation of PAR2

Analysis of β -arrestin as a modifier of G_q signaling and as a mechanism for PAR2 endocytosis and degradation was performed following PAR stimulation/activation. As previously described, cells were quiesced for 24 hours and treated with growth serum for 10, 30, or 60 minutes. After 10 minutes of serum stimulation, membrane fractions displayed a nonsignificant ($p=.15$) increase in total β -arrestin expression that was observed through 30 minutes ($p=.08$) and returned to baseline at 60 minutes (Figure 6A & B). Notably, nonsignificant ($p=.10$) reductions in cytosolic β -arrestin at 10 minutes and 60 minutes were observed (Figure 6C & D). In cells treated with SLIGRL or FSLLRY for 30 minutes, significant increases in total β -arrestin levels at the membrane were observed in both groups ($p=0.03$; $p<.001$, respectively) compared to nontreated cells (Figure 6E & F). No significant changes were observed in β -arrestin expression in cytosolic fractions in these groups; however, a marked ($p=.20$) reduction in cytosolic β -arrestin was observed in FSLLRY treated cells compared to controls (Figure 6G & H).

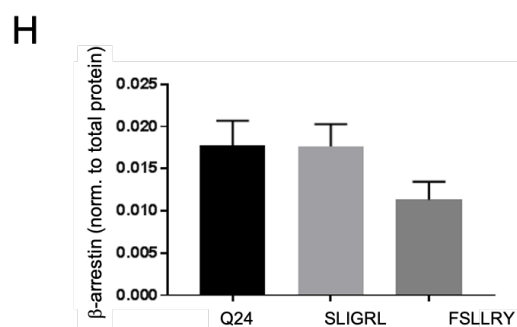
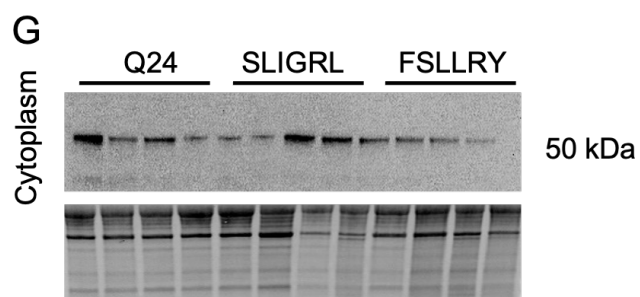
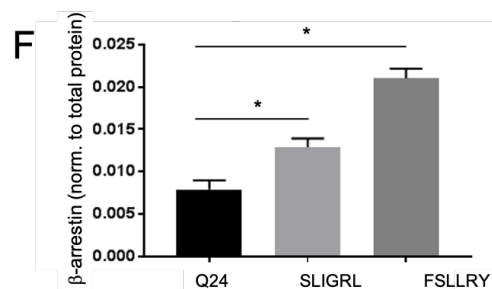
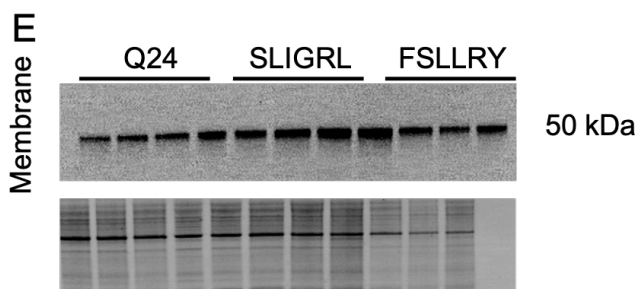
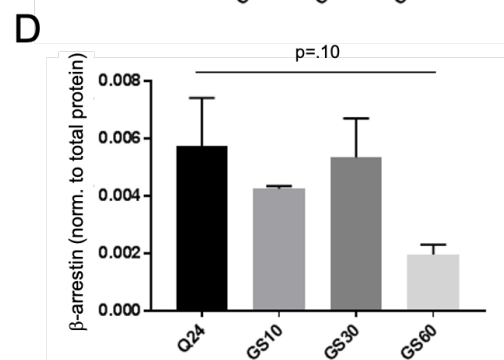
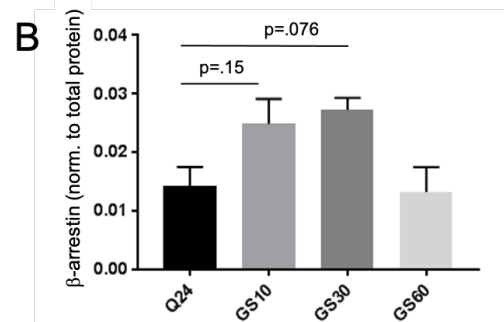
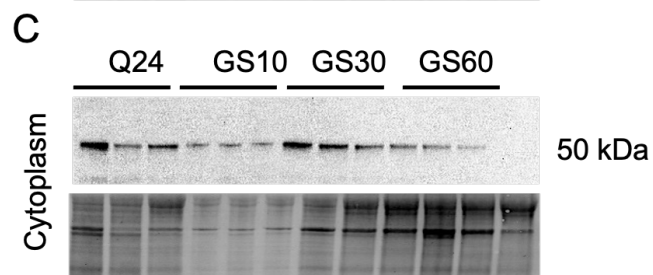
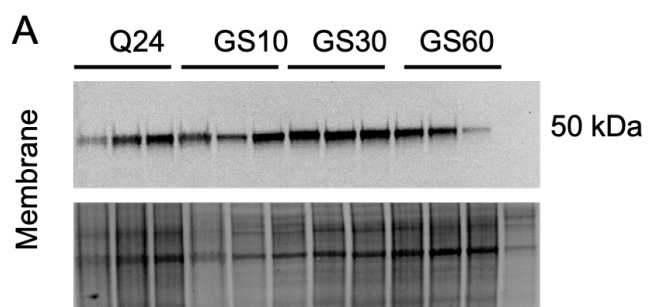


Figure 6: Beta-arrestin is induced at the plasma membrane following growth stimulation. Cells were quiesced for 24 hours (Q24) and treated with growth stimulation (GS) or PAR2 agonist (SLIGRL) or antagonist (FSLRY) and cellular fractionation lysates were collected. **A:** β -arrestin membrane fractionation and total protein **B:** β -arrestin is non-significantly increased at the plasma membrane after 10 and 30 minutes compared to quiesced cells and returned to baseline at 60 minutes. **C:** β -arrestin cytosolic fractionation and total protein **D:** No significant changes in cytosolic β -arrestin through 30 minutes; however, after 60 minutes a marked reduction is observed. **E:** β -arrestin membrane fractionation and total protein **F:** Membrane fractions exhibited significant increases in β -arrestin after treatment with SLIGRL or FSLRY. **G:** β -arrestin cytosolic fractionation and total protein **H:** No significant changes in β -arrestin in cytosolic fractions after treatment with SLIGRL or FSLRY. Protein expression normalized to total protein. N=3-6/group, *p<.05.

Preliminary data from our lab using BrdU incorporation as a measure of DNA replication suggested that VSMCs treated in 10% serum with SLIGRL for 24 hours displayed increased BrdU incorporation compared to untreated cells. To further investigate the role of PAR2 stimulation on DNA synthesis, cells were pretreated with kinase inhibitors and followed with treatment of SLIGRL for 24 hours. Cells treated with SLIGRL and concomitant inhibitors of PI3K, MAPK, or PKA experienced decreases in BrdU expression compared to those treated with SLIGRL alone (not shown). Following these early observations, PAR2 stimulation with SLIGRL also resulted in increased cell numbers compared to nontreated cells. When cells were also pretreated with inhibitors of PI3K, MAPK, or PKA, they displayed decreases in cell numbers compared to nontreated groups (data not shown).

GPR68 expression in vascular distension injury

Following our in vivo approach used to generate PAR2/4 data shown in Figure 3, balloon injury was performed on the LCA in male Sprague-Dawley rats to observe the influence of vascular trauma on arterial GPR68 protein expression. Left carotid arteries were harvested 30 minutes after injury or sham (naïve) operation, and whole vessel homogenates were prepared. Western blot analysis showed a significant (50%) decrease

in GPR68 protein expression in injured arterial homogenates compared to uninjured vessel homogenates (Figure 7A, B, &C).

