

**EXPRESSION OF ADIPONECTIN RECEPTORS BY
VASCULAR SMOOTH MUSCLE CELLS**

A Thesis presented to the Faculty of the Graduate School
of the University of Missouri

In Partial Fulfilment of the Requirement for the Degree of
Master of Science

by

MEREDITH J STEVENSON

William P Fay, MD, Thesis Supervisor

DECEMBER 2009

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EXPRESSION OF ADIPONECTIN RECEPTORS BY VASCULAR SMOOTH
MUSCLE CELLS

presented by Meredith J Stevenson,

a candidate for the degree of Master of Science

and hereby certify that, in their opinion, it is worthy of acceptance.

William P. Fay, MD

Virginia H. Huxley, PhD

Steven S. Segal, PhD

Paul J. Fadel, PhD

Douglas K. Bowles, PhD

To Gene, Shirley Jean, and Brian Eugene

Be the matter.

ACKNOWLEDGEMENTS

I would like to thank my adviser, Dr. Bill Fay, for his support. Also, the members of my thesis committee: Drs. Virginia Huxley, Steven Segal, Douglas Bowles, Paul Fadel, and Michael Rovetto, for their guidance, advice, and support.

To the members of the Fay lab during my time there, Elizabeth Grunz, Tammy Strawn, Alexander Lohman, and Jianbo Wu. Also thanks to Yuping Huang, Frank Xu, Nadish Garg, and Hongmin Sun. Thank you for your tolerance, humor, support, and commentary.

To a group of others without whom I would have been lost several times over: Darla Tharp, Anastasia Sacharidou, Chady Hakim, Sue Bingaman, Jianje Wang, Esteban Fernandez, Stephanie Wise, and Lisa Grauer and Saj Parathath from Dr Edward Fisher's Lab at NYU.

TABLE OF CONTENTS

Acknowledgements.....	ii
List of Figures.....	v
List of Abbreviations.....	vi
Abstract.....	viii
Chapter 1: Literature Review	
Adipose tissue as an endocrine organ.....	1
Adiponectin.....	2
Adiponectin receptors.....	4
APN in the vasculature.....	6
APN and thrombosis.....	8
APN and PAI-1.....	9
Summary.....	10
Chapter 2: Methods	
Cell culture.....	11
Reverse transcriptase PCR.....	13
Real-time PCR.....	13
Laser capture microdissection	15
Chapter 3: Results	
AdipoRs are expressed by VSMC <i>in vitro</i> and <i>in vivo</i>	18
APN does not alter PAI-1 expression by HCAVSMC.....	22
Summary.....	25

Chapter 4: Discussion.....	26
Chapter 5: Future Directions.....	30
Appendices	
1: Detailed cell culture protocol.....	34
2: Detailed RNA extraction protocol.....	37
3: Quantitation of real-time PCR data using the $2^{(-\Delta\Delta Ct)}$ method.....	40
4: Primer Sequences and PCR Product Sizes.....	41
5: Detailed protocol for laser capture microdissection.....	42
6: Relative PAI-1 expression in HCAVSMC using SM α A as housekeeping gene.....	47
7: Pursuit of a better housekeeping gene.....	48
8: Relative SM α A expression in HCAVSMC.....	49
9: Expression of adiponectin receptors by vascular smooth muscle cells.....	50
Literature Cited.....	66
Vita.....	71

LIST OF FIGURES

Figure.....	Page
1. APN isoforms binding to AdipoRs.....	5
2. Summary of experiment rationale.....	10
3. Pictographic representation of LCM.....	16
4. Example of porcine coronary artery viewed and tunica media cells collected by LCM.....	17
5. End-point PCR of HCAVSMC lysates.....	18
6. Western blot of HCAVSMC lysates.....	19
7. End-point PCR of homogenized pig coronary artery segment.....	20
8. End-point PCR of lysates from cells collected from mouse aorta by LCM.....	21
9. Effect of 10 µg/ml rhAPN on PAI-1 mRNA expression in cultured HCAVSMC.....	23
10. PAI-1 protein in HCAVSMC lysates after 24 hrs.....	24
11. PAI-1 protein in CM of HCAVSMC after 24 hrs.....	25
12. Example of real-time PCR data.....	40
13. Relative PAI-1 expression in HCAVSMC using SMαA as housekeeping gene.....	47
14. Relative SMαA expression in HCAVSMC treated with rhAPN.....	49
Figures of Appendix 9.....	55-61

LIST OF ABBREVIATIONS

AdipoR—Adiponectin receptor

AMPK—Adenosine monophosphate-activated protein kinase

APN—Adiponectin

BSA—Bovine serum albumin

CAD—Coronary artery disease

cAMP—Cyclic adenosine monophosphate

cDNA—Complimentary deoxyribonucleic acid

CM—Conditioned media

eNOS—Endothelial nitric oxide synthase

HCAVSMC—Human coronary artery vascular smooth muscle cell

HMW—High molecular weight

ICAM—Intracellular adhesion molecule

IL-6—Interleukin-6

LCM—Laser capture microdissection

LMW—Low molecular weight

MAPK—Mitogen-activated protein kinase

MMW—Mid molecular weight

mRNA/RNA—Ribonucleic acid

NF- κ B—Nuclear factor- κ B

NO—Nitric oxide

PAI-1—Plasminogen activator inhibitor type 1
PBS—Phosphate-buffered saline
PCR—Polymerase chain reaction
PDGF-BB—Platelet-derived growth factor-BB
PPAR—Peroxisome proliferator-activated receptor
RT—Reverse transcriptase
SDS—Sodium dodecyl sulfate
SM α A—Smooth muscle α -actin
TNF- α —Tumor necrosis factor- α
VCAM—Vascular cell adhesion molecule
VSMC—Vascular smooth muscle cell
WAT—White adipose tissue
WT—Wild type (mice)