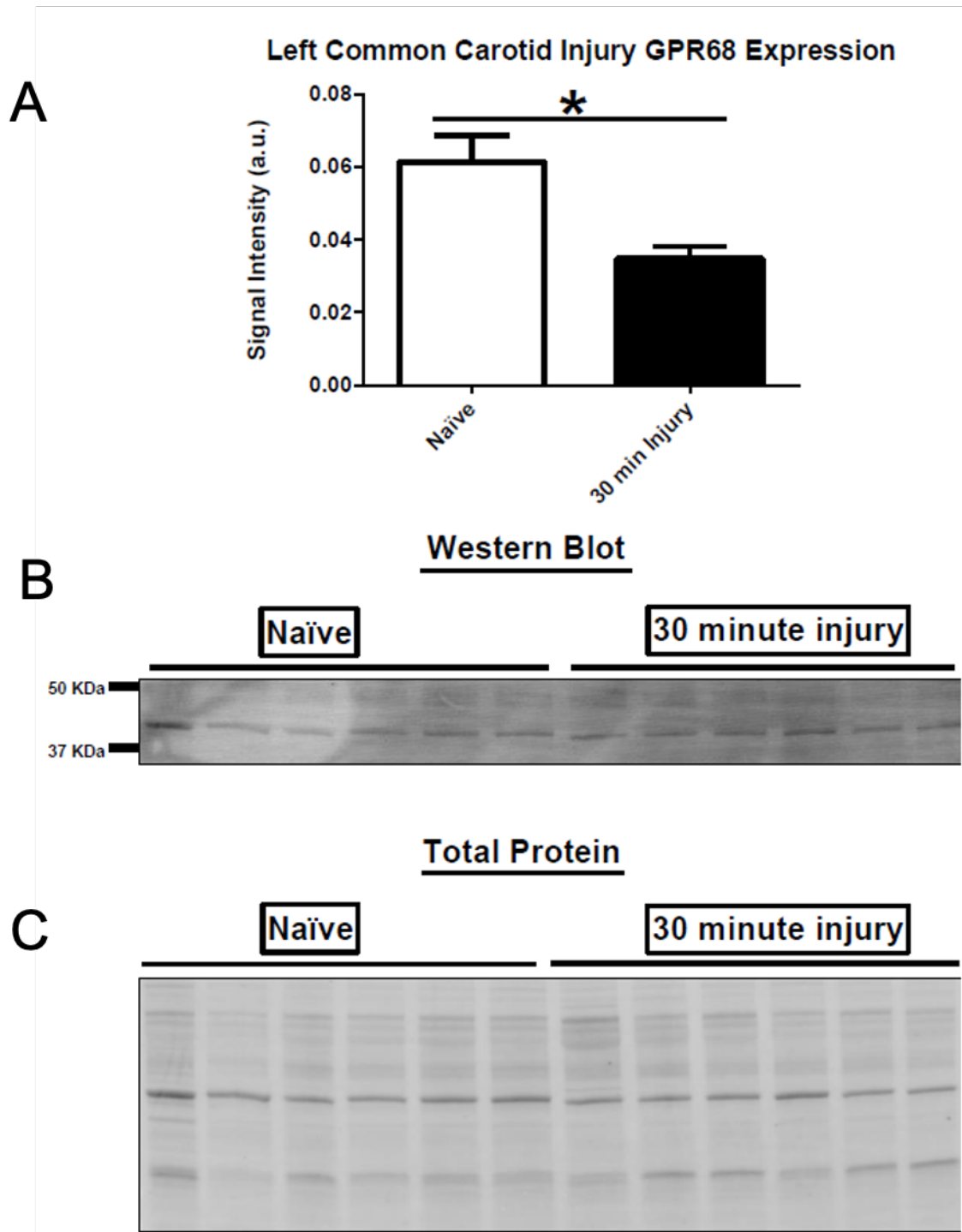
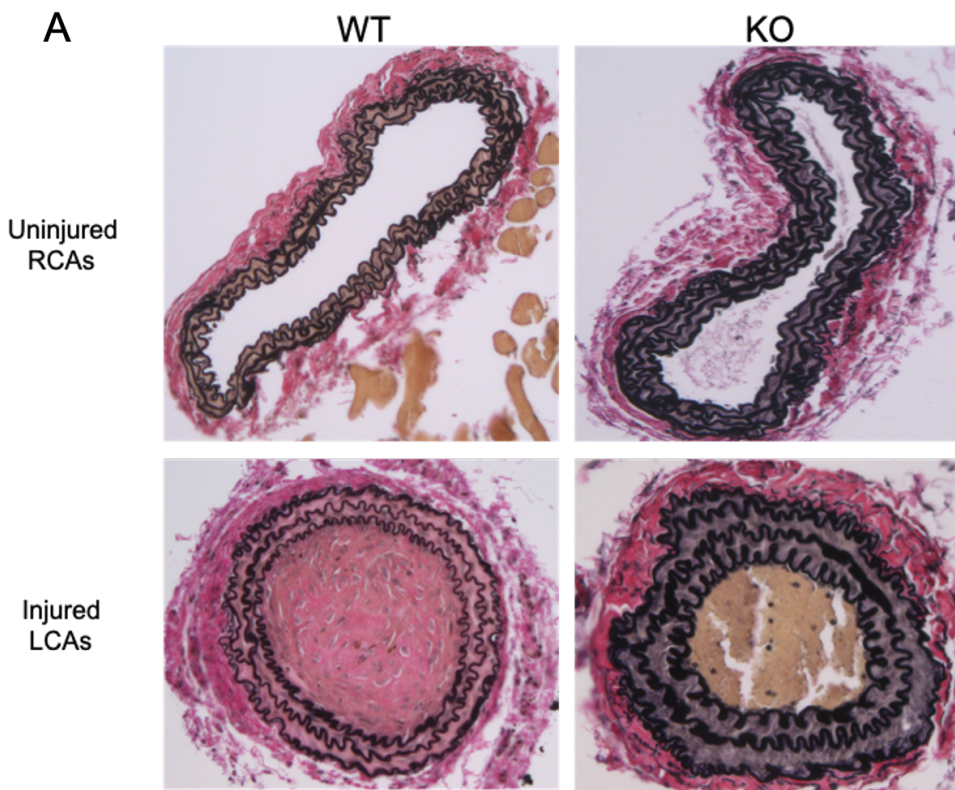


Figure 7: GPR68 is reduced following 30-minute balloon distension injury. **A:** GPR68 expression is significantly reduced in injured vessels compared to uninjured vessels. **B:** GPR 68 protein expression **C:** Total protein expression. Protein expression normalized to total protein. N=6/group, *p<.05.

GPR68 contributes to remodeling in the injured left common carotid artery

To more precisely explore the potential impact of GPR68 in vascular remodeling during induced ischemia in an in vivo setting, LCA ligations were performed in WT control, and GPR68 KO mice and tissues were harvested 4 weeks post-injury. Uninjured and injured vessels were assessed using histomorphometry for anatomic differences in the degree of vessel wall remodeling and neointimal development between WT and KO animals. Figure 8A displays VVG-stained vessel cross-sections of WT and KO injured and uninjured vessels. Both WT and KO uninjured vessels show a clear patent lumen area with no observable neointima and comparable medial wall areas. Injured WT vessels displayed robust, cellular-rich neointimal growth with near total occlusion of the lumen area, while the KO vessels showed little to no neointimal growth or lumen occlusion. Histomorphometry revealed a marked but nonsignificant ($p=.08$) reduction in neointimal area in KO vessels compared to WT vessels (Figure 8B). Of note, the mass located in the lumen of the injured KO vessel cross-section represents remnants of an acellular blood clot and is not neointimal tissue (based in part on lack of notable hematoxylin nuclear staining). No marked changes were observed in medial wall (MW) areas in both WT and KO vessels (Figure 8C), and in turn, MW areas were used to normalize neointimal areas on an animal-to-animal basis, and this neointimal to MW area normalization

showed a significant reduction in injured KO vessels compared to WT controls (Figure 8D). No significant changes in lumen area were observed in uninjured vessels; however, injured KO vessels showed a noticeable (non-significant) increase in lumen area compared to injured WT vessels (Figure 8E).



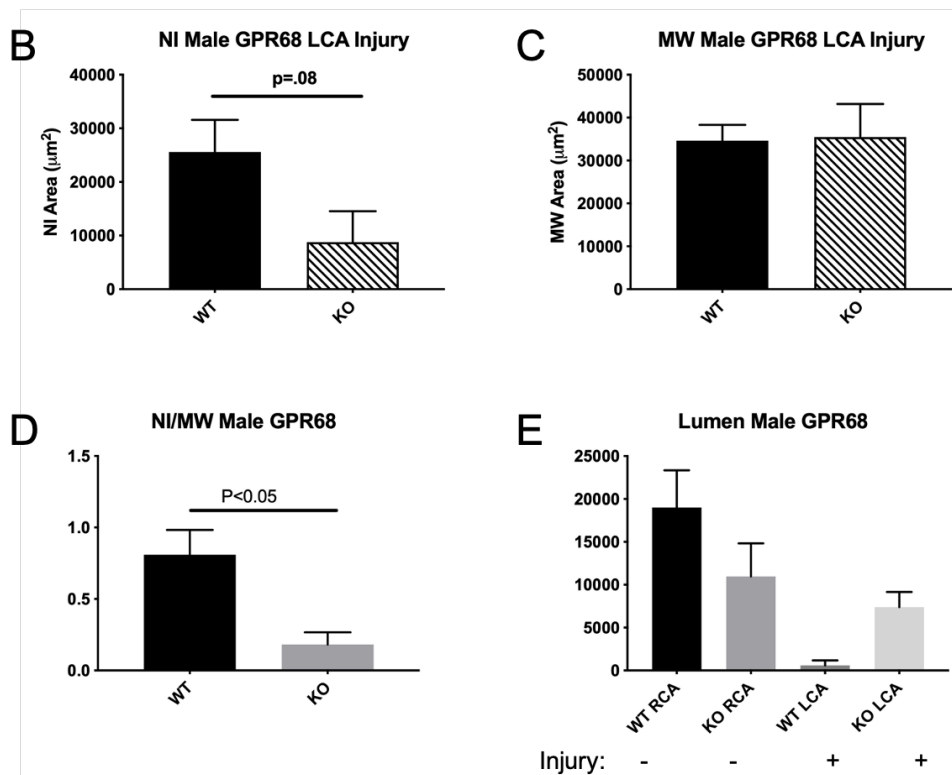


Figure 8: KO animals exhibit significantly less neointimal formation after carotid artery ligation. Both, WT and KO animal are treated with carotid artery ligation injury and tissues harvested four weeks post-injury. **A:** WT and KO injured vs uninjured Verhoeff van Gieson histological images. **B:** KO animals exhibit reduced neointimal(NI) formation compared to WT vessels. **C:** No differences exist between WT and KO medial wall (MW) size. **D:** KO animals display a significant reduction normalized NI area formation. **E:** WT injured vessels display a reduction in lumen area compared to uninjured and KO vessels. n=7 biological replicates per group; *p<.05 versus WT.

Phenotyping and genotyping GPR68 animals

Analysis of potential phenotypic differences between WT and GPR68 KO animals was performed to account for potential off-target systemic effects of GPR68 gene ablation and to provide confidence that the data observed using these models were a direct result of altered GPR68 expression. No marked changes in heart rate, mean arterial pressure,

systolic pressure, diastolic pressure, pulse pressure, or rate pressure product were observed across WT and KO groups (Figure 9A-F).

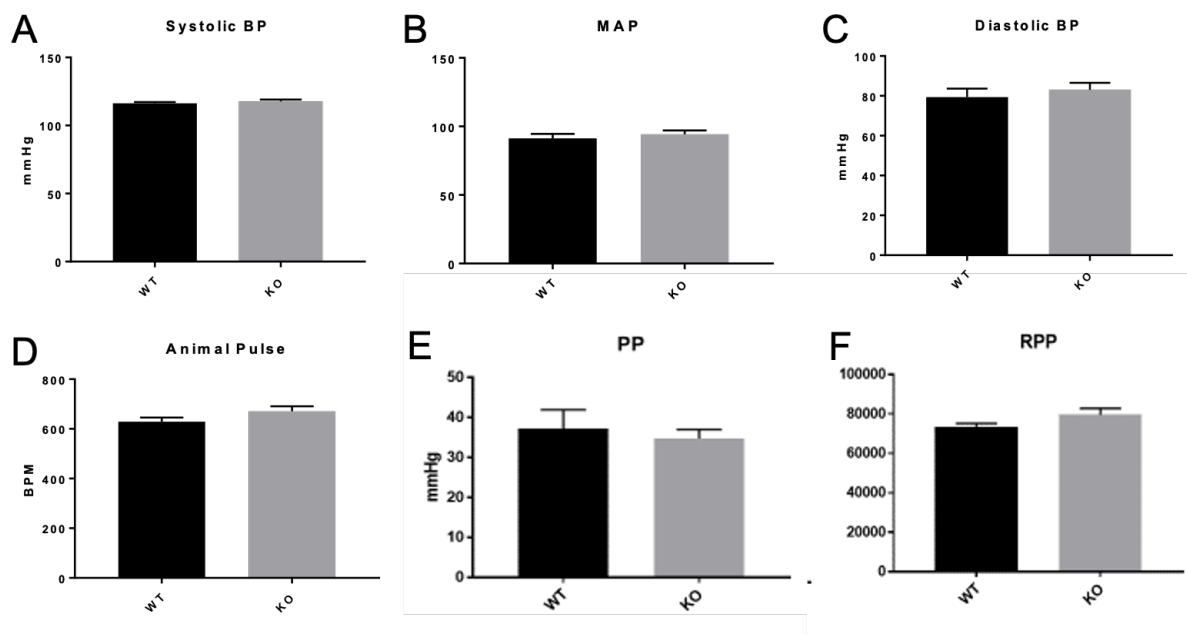


Figure 9: WT and KO animals exhibit no differences in cardiovascular phenotypes. WT and KO animals displayed no differences in cardiovascular phenotype readouts. n=3 biological replicates per group.

Complementing the phenotyping data, genotyping was performed by Transnetyx (an external genotyping vendor) to verify the genetic makeup of the WT and GPR68 KO mice. Tail snips from male and female mice, both WT C57BL/6J and GPR68 KO, were obtained and probed for GPR68 and neomycin genes. During the generation of the GPR68 KO animals, a neomycin cassette was inserted into the genome to serve as a positive control. When analyzed, GPR68 KO mice displayed a complete lack of GPR68 gene expression compared to marked GPR68 gene expression in WT animals (Figure 10). Conversely, WT animals showed an absence of neomycin gene expression while GPR68 KO animals

exhibited significant expression of neomycin. These data confirmed WT GPR68 gene expression and complete ablation of GPR68 in our GPR68 KO mice.

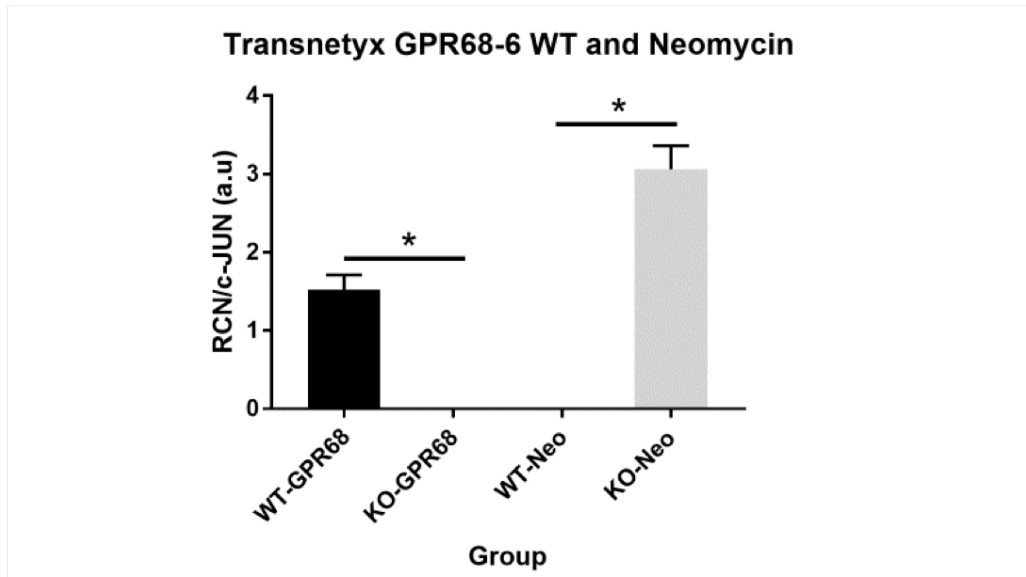


Figure 10: Genotyping data confirms knockout of GPR68. Using tail snips from WT and KO animals genotyping was performed via Transnetyx external vendor. A significant reduction in GPR68 expression is present in KO animals. A significant increase of neomycin, a positive control, is present in KO animals and absent in WT animals. Results are combined from tail snips and primary VSMCs. n=8-10 biological replicates per group; *p<.05

Cellular proliferation in WT and GPR68 KO VSMC culture

Cell numbers, as a direct measure of cellular hyperplasia/proliferation, as well as cellular viability, were analyzed using ViCell automated cell counting. Primary WT and KO VSMCs were initially observed with 10% growth serum for 0 through 48 hours. After 24 hours, WT and KO cells displayed no significant differences in cell numbers; however, after 48 hours, KO cells displayed a significant increase in cell numbers compared to WT

cells (Figure 11A). Viability was also assessed in WT and KO cells from 0 to 48 hours in 10% growth serum. Through 24 hours, no differences in cell viability were observed between WT and KO cells, but after 48 hours, WT cells displayed a significant reduction in cell viability compared to their counterpart KO cells (Figure 11B).

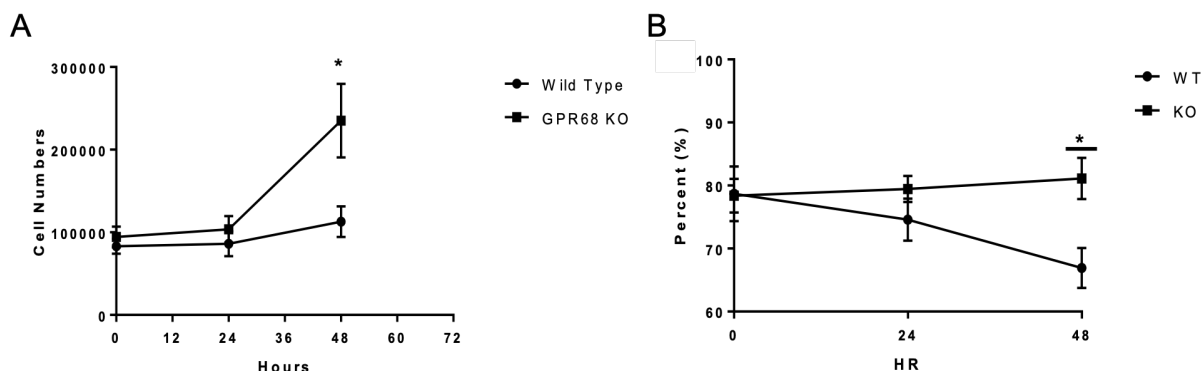


Figure 11: KO VSMCs display increased proliferation and cell viability. Cells were quiesced for 24 hours and treated with 10% growth stimulation through 48 hours and analyzed via ViCell automated cell counting. **A:** After 48 hours, KO cells exhibit a significant increase in cell numbers compared to WT cells. **B:** After 48 hours, KO cells exhibit a significant increase in cell viability. n=6-10 per group; *p<.05 versus.

To confirm the use of 10% serum as a growth stimulator, these experiments were repeated yet with 20% growth serum and with an extended time frame up to 72 hours. After 24 and 48 hours, no differences were observed in cell numbers between WT and KO cells; however, after 72 hours, KO cells displayed a significant increase in cell numbers compared to WT cells (Figure 12A). Cell viability was then assessed for 0 to 72 hours in 20% growth serum in both WT and KO cells. After 24 hours, no changes in cell viability were observed; however, after 48- and 72-hours, KO cells showed lower cell viability (Figure 12B).

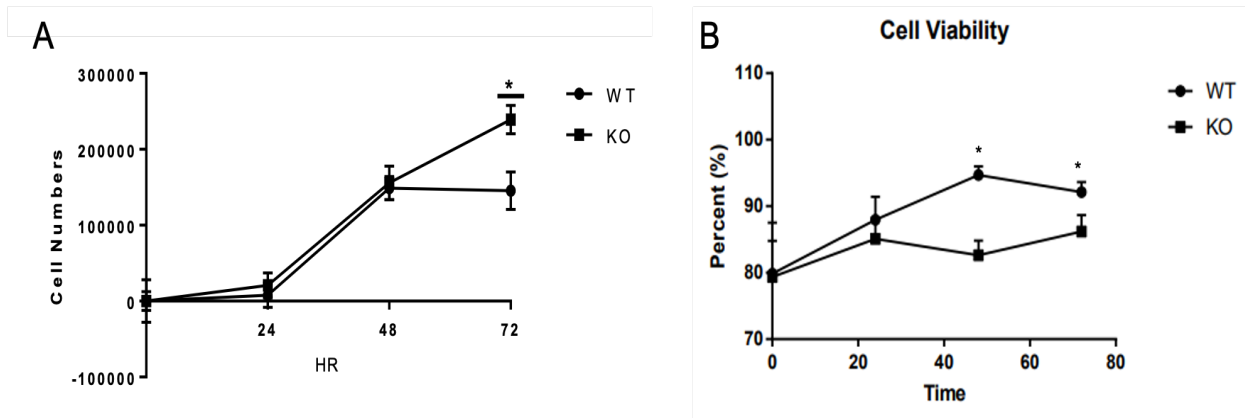


Figure 12: KO VSMCs display increased proliferation and cell viability after 72 hours. Cells were quiesced for 24 hours and treated with 20% growth stimulation through 72 hours and analyzed via ViCell automated cell counting. **A:** After 72 hours, KO cells exhibit a significant increase in cell numbers compared to WT cells. **B:** After 48 and 72 hours, WT cells exhibit a significant increase in cell viability. n=4-6 per group; *p<.05 versus.