ABSTRACT

Adiponectin (APN) is a 30-kDA insulin-sensitizing protein with strong effects on the vasculature. It binds three receptors: AdipoR1, AdipoR2, and T-cadherin. APN and AdipoRs are well-studied in the vascular endothelium, but studies describing the expression of AdipoRs in other vascular cells, particularly the vascular smooth muscle cells (VSMC), are lacking. In this work, I show that cultured human coronary vascular smooth muscle cells (HCAVSMC) express AdipoR genes and protein, and that AdipoR genes are expressed *in vivo* by porcine coronary artery and murine aortic VSMC. APN reduces plasminogen activator inhibitor, type 1 (PAI-1) expression by adipocytes, so I tested the functionality of the AdipoRs expressed by HCAVSMC by measuring the expression of PAI-1 mRNA expression in cells treated with recombinant human APN (rhAPN) or phosphate-buffered saline (PBS) control. There was no change in PAI-1 mRNA expression in rhAPN-treated cells vs. PBS-control treated cells over 48 hours (n=8, p=0.41). Therefore, I conclude that VSMC express AdipoRs but APN does not influence PAI-1 mRNA expression via a direct, receptor-mediated mechanism in HCAVSMC.

CHAPTER 1: LITERATURE REVIEW

Heart attack and stroke remain leading causes of death in the United States¹. Closely associated with these events is a growing epidemic of the metabolic syndrome and obesity characterized by increased adipose tissue mass and insulin resistance. Adipose tissue is not merely a storage facility for excess calories in the form of triglycerides; rather, it is a secretory organ that regulates energy metabolism and vascular function. When normal adipose functions are compromised in the obese state, severe complications can be seen such as type 2 diabetes mellitus, hypertension, and vascular dysfunction leading to coronary artery disease, and ischemic events.

Adipose tissue as an endocrine organ

In 1995 the first secretory product of white adipose tissue (WAT), leptin, was cloned, which led to the discovery and classification of a group of secretory products collectively termed “adipokines”². Proteins in this class include leptin, resistin, adiponectin, IL-6, TNF- α , angiotensinogen, and PAI-1. Adipose tissue is highly vascular, allowing these bioactive peptides to reach the systemic circulation and exert effects on energy metabolism, immunity, angiogenesis, blood pressure, and hemostasis²⁻⁵.

WAT contains a diverse cell population including mature adipocytes, stromal preadipocytes, immune cells, and endothelium⁵. Interestingly, anatomical location of adipose tissue appears to be important: expansion of visceral and abdominal WAT is more closely associated with pathological conditions than is expansion of subcutaneous WAT. Conversely, having very little WAT is also detrimental—a basal level of secretion of insulin-sensitizing and metabolism-influencing adipokines is required to avoid insulin resistance and other metabolic abnormalities.

In the progression toward obesity, adipocyte size increases rapidly and WAT is infiltrated to greater extent by macrophages, which secrete their own cytokines that regulate both the neighboring adipocytes and the peripheral tissues, resulting in a low-grade inflammatory state⁴. Adipocytes have abnormally high intracellular fatty acid content, which decreases insulin sensitivity, and aberrantly high availability of fatty acids in the periphery decreases skeletal muscle glucose uptake and strongly stimulates liver glucose secretion³.

Adiponectin

The focus of this work is on adiponectin (Adp, Acrp30, AdipoQ, herein referred to as APN). This 30-kDa protein is secreted almost exclusively from adipose tissue and consists of four distinct domains: an N-terminal signaling region followed by a variable sequence, an α -helical collagenous “stalk” region, and a C-terminal

globular head⁶. APN is one of the most abundant plasma proteins, present between 3-30 µg/mL. APN forms multimers in the plasma, circulating in a range of low-molecular-weight (LMW) trimers to mid-molecular-weight (MMW) hexamers to high-molecular-weight (HMW) 12-mers and 18-mers. Globular APN, a cleavage product of leukocyte elastase, also circulates in plasma at low levels⁷. APN mRNA expression is at least partly controlled by a peroxisome proliferator-activator receptor-γ (PPAR-γ) pathway as treatment with rosiglitazone, a PPAR-γ agonist, robustly increases APN mRNA in the WAT of mice and in cultured differentiated adipocytes^{8,9}.

APN first gained recognition as an insulin-sensitizing adipokine. In mice, globular APN increased fatty acid oxidation in skeletal muscle through activation of adenosine monophosphate-activated protein kinase (AMPK). Infusion of globular APN to wild-type (WT) mice increased free fatty acid (FFA) clearance from the plasma and reduced post-prandial plasma triglyceride and glucose levels¹⁰. This reduction of plasma glucose is due in part to a reduction in hepatic glucose synthesis due to decreased mRNA expression of glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PCK)^{11,12}. The ability of APN to at least partially control blood glucose and insulin levels *in vivo* is exhibited in APN-deficient (*Apn*^{-/-}) mice, which show moderate insulin resistance and glucose intolerance when fed a high-fat diet¹³. In lipotrophic mice, a model that has very little body fat, administration of globular and full-length APN (“rescue” of physiological plasma APN levels) reversed