Cell Cycle Progression

Analysis of cell cycle progression as a component of cell proliferation was performed via flow cytometry in 4-hour intervals starting at time 0 through 12 hours in the presence of 10% growth serum. At 0 hours, no significant differences between WT and KO cells for any cell cycle phase were observed; however, KO cells displayed a marked reduction in the number of cells in G₀/G₁ (p=.08) and a noticeable increase in the number of cells in G₂/M (p=.06) compared to WT controls (Figure 13A). After 4 hours, a significant reduction in KO cell numbers in the G₀/G₁ phase compared to WT cells was observed, with no changes evident across the other phases (Figure 13B). After 8 hours, once again, no significant differences were observed between WT and KO cells; however, KO cells displayed a marked increase in the G₂/M phase (p=.08) compared to WT cells (Figure

13C). After 12 hours, no significant differences were observed between WT and KO cells; however, KO cells displayed a noticeable increase in G₀/G₁ phase and markedly lower cell numbers in the S phase ($p=.14$) compared to WT cells (Figure 13D).

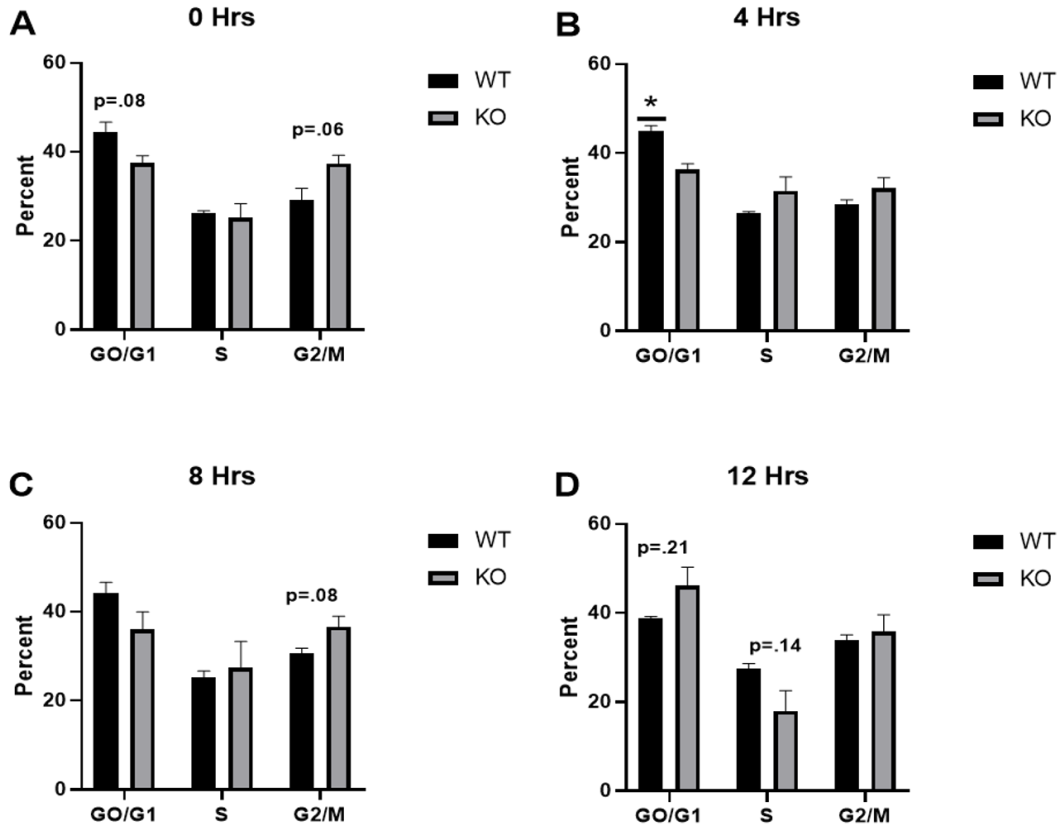


Figure 13: Cell cycle analysis suggest regulation of cell cycle via GPR68. Cells were analyzed in four-hour intervals through 12 hours via flow cytometry after treatment with 10% growth stimulation. **A:** Nonsignificant decrease in G₀/G₁ phase of KO cells with a nonsignificant increase in G₂/M phase of KO cells compared to WT cells at 0 hours **B:** A significant decrease in G₀/G₁ phase of KO cells with a nonsignificant increase in G₂/M phase of KO cells compared to WT cells at 4 hours **C:** Nonsignificant decrease in G₀/G₁ phase of KO cells with a nonsignificant increase in G₂/M phase of KO cells compared to WT cells at 8 hours **D:** : A significant increase in G₀/G₁ phase of KO cells with a nonsignificant decrease in S phase of KO cells compared to WT cells at 12 hours. $n=3$ per group; * $p<.05$ versus.

GPR68 signaling

To gain insight into the cellular signaling mechanisms behind GPR68 in light of its growth regulating properties, WT and KO VSMCs were treated with normal media (pH = 7.4-7.6) or acidic media (pH = 6.5-6.7) in the presence of IBMX, a broad phosphodiesterase (PDE) inhibitor, for 5 hours, after which whole cell lysates were prepared, and Western blotting was performed for Rap1A/1B, phosphorylated ERK1/2 (pERK), and total ERK1/2 (tERK) as plausible downstream effectors of GPR68. Wild-type cells treated with acidic media showed a significant reduction in Rap 1A/1B expression compared to WT cells in normal media. Knockout cells treated with acidic media showed a nonsignificant trend towards reduction of Rap 1A/1B expression compared to KO cells in normal media. When comparing WT to KO cells treated with acidic media, KO cells displayed significantly higher levels than WT cells (Figure 14A). Phosphorylated ERK/total ERK expression showed a noticeable but nonsignificant ($\sim 80\%$; $p = .12$) reduction in WT cells when treated with acidic media, while no significant differences were observed in KO cells in normal versus acidic media (Figure 14B).

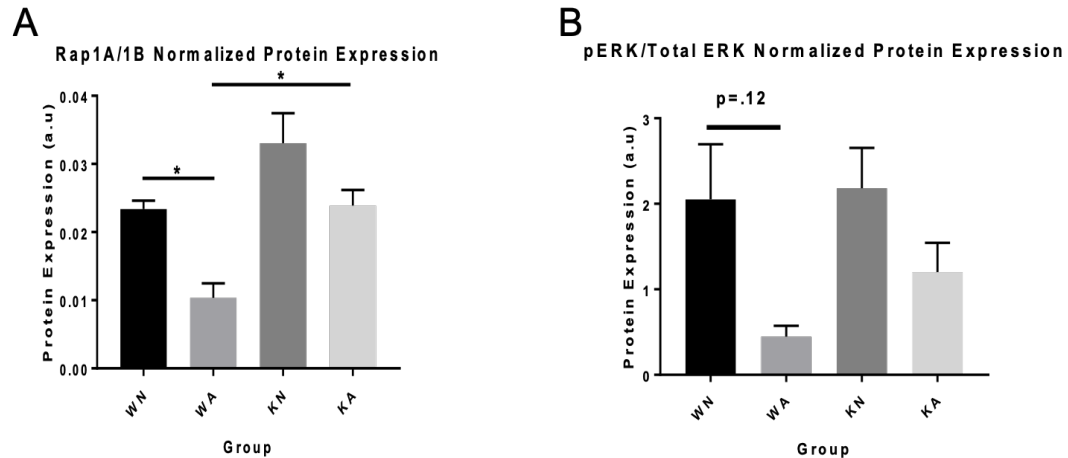


Figure 14: Treatment with acidotic media results in decreased expression of Rap1A/1B and ERK1/2. A: WT cells show a significant decrease in Rap1A/1B expression after treatment with acidotic media compared to normal media. **B:** WT cells show a nonsignificant decrease in ERK1/2 after treatment with acidotic media compared to normal media. n=3-6 per group; *p<.05 versus.

To look at potential changes in inflammatory interleukin-6 (IL-6) cytokine signaling following GPR68 activation, a Milliplex assay was performed on WT and KO VSMCs under normal or acidic media. Cells were treated with normal media or acidic media in the presence of IBMX for 5 hours, after which cells were harvested, and homogenates were prepared. Wild-type cells treated with acidic media showed a significant increase in IL-6 expression compared to all other groups, and notably, no differences were observed in KO cells under normal versus acidic conditions (Figure 15A). These results indicate a possible link between IL-6 and GPR68 signaling in the presence of acidosis. A Milliplex assay was then performed on whole tissue homogenates to further examine an IL-6-GPR68 relationship under in vivo injured conditions. Tissue homogenates were created for WT and KO LCAs following a 24-hour arterial ligation injury. Results showed

no marked changes in IL-6 LCA homogenates between injured WT and KO vessels (Figure 15B).

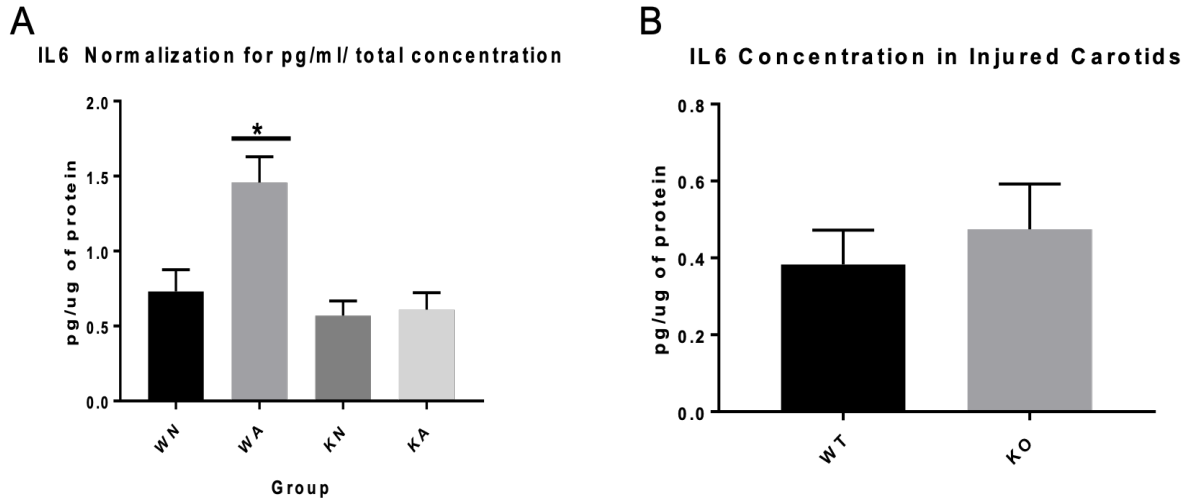


Figure 15: IL-6 increases in WT cells after treatment with acidotic media. Cells quiesced and treated with normal or acidic media for 5 hours and harvested. Vessels were treated with carotid artery ligation and harvested 24 hours post-injury. **A:** WT cells treated with acidotic media displayed a significant increase in IL-6 expression. **B:** No changes were observed between WT and KO injured vessels. n=3-6 per group; *p<.05.

GPR68 proliferation and signaling under hypoxia

Under a setting of hypoxia incubation (compared to normoxia controls), cell numbers were used again as a measure of proliferation and were analyzed using ViCell automated cell counting with trypan blue exclusion staining. Primary WT and KO VSMCs were observed following incubation in 20% growth serum for 0 to 72 hours under hypoxic (1% O₂) conditions. After 24 and 48 hours, WT and KO cells displayed no marked differences in cell numbers; however, after 72 hours, KO cells displayed a significant increase in cell numbers compared to WT cells (Figure 16B). Figure 16A displays data for cells grown in

normoxic conditions (copied Figure 12A) for comparison. Our data show that in hypoxia, WT cells result in marked increased proliferation compared to their normoxic counterparts, with no differences observed between KO cells.

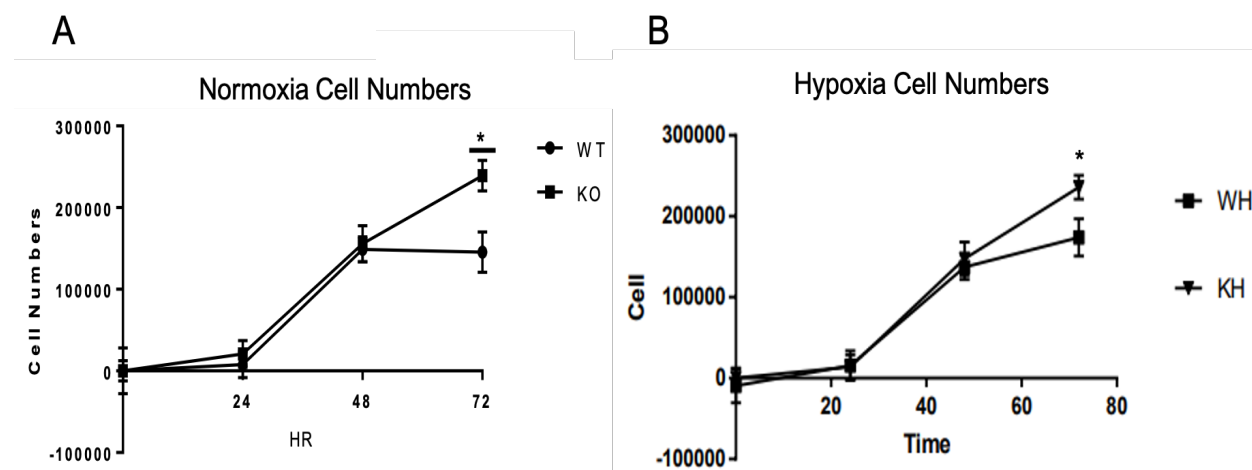


Figure 16: KO cells in hypoxia show increased cell numbers. Cells were quiesced for 24 hours and treated with 20% growth stimulation in hypoxia through 72 hours and analyzed via ViCell automated cell counting. In hypoxia, KO cells exhibited a significant increase in cell numbers at 72 hours compared to WT cells with no changes at 24 or 48 hours. n=4-6 per group; *p<.05

To analyze changes in GPR68 signaling in the presence of hypoxia, WT and KO cells were treated with 20% growth serum for 0 to 48 hours under normoxic or hypoxic conditions. At 24 and 48 hours, cell lysates were obtained, and Western blot analysis for phosphorylated ERK1/2, total ERK1/2, and β -arrestin was performed. At 24 hours, WT cells treated under hypoxic conditions showed a significant reduction in pERK/tERK expression compared to WT cells under normoxic conditions. Alongside, KO cells under hypoxic conditions showed a significant reduction in pERK/tERK expression compared to KO cells under normoxic conditions for 24 hours. Under hypoxic conditions, KO cells also

exhibited a significant increase in pERK/tERK expression compared to WT cells (Figure 17A). After 48 hours, no significant changes were observed in pERK/tERK expression between WT groups in normoxia versus hypoxia, although hypoxic WT cells displayed markedly (50%) lower levels of pERK/tERK compared to normoxic cells (Figure 17B). This expression pattern held true for the KO group comparisons. Under normoxic conditions after 48 hours, KO cells displayed a significant increase in pERK/tERK expression compared to WT cells, while under hypoxia, KO cells displayed a noticeable but nonsignificant ($p<.08$) increase in pERK/tERK expression compared to their WT counterparts.

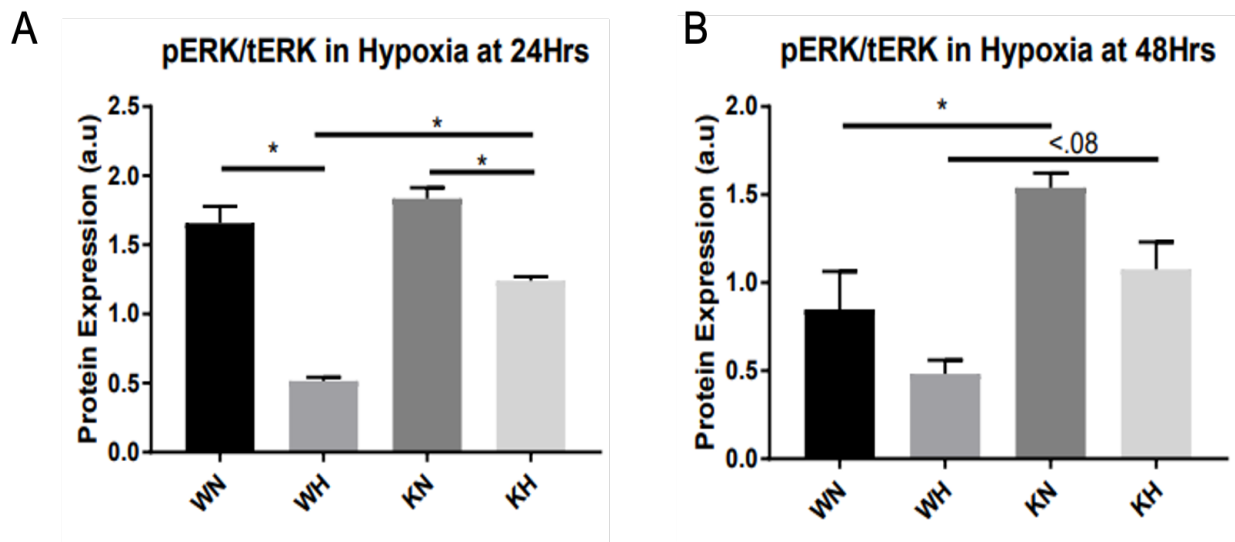


Figure 17: Treatment in hypoxia results in decreased expression of ERK1/2. **A:** WT cells show a significant decrease in ERK1/2 expression after treatment with hypoxia compared to normoxia at 24 hours. **B:** WT cells show a nonsignificant decrease in ERK1/2 after treatment with hypoxia compared to normoxia at 48 hours. $n=3$ per group; * $p<.05$ versus.

Analysis of β -arrestin as a mechanism for GPR68 endocytosis and degradation was conducted as a potential pathway of GPR68 regulation. Cells were collected after 48 hours of incubation in 20% serum, and cell homogenates were prepared and probed for total β -arrestin. No significant changes were observed across all WT and KO groups under normoxic or hypoxic conditions. Compared to other groups, WT cells under normoxic conditions displayed markedly (but not significantly) lower levels of β -arrestin (Figure 18).

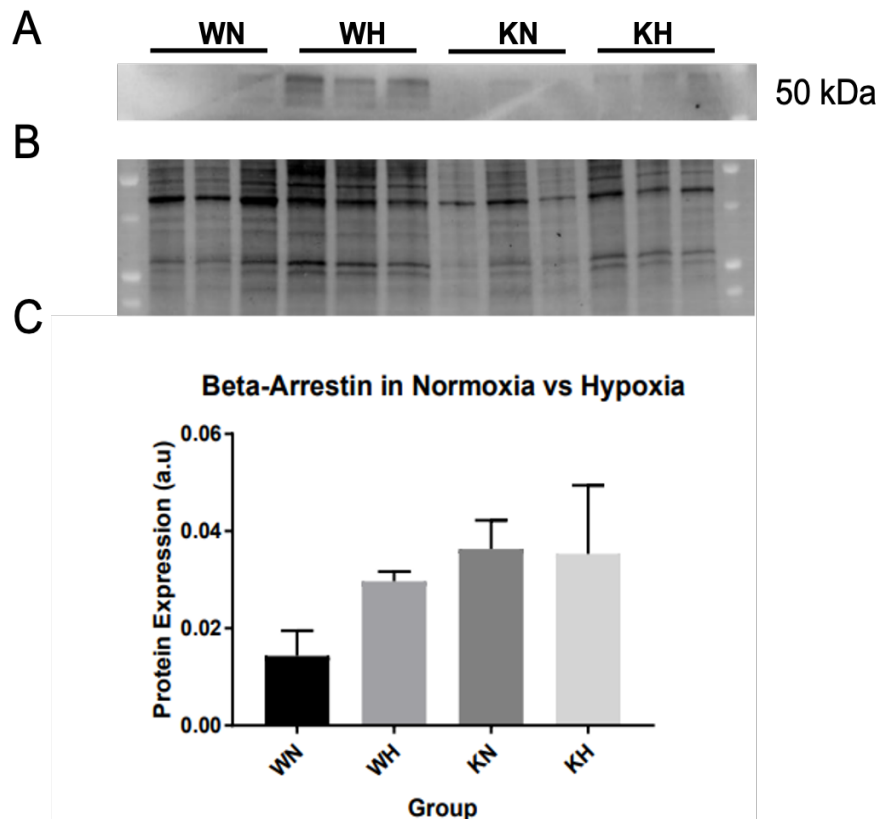


Figure 18: Beta-arrestin changes in normoxia vs hypoxia cells. **A:** β -arrestin protein expression **B:** Total protein expression **C:** WT cells show a non-significant, increase in β -arrestin after treatment with hypoxia compared to normoxia. KO cells in both hypoxia and normoxia show a non-significant, increase in β -arrestin compared to WT cells in normoxia. n=3 per group; *p<.05 versus.