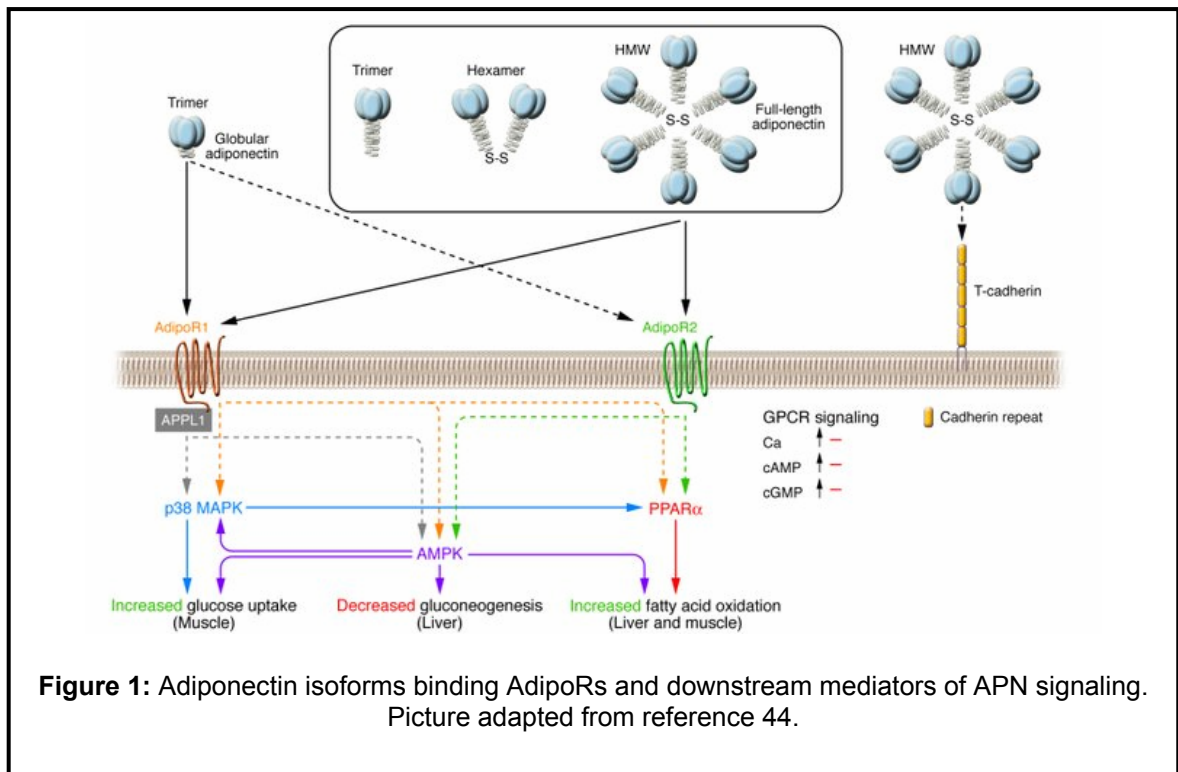
hyperglycemia and hyperinsulinemia characteristic of this model, though it did not completely restore blood glucose or insulin to physiologic levels⁸.

Adiponectin receptors

APN exerts many of its effects by binding to its receptors (AdipoRs): AdipoR1, AdipoR2, and T-cadherin. AdipoR1 and AdipoR2 are commonly referred to as “typical” APN receptors and share significant (67%) homology⁷. AdipoR1 and AdipoR2 are integral membrane proteins with seven transmembrane domains, but the N-termini of both receptors are internal while the C-terminus is external, thus they are opposite the topology of traditional G-protein coupled receptors. Also, the C-terminal regions are short, much more so than is typically seen with other peptide hormone receptors. Just as APN may form multimeric structures, so can its receptors; they form both homo- and heteromultimers in rafts on the plasma membrane.

AdipoR1 was first discovered using a human skeletal muscle cDNA library to scan for globular APN-binding molecules¹⁴. AdipoR1 is expressed ubiquitously but is concentrated in the skeletal muscle and preferentially binds globular- and lower-molecular-weight APN. AdipoR2, discovered by its sequence homology to AdipoR1, is expressed predominantly in the liver. AdipoR2 preferentially binds full-length and higher-molecular-weight APN¹⁵. Downstream intracellular signaling mediators common to these two receptors include cAMP, AMPK, PPAR- α , and p38 MAPK⁷.

T-cadherin, also called H-cadherin or cadherin 13 (CDH13), is structurally and physiologically distinct from AdipoR1 and AdipoR2. It has no membrane-spanning regions; T-cadherin is tethered to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety. This underscores its differences from other cadherins in terms of spatial location and function¹⁶. T-cadherin is not simply a cell-cell adhesion molecule with an intracellular signaling domain sequestered at cell-cell junctions as are most cadherins; it is more widely distributed throughout the plasma membrane and acts as a signal-transferring molecule through currently poorly-understood mechanisms. Interestingly, T-cadherin's amino acid sequence is 92% conserved from mice to humans,



significantly higher than other cadherin sequences, possibly indicating a more important biological function than others of its class¹⁷.

APN in the vasculature

APN is well studied in the vasculature, particularly in the endothelium as related to the development of atherosclerosis and neointima formation¹⁸⁻²⁴. Addition of purified HMW APN from human plasma resulted in increased nitric oxide (NO) release in the cultured media of human umbilical endothelial cells (HUVEC). This increase in secreted NO was accompanied by an increase in endothelial nitric oxide synthase (eNOS) activity and an increase in phosphorylated AMPK¹⁸.

Globular APN similarly increases NO production and eNOS activity *in vitro*. Treatment of bovine aortic endothelial cells with globular APN resulted in dose- and time-dependent increase in NO concentration in the conditioned media. HUVECs treated with globular APN had increased eNOS phosphorylation and thus activity as well¹⁹.

In the early stages of atherosclerosis, circulating inflammatory mediators induce the endothelium to express adhesion molecules that trigger monocyte adhesion. APN reduces TNF- α -induced vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, and E-selectin expression in human aortic endothelial cells (HAECs) as well as TNF- α -induced monocyte adhesion to HAEC monolayers, presumably through activation of NF- κ B²⁰. HMW APN dose-dependently inhibits TNF- α -induced NF- κ B activation¹⁸. This inhibition

is enhanced with longer exposure to HMW APN prior to TNF- α challenge. Adhesion molecule expression and leukocyte adhesion to the endothelium are enhanced in *Apn*^{-/-} mice, also through TNF- α -mediated signaling²¹.

APN reduces monocyte infiltration and inhibits macrophage lipid loading and thus formation of monocyte-derived foam cells⁶. Human monocyte-derived macrophages treated with APN have reduced intracellular cholesterol content and lipid droplet formation *in vitro*²². A reduction in foam cells could reduce neointima formation. This is reflected *in vivo* using apolipoprotein-E-deficient (*ApoE*^{-/-}) mice. These mice spontaneously form fatty aortic lesions when fed a high-fat diet. High-fat-fed *ApoE*^{-/-} mice infected with adenoviral APN showed reduced lipid droplets in fatty lesion formation as compared to control animals infected with adenoviral β -galactosidase²³. APN reduces high density lipoprotein (HDL)-mediated cholesterol efflux from THP-1 macrophage foam cells, which coincides with increased PPAR- γ expression and decreased scavenger receptor-A1 (SR-A1) expression²⁴.