Discussion

Cardiovascular disease remains the number one cause of morbidity and mortality in the United States and worldwide [4, 7, 8, 64]. Cardiovascular disease is responsible for over 800,000 deaths in the United States and around 18 million deaths worldwide annually [4, 7, 64]. The morbidity and mortality rates of CVD are also accompanied by a significant economic burden [4]. The direct and indirect costs of CVD currently exceed \$350 billion annually and are expected to rise above \$700 billion annually by 2035 [4]. According to the American Heart Association, the prevalence of CVD related illnesses in adults over the age of 20 is approximately 50% [4]. The frequency of CVD in men and women continues to rise with age; however, the prevalence of CVD is highest amongst men [4]. Given the national and international impact of CVD, it is essential to continue to elucidate the mechanisms behind CVD.

The development and progression of CVD is highly complex and intricate, consisting of numerous pathways and resulting in abnormalities in an otherwise normally functioning system. Cardiovascular disease complexities stem from multiple dysfunctions: genetic makeup, cellular structure, and cellular signals. A central facet of CVD pathogenesis is unregulated and abnormal VSMC growth resulting in vascular stenosis and downstream tissue ischemia [1, 2, 3, 14, 15, 65, 66]. Research continues to produce valuable studies that reveal therapeutic targets that can be used to treat CVD effectively; however, lack of complete understanding of cellular pathways and signaling mechanisms behind VSMC proliferation hinders clinical progression.

Currently, approximately 34% of FDA-approved drugs target GPCRs, as this highly diverse family of receptors contributes to numerous pathophysiological functions [9, 10, 14]. These integral membrane receptors are comprised of seven transmembrane segments connected via intracellular and extracellular loops [11]. Functionally, these receptors act as second messengers to activate a wide range of complex downstream pathways that control numerous cellular functions in multiple different environments. One specific GPCR subfamily is the protease-activated receptors, which have been studied widely and are gaining interest as possible contributors to CVD progression [50-52]. The first discovered was PAR1, which gained interest through its involvement in platelet aggregation and thrombogenesis [44, 52]. A partnership between PAR1 and PAR2 has been implicated in abnormal or diseased VSMCs and is thought to contribute to pathologic proliferation and inflammation [50, 67, 68]. While PAR2 and PAR4 have been linked to various VSMC disorders, their involvement in the pathology of VSMC growth lacks full understanding [45, 50, 55, 56, 69]. Proteases have been identified as critical contributors to vascular physiology, noted to contribute to cellular proliferation, migration, ECM remodeling, inflammation, and injury [44]. During vascular distress, liberated proteases have the capacity to activate PARs and stimulate GPCR signaling. Numerous intracellular G protein signaling pathways have been identified for GPCRs and the PARs including stimulatory G_s (adenylate cyclase/cAMP activation), inhibitory G_i (adenylate cyclase/cAMP inhibition), G_q (PLC/DAG/IP₃; MAPK/PI3K; Ca²⁺ stimulation), and $G_{12/13}$ (RhoA/Ras) [14, 70].

First, in this study, in vivo experiments were performed to determine changes in PAR2 and PAR4 expression in the presence of vascular injury, highlighting a potential role for PARs in vascular growth during distress. Male rat carotid arteries were injured using balloon-induced medial wall distension, and arterial homogenates were generated 30 minutes post-injury and probed for PAR2 and PAR4 expression [63] and Erk levels as a readout of PAR activity. Both PAR2 and PAR4 displayed increases in protein expression compared to uninjured vessels, yet a significant increase was only observed for PAR2 (see figure 3). Additional comparisons revealed that absolute PAR4 expression was significantly higher than that for PAR2 in both uninjured and injured vessels. Previous reports have shown changes in PAR2 expression in the presence of vascular injury; however, the focus of these studies was mostly on PAR1 [71-74]. Compared to previous literature, our results confirm induction of arterial wall PAR2 following vascular injury [75]. When analyzing PAR2, the previous study used immunostaining for PAR2 within cells to view differences in uninjured vs. injured vessels; however, this method is highly subjective and does not provide quantitative data compared to that obtained via Western blotting used in our study. Further, in this previous study [75], PAR2 was analyzed at longer time points, whereas our study used shorter time points to effectively elucidate acute PAR2 signaling as a potential mechanistic controller of injury-induced growth. Previously, phosphorylated Erk 1/2 to total Erk 1/2 has been used as an indirect measure of PAR activity [81, 82]. In this line, using p-Erk1/2 normalized to total Erk1/2 as an estimate of PAR2/4 activity, results showed that 30 minutes post-injury arterial homogenates displayed (nonsignificant) increases in PAR activity (not shown). It should be noted that

in vivo homogenates include multiple cell types outside of VSMCs, such as vascular endothelial cells that be involved in these observations in an intact setting.

After the discovery of significant PAR2 induction in the presence of in vivo vascular injury, in vitro experiments using rat primary VSMCs were conducted to analyze the induction of PAR2 in a controlled, growth-stimulating environment. As shown in Figure 4, quiesced cells were treated with growth-stimulating (10%) serum, and cellular fractions were analyzed at acute time points (10-60 minutes). Within 10 minutes, PAR2 expression increased significantly at the plasma membrane compared to non-treated (only quiesced) cells, and these levels returned to baseline after 30 and 60 minutes (see Figure 4B). These observations give reason to believe that after growth stimulation, PAR2 is quickly activated and shuttled to the membrane (where it can exert its receptor function), followed by rapid downregulation and internalization (perhaps by β -arrestin, discussed later). These observations support our in vivo data that show that arterial PAR2 is induced acutely (30 minutes) after growth-stimulating vascular injury.

To further validate these findings, a selective pharmacologic PAR2 agonist, SLIGRL, and a selective PAR2 antagonist, FSLLRY, were used in VSMCs to analyze PAR2 activity and regulation in the context of serum-induced growth. Our study confirmed that VSMCs treated with PAR2 agonist, SLIGRL, generated increases in phosphorylated ERK 1/2 to total ERK 1/2 in a time-dependent manner (Figure 5A), with robust induction of Erk after 10 minutes of PAR stimulation ($R^2 = 0.89$) at all concentrations used. Next, in VSMCs treated with SLIGRL, a significant increase in PAR2 expression is exhibited in cytosolic

fractions (see Figure 5D & E) with no changes seen in the plasma membrane (see Figure 5B & C). No changes were observed in PAR2 expression of cells treated with FSLLRY in both cytosolic and membrane fractions. This is consistent with previous reports, given that SLIGRL and FSLLRY are not biological molecules that respond in the same manner as proteases [76-80].

As previously shown, expression of GPCRs including PARs at the plasma membrane is both induced and downregulated quickly, likely due to β -arrestin modulation and control [9, 10]. To assess the possibility of β -arrestin as a regulator of PAR activity in our rat primary VSMCs, experiments were repeated in the presence of growth serum or following incubation with pharmacologic PAR modulators, and VSMC fractions were probed for β -arrestin expression via Western blot analysis. After 10 and 30 minutes of growth stimulation, cells displayed a nonsignificant (~doubling) increase in β -arrestin expression at the plasma membrane followed by a return to baseline levels after 60 minutes (see Figure 6A & B). These results provide evidence that shortly following growth stimulation and PAR2 membrane localization (Figure 4A, B), β -arrestin is quickly induced to downregulate PAR2 activity. In complement, we found that treatment with SLIGRL or FSLLRY for 30 minutes resulted in significant increases in β -arrestin at the plasma membrane (see Figure 6C & D). Previous literature has reported β -arrestin induction at the plasma membrane in HEK293 cells following treatment with SLIGRL; however, our data shows β -arrestin recruitment to the plasma membrane following independent pharmacologic agonism or antagonism in VSMCs [83, 84]. Blockade of ligand binding via FSLLRY, has also exhibited decreases in β -arrestin within cytosolic fractions. These

combined data suggest that PAR2 activation occurs quickly, promoting receptor localization to the plasma membrane, which is then rapidly downregulated via β -arrestin through internalization and degradation. Along with this, preliminary data (not included here) suggest that PAR2 agonism with SLIGRL results in increased BrdU incorporation and cell numbers. When PAR2 stimulated cells were treated with select kinase inhibitors, decreased PAR activity occurred in groups that corresponded to PKA, PI3K, or MAPK pathways. These data highly suggest that PAR2 stimulation and activity with respect to VSMC growth control are occurring through these kinase signaling pathways.

Following our study on PARs, we began to look at other complications that occur during the pathogenesis of CVD and other GPCRs that may be involved. As discussed, acidosis as a possible contributor to the pathogenesis of CVD led to the identification of proton-sensing GPCRs, including GPR68 [29-32]. Luminal occlusion contributes to reduced blood flow and ischemia of downstream tissues resulting in extracellular acidosis [1, 2, 3, 14-18]. Ischemia of tissues generates a metabolic shift, ultimately leading to proton (H^+) accumulation [19-21]. The role of pH in cardiovascular function has been well known for over a century [25], although its regulatory influence on the pathophysiology of CVD remains unclear. In this regard, the second subfamily of GPCRs was identified as a possible contributor to CVD progression; however, information is just now emerging on these proton-sensing GPCRs, and of specific interest in our research is GPR68.

To explore this concept of proton sensing GPR68, our initial experiments investigated in vivo responses to vascular injury. In this study, the rat carotid artery balloon injury and

the mouse carotid artery ligation were used as our vascular injury models. Our first model used rat carotid artery balloon distension as a follow-up to our initial experiments assessing PAR expression (see Figure 3), and we are able to analyze VSMC proliferation and migration following mechanical distension of the vessel [59, 63]. Previous literature demonstrated an increase of GPR68 in the presence of mechanical distension [85]. This surgical procedure consists of isolating the common carotid artery followed by insertion of a balloon catheter through an arteriotomy and then balloon inflation to generate vessel wall distension. Our results from this procedure showed a significant decrease in carotid artery protein expression of GPR68 compared to uninjured vessels after 30 minutes (see Figure 7). First, it should be noted that while this decrease in GPR68 expression was observed at an acute time point, this could represent a regulatory response, and GPR68 expression may indeed be upregulated at longer time points. Secondly, to our knowledge, distension injury of the vessel wall has not been linked to acidosis, and in our hands, we did not evaluate vessel wall or VSMC acidosis following surgical balloon distension. Finally, antibodies have been shown to be unreliable at effective detection of GPCRs [86]. This unreliability is likely due to the high transitory nature of active GPCRs and the homology between other GPCRs, including known epitopes. The activity of β -arrestin has been highlighted previously to quickly internalize and/or degrade GPCRs within minutes following stimulation [87].

In the mouse arterial ligation model, blood flow is stopped in the carotid artery to generate an ischemic and acidotic microenvironment in tissues downstream of the ligature [59]. This model allows us to provoke reduced flow and induction of vessel wall acidosis and

subsequently analyze these phenomena as a potential contributor to GPR68 activation and control of vascular cell proliferation. Using our WT and GPR68 KO mice, ligation of the left carotid artery was performed, and tissues were harvested after four weeks. From histomorphological analyses, we were able to demonstrate that KO animals displayed significantly less neointimal growth compared to WT vessels (see Figure 8). In these photomicrographs, injured WT vessels show a robust, cell-rich (based on nuclear staining) neointima with almost total lumen occlusion, while the injured KO vessels lack noticeable neointima (and instead, have an occlusive, cell-depleted thrombus). These observations suggest that GPR68 may positively contribute to VSMC proliferation in an in vivo ischemic/acidotic environment; however, in this context, we must consider the role of other cell types within the tissue or circulation in a whole-body setting as a potential influence. It is also important to note the difference in the time frame used in our in vivo and in vitro experiments. Our left carotid artery ligation tissues were harvested after four weeks, an optimal time to evaluate vessel wall remodeling from ligation [59] whereas our in vitro experiments generally ranged between 5 and 72 hours. The VSMC neointimal formation may result from a longer time frame in combination with influence from other cell types. The short time frame in vitro may lead to short-term GPR68 signaling that may initially regulate VSMC proliferation. In contrast, at longer time points, we see that this signaling cascade becomes altered in the continued presence of persistent ischemia, resulting in increased proliferation and neointimal occlusion. Interestingly, GPR4, a proton sensing receptor found in endothelial cells, has also been shown to heterodimerize and contribute to GPR68 signaling [110]. This study showed that GPR4 formed strong and weak homo and heterodimers with multiple types of receptors, and the authors concluded

that it is crucial to consider the status of associated receptors when studying a particular receptor of interest.

The use of KO animals allows us to analyze the effects that result from the exclusion of a protein of interest, in this case, the GPCR GPR68. In an effort to ensure the validity of our in vivo results and to rule out systemic, off-target effects of otherwise targeted gene ablation, differences in cardiovascular phenotypes between WT and KO mice were considered. It was essential for us to study these potential changes as previously WT and GPR68 KO mice have exhibited differences in osteoblasts and osteoclasts in the context of bone density as well as changes in insulin levels [41, 88]. Our measures included heart rate, systolic blood pressure, diastolic blood pressure, and mean arterial pressure (the arterial pressure within a cardiac cycle), and from these values, pulse pressure (the difference in systolic and diastolic blood pressure) and rate pressure product (a measure of cardiac work) were calculated for each individual animal. Evaluating these parameters allows us to identify potential systemic confounders and visualize vascular and cardiac work as potential contributors to our observations [89, 90]. Our results displayed no significant differences in cardiovascular phenotypes (based on these parameters analyzed) between WT and KO animals (see Figure 9), suggesting that our data is likely due solely to the genetic knockout of GPR68 and not to other systemic confounders.

Further validation of our results was obtained through the use of Transnetyx, an external genotyping vendor, to ensure that WT animals exhibited physiologically observable levels of GPR68 while KO tissues exhibited a lack of GPR68. Genotyping data revealed marked

GPR68 gene expression in WT animals (normalized to c-jun) while no transcript levels were detected in KO animals (see Figure 10). Moreover, during the generation of the KO animal colony, neomycin was inserted into the genome to serve as a positive control. Results showed that WT animals exhibited a complete lack of neomycin gene expression (normalized to c-jun) compared to KO animals that showed significant levels of neomycin.

Following in vivo results and confirmation of the genetic makeup of our mouse colonies, we analyzed the potential influence of GPR68 on in vitro VSMC proliferation and cell viability. Literature has shown that VSMC proliferation normally remains quiescent in mature vasculature, but in the presence of CVD, VSMC proliferation becomes uncontrolled due to a shift away from quiescence and towards a synthetic phenotype [3]. To conduct these studies, VSMCs were quiesced for 24 hours (in order to confirm all cells to a G₀ resting phase), and then cells were treated with 10% growth serum. After 24 hours of growth stimulation, no differences were observed in cell numbers or cell viability between WT and KO cells (see Figures 11A & B). However, after 48 hours, a significant increase was observed in KO cell numbers compared to WT cell controls. Alongside an increase in KO cell numbers, KO cells also displayed a significant increase in cell viability versus WT cells. These findings suggest that WT cells may not be progressing as well as the KO cells, perhaps due to insufficient growth stimulation; however, historically, our WT cells have responded well to 10% growth serum with an approximate doubling in population every ~24 hours through at least 96 hours post-plating. Nonetheless, considering these data and the relatively low numbers of our WT cells, we repeated these experiments in the presence of 20% growth serum over an extended period of time. After

24 and 48 hours, no differences in cell numbers were detected between WT and KO cells; however, after 72 hours, a significant increase in cell numbers was observed in KO cells compared to WT controls. Interestingly, it appears that the WT cell numbers remained relatively constant between 48 and 72 hours while the KO cells continued to increase in number through 72 hours. In terms of cell viability, while WT and KO cells showed significant differences at 48 and 72 hours, it should be noted that both WT and KO cells showed higher cell viability and a smaller gap between the groups after incubation in 20% serum versus 10% serum. A consistent increase in KO cell numbers compared to WT cell numbers suggests that GPR68 plays a cytostatic, regulatory role under growth-provoked, in vitro conditions. This growth inhibitory role for GPR68 then seems to be lost with genetic removal of GPR68, in turn leading to increased cell numbers witnessed in these data.

In an attempt to further discern WT and KO responses to in vitro growth stimulation, we analyzed cell cycle progression. In order to assess cell cycle progression, we utilized flow cytometry to examine WT and KO cells in four-hour intervals under 10% growth stimulation. Cell cycle progression allows us to account for the percentage of cells in three phases: G_0/G_1 (G_0 is a cytostatic, quiescent phase; G_1 is the first gap phase of initial cell growth), S (the synthesis phase responsible for DNA replication), and G_2/M phase (G_2 : second gap phase; M: mitotic phase; responsible for ensuring proper cell growth and mitosis) [91]. Our data reveal that WT cells initially show a higher percentage of cells in G_0/G_1 phase compared to KO cells beginning at 0 hours (see Figure 13A), while KO cells remain higher in the G_2/M phase. This trend continues through 4 and 8 hours (see Figure 13B & C). It is also worth noting that KO cell percentage in the S phase generally trends

higher than WT cells from 0 to 8 hours. We then see that after 12 hours, these trends reverse to a lower percentage of WT cells in G₀/G₁ phase and G₂/M phase with higher WT cell numbers in the S phase (see Figure 13D). These cell cycle data combined with cellular proliferation data give reason to believe that KO cells progress more quickly through the cell cycle compared to WT cells. We have previously described that under normal conditions, vascular proliferation is held in check; however, in the presence of a disease state, VSMC proliferation becomes uncontrolled due to a shift towards a synthetic phenotype [3]. In comparison, increased proliferation in KO cells has been shown in different cell types, such as caveolin-2 KO mice, who displayed increased cell numbers after 96 hours compared to WT controls [111]. Studies have also shown that KO of GPR68 resulted in an increased proliferation of epithelial and breast cancer cells [112, 113]. Another consideration is that GPR68 potentially halts cell cycle progression, as shown with GPR132, another reported proton-sensing GPCR [114]. Increases in cell numbers and viability among KO cells, along with a shift to S and G₂/M phases from the time of plating, suggest that GPR68 is involved in the inhibitory regulation of VSMC proliferation. This further provides evidence that knockout of GPR68 also results in increased proliferation and that GPR68 may be responsible for cytostatic control of proliferation in normal, quiescent conditions.

Based on our in vivo and in vitro results, we began to analyze particular proteins of interest that may be involved in intracellular GPR68 signaling in primary VSMCs. We know that GPR68 becomes maximally activated under acidic conditions around pH 6.8; however, normal blood pH ranges from 7.35 to 7.45 [38, 92]. In an effort to replicate acidotic

conditions in vitro, acidic media (pH 6.5-6.7) was specially prepared, and cells were incubated in either acidic media or normal, complete media (pH 7.4-7.6) for varying amounts of time. A theorized signaling pathway for many cellular signals for GPR68 involves G_s /cAMP. This pathway likely occurs following stimulation of GPR68 that increases the activity of adenylate cyclase (AC) to synthesize cAMP from ATP. The activity of cAMP proceeds through a family of cAMP-dependent kinases or through exchange protein activated by cAMP (EPAC), or the cyclic nucleotide is degraded via breakdown by PDEs. Much of the current literature points to the role of cAMP to decrease cellular proliferation, thus prompting our interest [93-97]. To explore the potential regulatory role of GPR68, we analyzed intracellular proteins of interest that may be involved in the cAMP pathway. The large body of data points to cellular proliferation occurring through G_s signaling, which is thought to be the pathway of interest for GPR68 [29, 57, 98]. Previous data in our lab, not shown in this study, indicated that WT cells treated with acidic media for 5 hours showed lower levels of cAMP compared to WT cells treated with normal media. These data also showed that GPR68 KO cells failed to respond to incubation in acidic media (compared to normal media) and that KO cells treated with normal media had reduced cAMP levels compared to WT cells in normal media.