APN is deposited in the subendothelial space after arterial injury²⁵ and reduces vascular smooth muscle cell (VSMC) migration and proliferation. *In vitro*, APN binds plasma growth factors such as platelet-derived growth factor (PDGF)-BB and endothelial growth factor (EGF)²⁶ and reduces growth factor-induced DNA synthesis and migration of human aortic VSMC²⁷. These *in vitro* observations are reflected *in vivo*, in that *Apn*^{-/-} have increased neointima formation after femoral artery wire injury²⁸. The neointima of *Apn*^{-/-} mice showed

increased staining for smooth muscle α -actin and BrdU as compared to WT controls, all of which were reversed with adenoviral APN infection.

APN and thrombosis

Plasma APN concentration is decreased paradoxically in obesity, diabetes, metabolic syndrome, and coronary artery disease (CAD)⁷. As these diseases are also associated with higher thrombosis rates, reduced plasma APN levels may increase susceptibility to cardiovascular events such as heart attack and stroke. APN may reduce thrombosis through several mechanisms; the most-studied of which is by increasing NO synthesis and release from the endothelium.

Increased synthesis and release of NO from the endothelium encourages unrestricted blood flow and reduces the occurrence of flow-related endothelial injury. NO also reduces platelet aggregation which reduces blood clot formation. This dual action of increased circulating NO maintains a healthy vascular environment less conducive to thrombotic events.

Recombinant APN treatment reduces agonist-stimulated platelet aggregation *in vitro* but the mechanism(s) by which it exerts this effect is/are unclear. Platelets from *Apn*^{-/-} mice show enhanced agonist-stimulated aggregation *in vitro*, which is reversed with addition of recombinant APN to platelet-rich *Apn*^{-/-} mouse plasma⁹. Also, thrombi formed over a collagen-coated surface were larger and thicker using *Apn*^{-/-} mouse whole blood as compared to WT controls.

Apn^{-/-} mice show accelerated thrombus formation⁹. This response is attenuated by infecting mice with an adenovirus coding for APN that increases plasma APN concentration to supraphysiological levels. AdipoR1 and AdipoR2 are found on platelets, suggesting that APN has receptor-mediated effects on platelets that have yet to be discovered.

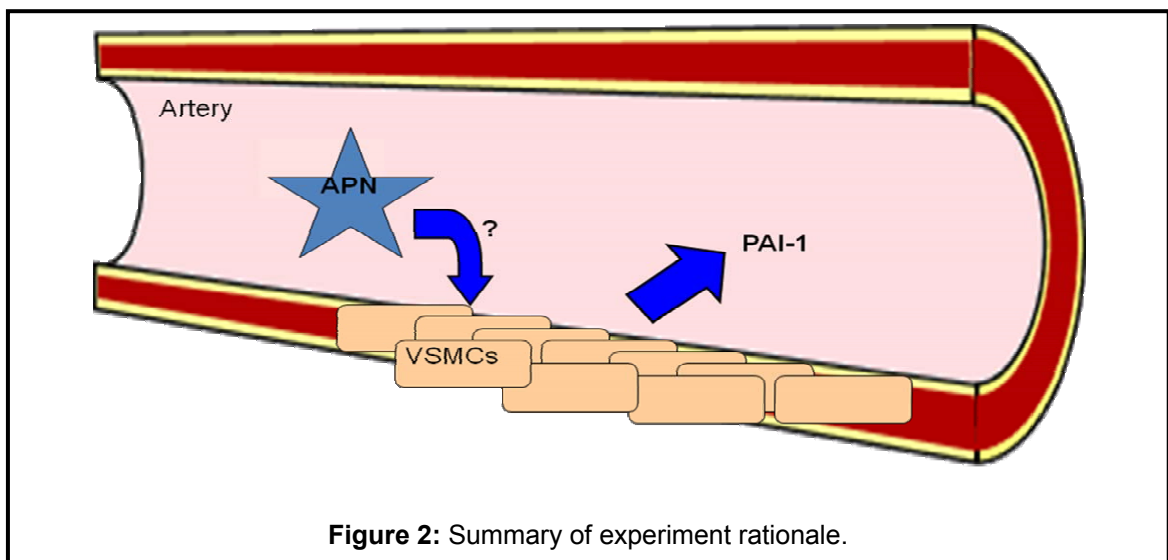
APN and PAI-1

Plasminogen activator inhibitor type-1 (PAI-1) inhibits tissue-type plasminogen activator (tPA) and urinary-type plasminogen activator (uPA), enzymes that convert plasminogen to plasmin, a serine protease that degrades fibrin. PAI-1 is secreted by adipocytes, platelets, the vascular endothelium and smooth muscle cells, and other cell types.

PAI-1-deficient mice are protected from arterial thrombosis, suggesting a pro-thrombotic role for PAI-1²⁹. Indeed, increased plasma PAI-1 levels are a risk factor for thrombosis in humans³⁰. Obese individuals have increased plasma PAI-1 levels, partly from increased adipose tissue mass in general but also from increased PAI-1 mRNA and protein expression in the adipose tissue. APN decreases PAI-1 mRNA and protein released in the conditioned media of cultured adipocytes³¹, but the effect of APN on other cells that express and secrete PAI-1, including the VSMC, is unknown.

Summary

Adiponectin (APN) is produced by adipocytes and influences insulin sensitivity in peripheral tissues. In obesity, a paradoxical decrease of plasma APN is accompanied by an increase in circulating PAI-1. APN reduces the expression of PAI-1 by the adipocyte, but its effects on other cell types that secrete PAI-1, such as the VSMC, are unclear. APN has strong vascular effects that are well-studied in the endothelium, however, studies examining APN's effect on other vascular cells, such as VSMC, are lacking. The studies presented herein test the working hypotheses that 1) AdipoRs are expressed *in vitro* by human coronary artery vascular smooth muscle cells (HCAVSMCs) and *in vivo* by murine aortic smooth muscle cells. Also, 2) APN exerts a direct, receptor-mediated effect on HCAVSMC to reduce PAI-1 expression *in vitro*.



CHAPTER 2: METHODS

Cell Culture

Human coronary artery vascular smooth muscle cells (HCAVSMC) were purchased from Cascade Biologics and grown under standard conditions using Media 231 with added Smooth Muscle Cell Growth Supplement, also from Cascade Biologics. The media was further supplemented with Amphotericin-B (Fungizone, Invitrogen) and Gentamicin (Abraxis Pharmaceuticals). Cells were passaged at 80% confluency using TrypLE solution from Gibco. Cells of passage 3-6 were used in experiments. A detailed protocol for cell culture is provided in Appendix 1.

For experiments in which I examined AdipoR expression, confluent monolayers of HCAVSMC were lysed using TRIzol reagent (Invitrogen) or ice-cold RIPA buffer (Pierce) containing protease inhibitors (Complete EDTA-free, Roche). TRIzol lysates were frozen at -80°C until they could be used for subsequent PCR reactions.

RIPA lysates of HCAVSMC were centrifuged to pellet insoluble material and resulting supernatants were stored on ice. Protein concentration of RIPA lysates was estimated using a BCA kit from Pierce. Samples of lysates (up to 50

µg total cellular protein) were heated in a loading buffer containing SDS and β-mercaptoethanol (both Sigma) and electrophoresed through 10% agarose gels that contained SDS. Proteins were transferred to nitrocellulose membranes and blocked with 2.5% nonfat milk and probed with appropriate primary and HRP-tagged secondary antibody (all from Santa Cruz). Membranes were developed with ECL substrate (Pierce) and imaged on x-ray film.

For experiments in which I measured relative PAI-1 expression in HCAVSMC, 100% confluent cell monolayers were serum-starved overnight before treatment. APN is present in serum, and as the growth media contained serum, I wanted to reduce the effect of “endogenous” APN from the serum in the growth media as much as possible. Therefore, the afternoon previous to treatment, cells were washed three times with warm sterile PBS before serum-free media was added. The serum-free media contained Media 231 and antibiotics only; no growth supplement or bovine serum albumin (BSA) was present. A group of cells termed “Time 0” cells were lysed the following morning and served as controls. To treat the remaining cells, serum-free media was aspirated and treatment media—Media 231 with added rhAPN (10 µg/mL from R&D Biosystems), PBS vehicle (same volume as rhAPN), D-glucose (27.5 mM, from Sigma), or rosuvastatin (10 µM, from Toronto Research Chemicals) —was applied to the cells. After 24 or 48 hours, cells were lysed with TRIzol reagent or RIPA buffer with protease inhibitors for PCR or Western analysis of relative PAI-1 expression.

Reverse transcriptase PCR

Genomic end-point PCR is an effective means of analyzing the presence of a gene within a specific genome. It is commonly used in genotyping of laboratory animals and in semi-quantitative analysis of gene expression. However, it is ineffective for quantitative analysis of gene expression. To achieve this, I extracted mRNA from TRIzol lysates of cultured cells. After chloroform phase separation, total cellular RNA was precipitated with 3M sodium acetate (Sigma), 100% ethanol (Acros Organics), and glycogen (Roche). A detailed protocol for RNA extraction is provided in Appendix 2.

I quantitated total cellular RNA using a NanoDrop 1000 from Thermo Scientific and subjected it to reverse transcriptase PCR using the High Capacity cDNA Synthesis Kit from Applied Biosystems. Negative control reactions lacking reverse transcriptase were included for each sample. By using carefully designed primers based on the cDNA sequence and spanning an intron in the genomic DNA, I could indirectly examine the expression of a gene either by end-point PCR or measure relative mRNA abundance using real-time PCR.

Real-time PCR

Real-time PCR is an effective means of quantitatively measuring gene expression. I used SYBR green in my experiments, a reporter dye that

intercalates into double-stranded PCR product. Quantitation of results can be achieved by a few different methods; I performed relative quantitation using the $2^{(-\Delta\Delta Ct)}$ equation as described by Livak and Schmittgen^{32,33}.

Using this method, gene expression is estimated by assigning a value to the PCR cycle in which a reporter dye (SYBR green) reaches an arbitrary fluorescence threshold in the exponential phase of the PCR reaction, called the threshold cycle (Ct). The Ct of a target gene is then normalized to a control “housekeeping” gene in each sample, which serves as a loading control. For this set of experiments, the Ct of the housekeeping gene (RPL13a) was subtracted from the Ct for the target gene (PAI-1), producing a ΔCt . The ΔCt for PBS vehicle-treated cells was then subtracted from the ΔCt of rhAPN-treated cells, producing the $\Delta\Delta Ct$. Real-time PCR data is measured during exponential phase during which each copy of PCR product is doubled, so 2 is raised to the power of the negative $\Delta\Delta Ct$ yields the relative PAI-1 abundance in fold change of PAI-1 mRNA expression in rhAPN-treated cells from control-treated cells.

Briefly, relative gene expression = $2^{(-\Delta\Delta Ct)}$

where $\Delta\Delta Ct = \Delta Ct_{(treated\ group)} - \Delta Ct_{(control\ group)}$

and $\Delta Ct = Ct_{(target\ gene)} - Ct_{(housekeeping\ gene)}$

For further detail on the mathematics involved with my experiments, please see Appendix 3 or reference 32.

Samples of cDNA from prior reverse transcriptase PCR reactions were used in real time PCR reactions. The cycle conditions were as follows: an initial hold at 95°C for 3 minutes to activate the heat-stable Taq polymerase, followed by 45 cycles of 95°C for 30 seconds and annealing temperature for 20 seconds. Many of my primer sets had similar, but not identical, annealing temperatures, but all annealing temperatures were between 55-60°C. A melt curve was conducted after real-time PCR to assess purity of product. During optimization experiments, samples of cDNA were subjected to end-point PCR to ensure that the resulting PCR product was of the expected size. A list of PCR primers and product sizes for these experiments is presented in Appendix 4. I performed paired t-tests and 2-way ANOVAs to test the significance of the results of real-time PCR experiments. Power analysis and sample size calculations were performed using software from DSS Research.

Laser capture microdissection (LCM)

LCM is an effective means of collecting a specific cell type *in vivo*. In the experiments described in Chapter 3, the smooth muscle layer of mouse aorta was excised to study expression of AdipoRs. Male wild-type (WT) mice 10-16 weeks of age on a C57BL6/J background were anesthetized by inhalation of a mixture of room air and 4% isoflurane. The thoracic aortas were harvested from the aortic arch to the diaphragm through an abdominal incision that included opening the rib cage. Aortas were cut into <1-cm sections, then placed in screw-

top cryopreservation tubes, and snap-frozen in liquid nitrogen. Frozen vessel segments were then embedded on OCT compound (Tissue Tek) and cryo-sectioned into 6µm cross-sections. I stained the slides with HistoGene stain (Arcturus) and then dehydrated them in increasing concentrations of ethanol with a final dehydration in xylenes. I dried the slides in a fume hood and kept them in a desiccator with Drierite (Fisher) until use, which was within 1 hour of staining

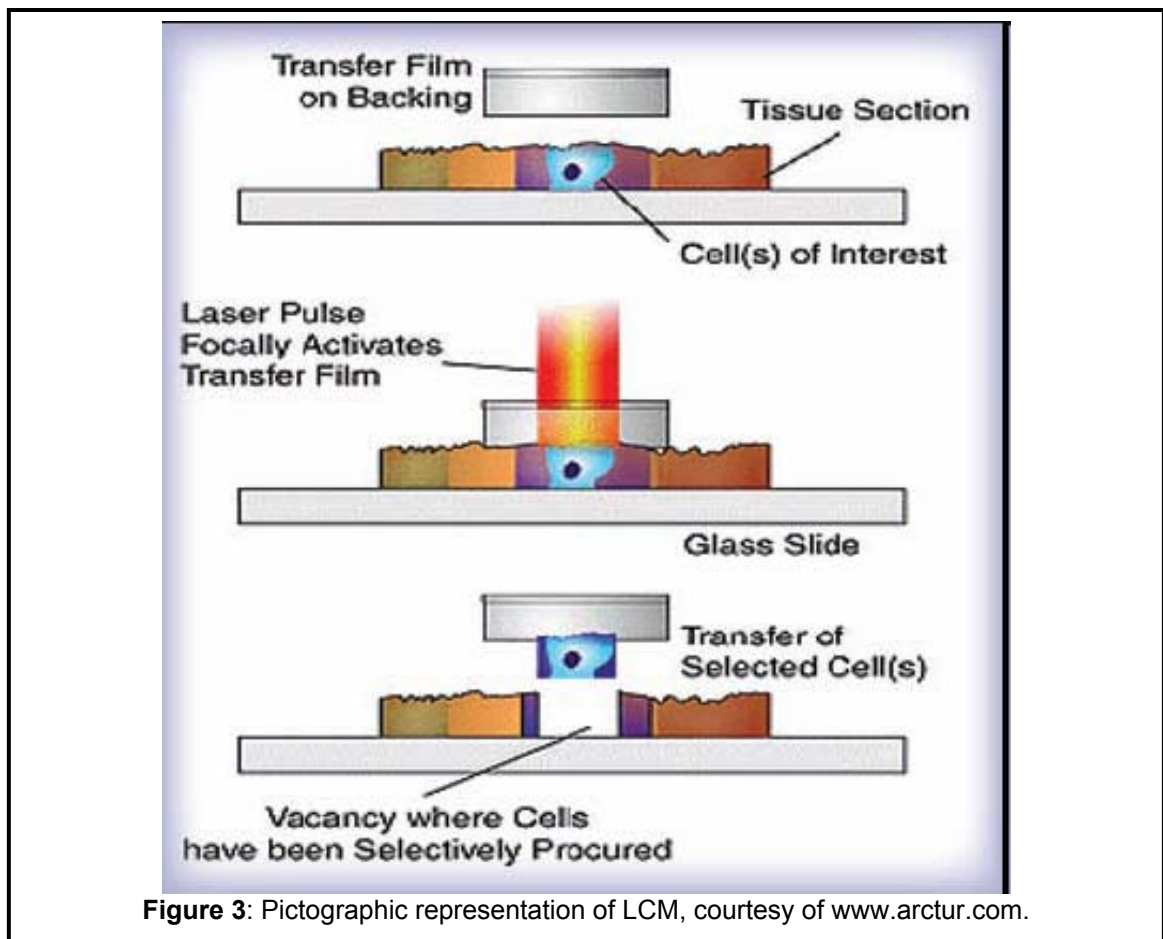


Figure 3: Pictographic representation of LCM, courtesy of www.arctur.com.

and dehydrating to reduce RNA degradation.

In LCM, a microscope is used to identify cells in a tissue section. A cap that holds a thermoreactive film is placed over the cells of interest, and the film is melted by an infrared laser guided by a computer that interfaces the microscope

and laser. As the cap is lifted from the tissue, the desired cells adhere and these “captured” cells can be lysed and used in further analysis such as reverse transcriptase and end-point PCR. I used a Molecular Devices Arcturus system located in the Molecular Cytology Core at the University of Missouri. Media cells were collected using CapSure Macro LCM caps (Arcturus). RNA from freshly-captured cells was extracted using the PicoPure RNA Isolation Kit (Arcturus).

A pictographic representation of the LCM process is shown in Figure 3 and representative images of porcine coronary artery cells I captured using LCM are displayed in Figure 4. A detailed protocol for LCM is provided in Appendix 5.