It has been documented in different cell types that cAMP signaling may occur through EPAC as an effector protein to influence cell proliferation [99-102]. During our experiments, we experienced an inability to effectively probe for EPAC protein via numerous antibodies and Western blot analyses. Previous studies have highlighted the

role of the Ras-associated protein, Rap1A/1B, as a downstream target of EPAC, and Rap1A/1B has been indicated in growth signaling [103, 104]. In turn, we found that WT cells treated with acidic media for 5 hours in the presence of IBMX, a PDE inhibitor, resulted in a significant reduction in Rap1A/1B (see Figure 15A). A reduction in Rap1A/1B expression was also observed in KO cells treated with acidic media compared to KO cells in normal media; however, this reduction was nonsignificant. Further, in acidic media, KO cells showed significantly higher Rap1A/1B protein expression compared to WT cells. These data suggest that GPR68 signaling occurs at least in part through cAMP and EPAC to influence Rap1A/1B stimulation.

Following our studies of Rap1A/1B, we analyzed extracellular signal-regulated protein kinases 1 and 2, Erk 1/2, as a downstream target of Rap1A/1B. This Erk pathway can result in nuclear translocation and has been linked to transcriptional control of cell cycle progression and cellular proliferation. Further, ERK1/2 has also been shown to be linked to cAMP signaling in response to Rap1A/1B stimulation to influence VSMC proliferation [105]. We found that following treatment with acidic media, WT cells exhibited markedly lower levels of ERK1/2 compared to WT cells treated with normal media (see Figure 14). We also observed a nonsignificant reduction in ERK1/2 expression in KO cells treated with acidic media compared to KO cells in normal media. This protein expression data in VSMCs suggests that GPR68 likely occurs through G_s signaling but may also be a result of biased signaling [36]. Biased GPCR signaling can involve a variety of receptor and/or G protein modifications and can include, for example, a particular receptor switching between G protein subunits or a G protein changing its downstream intracellular

effector(s), all in a highly context-specific fashion. Our observations suggest that prolonged exposure to acidic media may result in biased signaling of GPR68, either through mitigated G_s signaling and/or enhanced G_i signaling, to reduce cAMP [36]. Studies analyzing GPR68 in VSM found that the presence of acidotic media resulted in increased expression of cAMP [43]. A second study looking at cancer-associated fibroblasts (CAFs) showed a decrease in cAMP levels in acidic media when GPR68 was knocked down via siRNA [115]. This study also showed an increase in cAMP after treatment with acidic media in control cells [115]. Following cAMP, EPAC has also been highlighted in the literature to both contribute and inhibit proliferation in a variety of cell types [99-102, 116]. Previous studies have highlighted the role of the Ras-associated protein, Rap1A/1B, as a downstream target of EPAC, and Rap 1A/1B has been indicated in growth signaling [103, 104]. Signaling of Rap1A/1B has also been linked to Erk 1/2 in the context of proliferation [105]. In WT cells treated under acidic conditions, we see reductions of cAMP, Rap1A/1B, and Erk 1/2, yet these changes are absent in the GPR68 KO cells, suggesting that GPR68 signaling likely occurs through a cAMP-EPAC pathway.

In addition to aberrant growth, inflammation plays a central role in the pathogenesis of CVD. In this respect, a signaling cascade of interest in our lab has been the cytokine interleukin 6 (IL-6), which has been shown to be linked to GPR68 [33]. Interleukin 6 has been identified to be induced under acidic conditions, and its expression may be increased through ERK1/2 signaling [33]. Interleukin 6 has been reported to be involved in immune and inflammation responses, with studies emphasizing that an increase in IL-6 presents an increased risk for myocardial infarction [103, 104]. In CVD, IL-6 has been

connected to atherosclerosis and ischemia/hypoxia in human coronary VSMCs [14, 108, 109]. Results from our in vitro studies displayed a significant increase of IL-6 expression in WT cells treated with acidic media compared to WT cells in normal media (see Figure 16A). In comparison, KO cells treated with either acidic or normal media showed no changes in IL-6 expression (see Figure 15B). These findings suggest that in the presence of acidosis, GPR68 stimulates the upregulation of IL-6 in VSMCs. Additionally, under in vivo conditions, WT and KO animals treated with LCA ligation injury displayed no marked differences in IL-6 expression. It should be noted that our in vitro cells were treated with acidic media for 5 hours while our in vivo tissues were observed 24 hours post-injury, a much longer time point in which any acute induction of IL-6 may have been returned to baseline. These data were also generated in whole arterial homogenates and do not take into consideration possible secretion of IL-6 and circulating IL-6 following tissue injury.

Lastly, to provide a more biologically relevant in vitro model that would mimic ischemic/hypoxic/acidotic conditions for analysis of WT and GPR68 KO comparisons, a hypoxia chamber was used. Reduced blood flow results in reduced oxygen in downstream tissues, ultimately leading to extracellular acidosis, as discussed [19-21]. To attempt to replicate conditions of ischemic tissues, following 24-hour quiescence, WT and KO cells were treated with 20% growth stimulation and placed inside a self-contained hypoxia chamber for 24, 48, or 72 hours, after which cell numbers were evaluated.

Our results mimic those observed earlier under acidic conditions (see Figures 11, 12) and showed that GPR68 KO cells continue to exhibit a significant increase in viable cell

numbers after 72 hours with no changes between groups at 24 or 48 hours (see Figure 16B). For ease of comparison, Figure 16A is copied from Figure 12A and shows data from cells grown under normoxic conditions. Next, cells were incubated in hypoxia (as described) and harvested and probed for ERK1/2 expression via Western blotting. After 24 hours, WT cells in hypoxia showed significantly reduced levels of ERK1/2 compared to WT cells in normoxia (see Figure 18A). Similarly, KO cells in hypoxia exhibited significantly reduced ERK1/2 induction compared to KO cells in normoxia. After 48 hours, a nonsignificant reduction between normoxia and hypoxia is observed between the WT groups as well as the KO groups (see Figure 17B). We also observed a significant increase in levels of ERK1/2 induction within normoxic KO cells compared to normoxic WT cells, thus supporting previous findings in this study. Lastly, after 48 hours, cells were probed for β -arrestin as a potential negative regulator of GPR68 activity. As seen in Figure 18, results displayed nonsignificant increases of β -arrestin expression in hypoxic WT cells compared to normoxic WT cells. However, KO cells failed to alter β -arrestin expression between normoxic and hypoxic conditions. These data suggest that β -arrestin is upregulated in KO cells and in the presence of hypoxia as a regulatory response and that this is at least partly dependent upon the presence of GPR68. It is worth noting that these data represent a prolonged time point (48 hours) and expression of total β -arrestin, which does not necessarily represent β -arrestin activity.

In conclusion, this thesis has served to highlight the roles of several understudied yet likely important GPCRs in VSM in the context of CVD pathogenesis: PAR2, a protease-activated receptor, and GPR68, a proton sensing receptor. Through this study, we have

been able to shed light on possible roles and signaling mechanisms behind PAR2 and GPR68 in VSM. With these data, foundations regarding potential mechanisms of PAR2 and GPR68 activation have been established, and further research should allow more in-depth analyses of these critical aspects underlying aberrant VSM in CVD.

We were able to confirm our hypothesis that PAR2 is induced in the presence of vascular distress and following in vitro growth stimulation. Following arterial balloon injury, PAR2 expression is significantly increased, which supports our theory. In vitro, we observed that PAR2 resulted in increased BrdU incorporation and cell numbers after serum stimulation. We also found that proliferation of VSMCs via PAR2 stimulation may occur through PKA, PI3K, or MAPK signaling pathways. It also appears that downregulation of PARs, and other GPCRs, may be achieved and largely modified by β -arrestin signaling. This information highlights the potential biological importance of PAR2 as a contributor to VSMC growth and may serve as a future therapeutic target to regulate proliferation in the context of CVD.

Similar to the model used for PAR comparisons, we used a balloon distension injury model and yet found decreased GPR68 expression following injury. We were then able to explore the role of acid-sensing GPR68 in the presence of in vivo vascular ischemia. Our data allowed us to observe that in the presence of an ischemic ligation model, lack-of/knockout of GPR68 results in decreased neointimal formation while WT animals display near total luminal occlusion. With no observable differences between animal

phenotypes and confirmation of KO genotype, our hypothesis that GPR68 stimulation leads to increased proliferation in the presence of vascular injury was validated.

Following our *in vivo* data, in a controlled *in vitro* environment, we discovered that following growth stimulation, KO cells experienced consistently increased proliferation and cell viability compared to WT cells. Further analysis of WT and KO cells showed an increased rate of cell cycle progression. This information initially conflicted with our hypothesis; however, literature has provided context that GPCRs may be able to operate through biased signaling based on their environment. Mechanisms of GPR68 have also been explored, pointing towards G_s signaling. In the presence of acidosis, WT cells have been shown to exhibit decreased levels of cAMP, Rap1A/1B, and Erk 1/2, suggesting that GPR68 signaling likely occurs through a cAMP-EPAC pathway. It is possible that in the presence of pathologic acidosis/ischemia/hypoxia, WT cells downregulate cAMP and lead to increased proliferation, but in the presence of normal/normoxic conditions, GPR68 regulates proliferation through stimulation of G_s -cAMP pathway. Data from our lab also suggest an upregulation of β -arrestin in cells treated with ischemia/hypoxia may be involved in the regulation of GPR68 as shown for PAR2. This information gives us reason to believe that in the presence of ischemia/acidosis, GPR68 may contribute to increased proliferation via downregulation of G_s pathways. It is likely that under cytostatic, quiescent conditions, cellular proliferation may be limited via G_s -cAMP pathway. This information highlights the potential biological importance of GPR68 as a contributor to VSMC growth and may serve as a future therapeutic target to regulate proliferation in the context of CVD.

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Appendix: Animal Care and Use Approval



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252-744-2436 office | 252-744-2355 fax

Animal Care and Use Committee

May 20, 2020

Dave Tulis, Ph.D.
Department of Physiology, ECU

Dear Dr. Tulis:

Your Animal Use Protocol entitled, " NO-independent cGMP regulation of vascular remodeling in mice" (AUP #Q261d) was reviewed by this institution's Animal Care and Use Committee on 05/20/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

A handwritten signature in black ink that reads "S. McRae".

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure



May 19, 2020

Dave Tulis, Ph.D.
Department of Physiology, ECU

Dear Dr. Tulis:

Your Animal Use Protocol entitled, "NO-independent cGMP regulation of vascular remodeling in rats " (AUP #Q360) was reviewed by this institution's Animal Care and Use Committee on 05/19/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

A handwritten signature in cursive script that reads "S. McRae".

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure