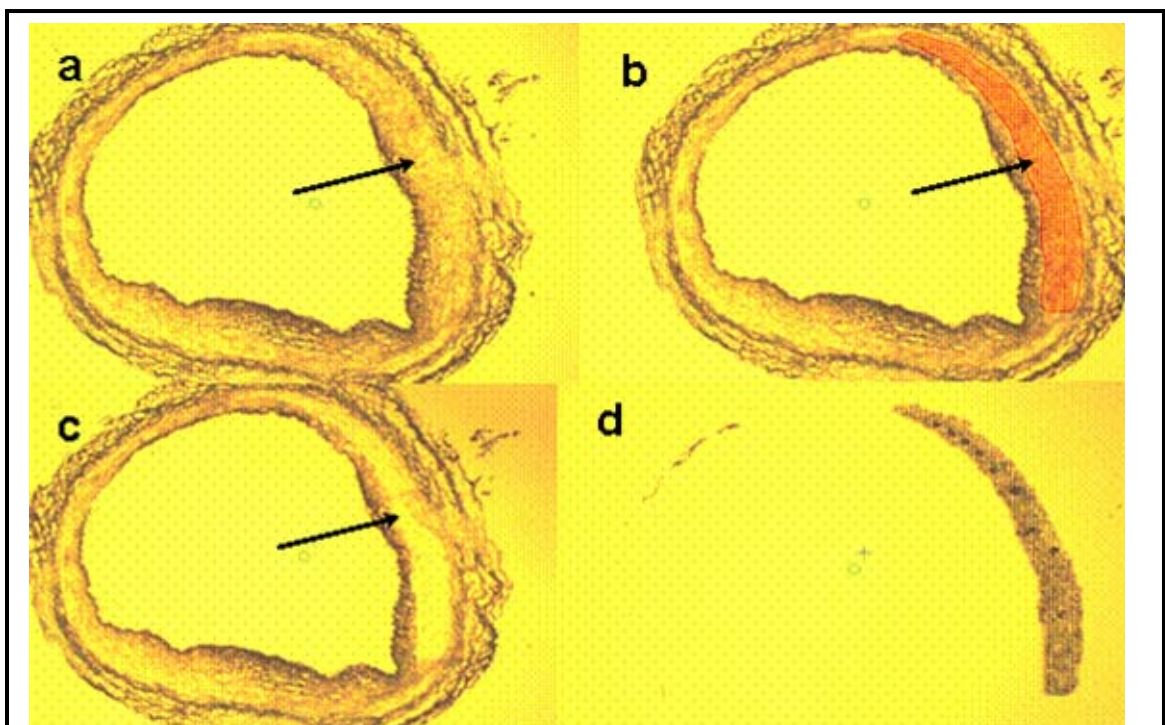


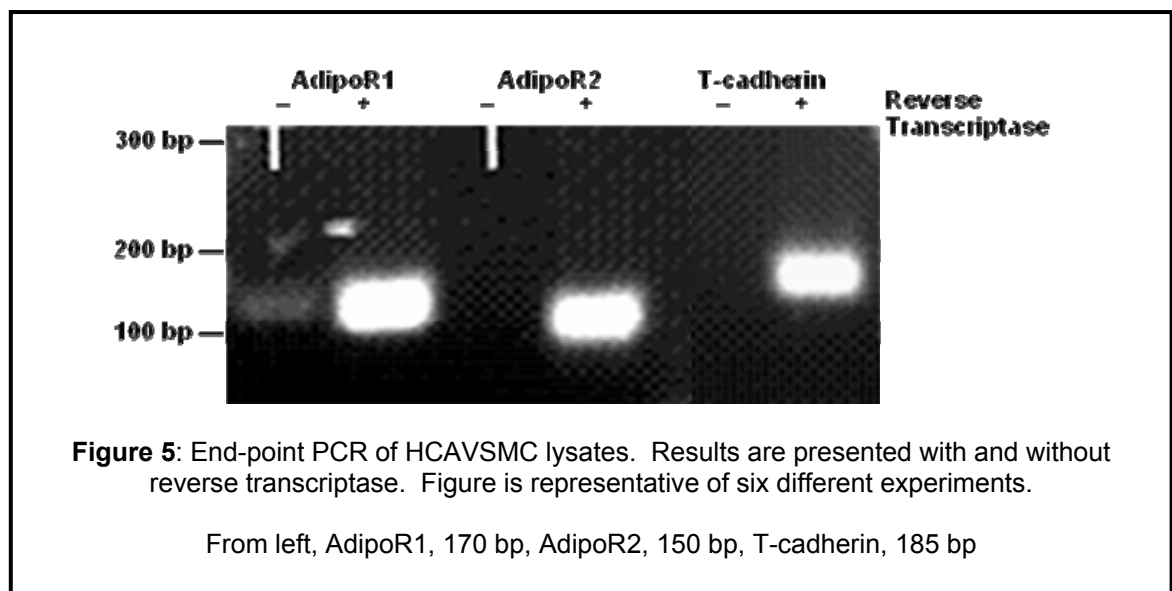
Figure 4: Example of porcine coronary artery viewed and tunica media cells collected by LCM microscope.

A) tissue section still intact, black arrow designating smooth muscle cell-rich tunica media layer; b) area to be collected is designated by red hatched region; c) tissue section after collection, note the absence of cells previously marked by red hatched region in b; and d) a view of the cap after cell collection showing attached cells.

CHAPTER 3: RESULTS

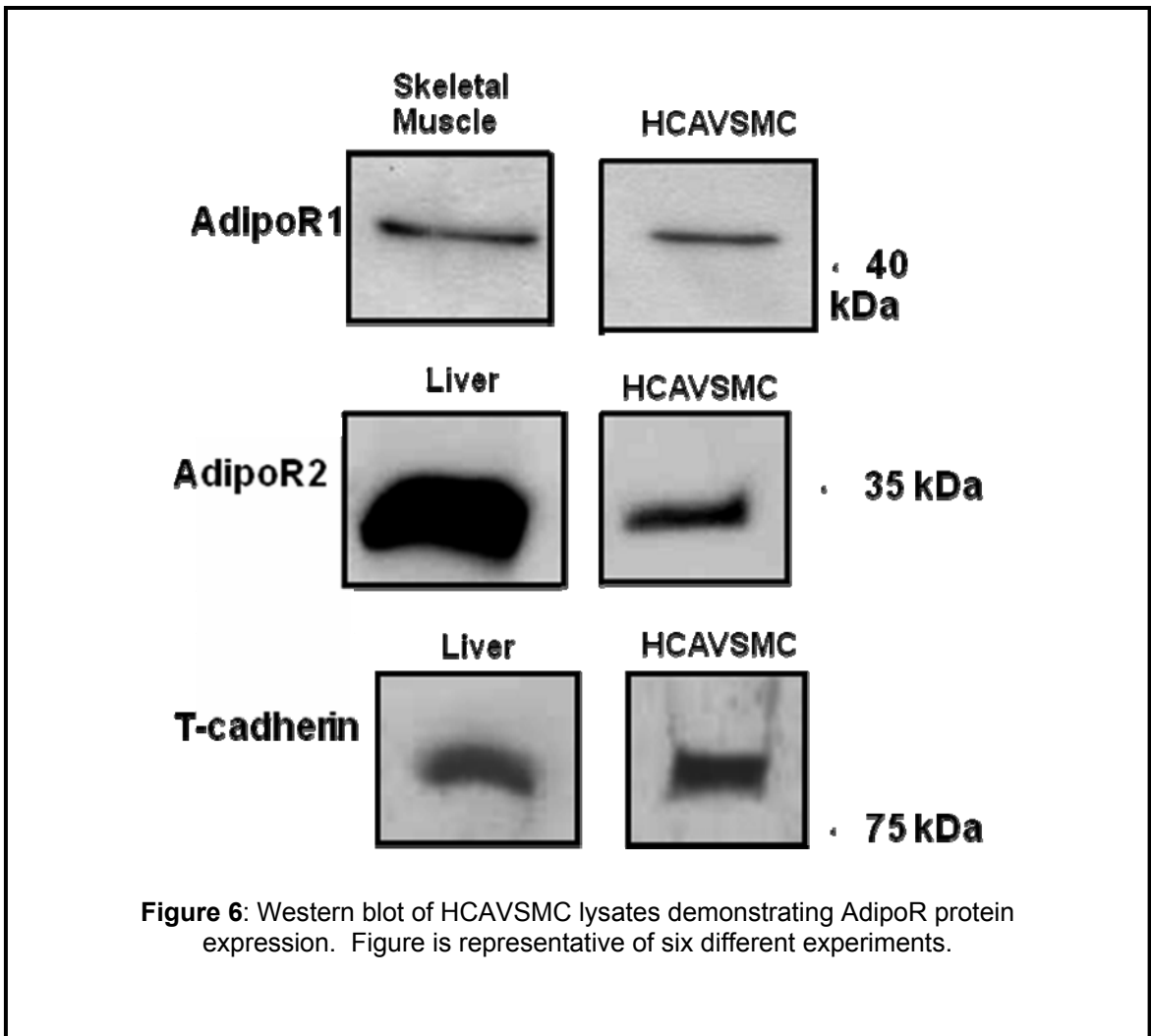
AdipoRs are expressed by VSMC *in vitro* and *in vivo*

To examine the expression of AdipoRs by VSMC, lysates of cultured HCAVSMC were prepared and subjected to reverse transcriptase and end-point PCR. Robust bands of predicted sizes were seen for each set of AdipoR primers. Very faint or no bands were seen in reactions lacking reverse transcriptase, demonstrating very little to no genomic DNA contamination (Figure 5). Sequencing of PCR products showed the products were identical to corresponding AdipoR1, AdipoR2, and T-cadherin genomic sequences. These results suggest the gene for each APN receptor is expressed in cultured



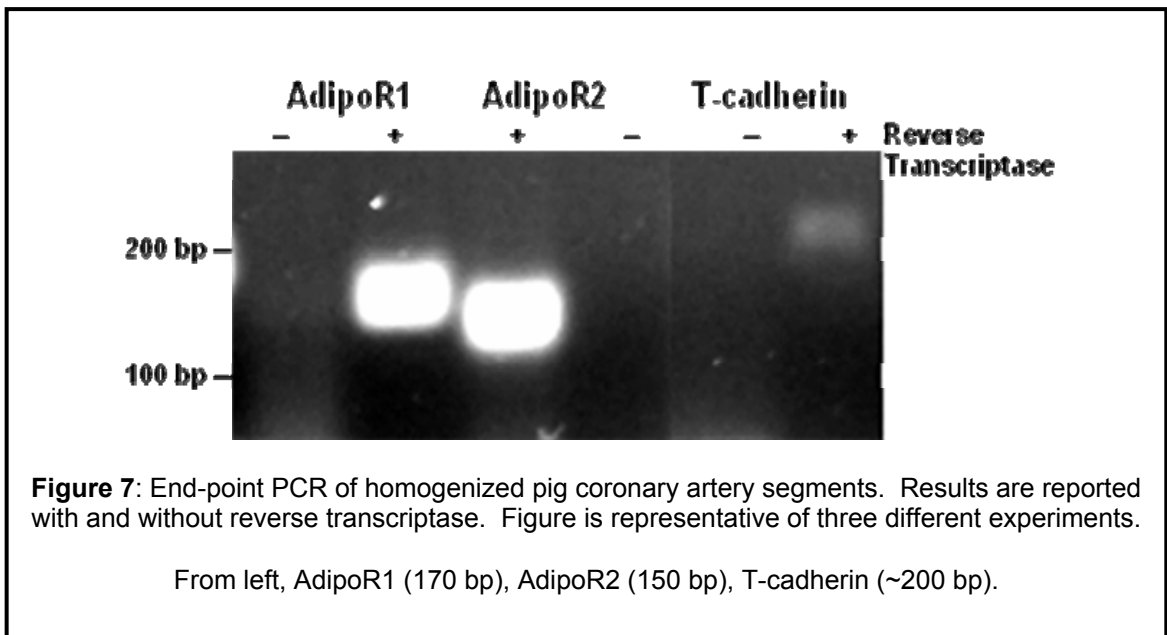
HCAVSMC.

To examine the expression of each AdipoR at the protein level, HCAVSMC lysates were subjected to SDS-PAGE/Western blotting using antibodies specific for each receptor. Cell lysates prepared from mouse skeletal muscle and liver were used as positive controls as each of the polyclonal primary antibodies used is known to cross-react with murine and human AdipoR1, AdipoR2, or T-cadherin. Bright bands of the expected size were observed for



each receptor in HCAVSMC lysates, consistent with expression of all three receptors in cultured HCAVSMC (Figure 6).

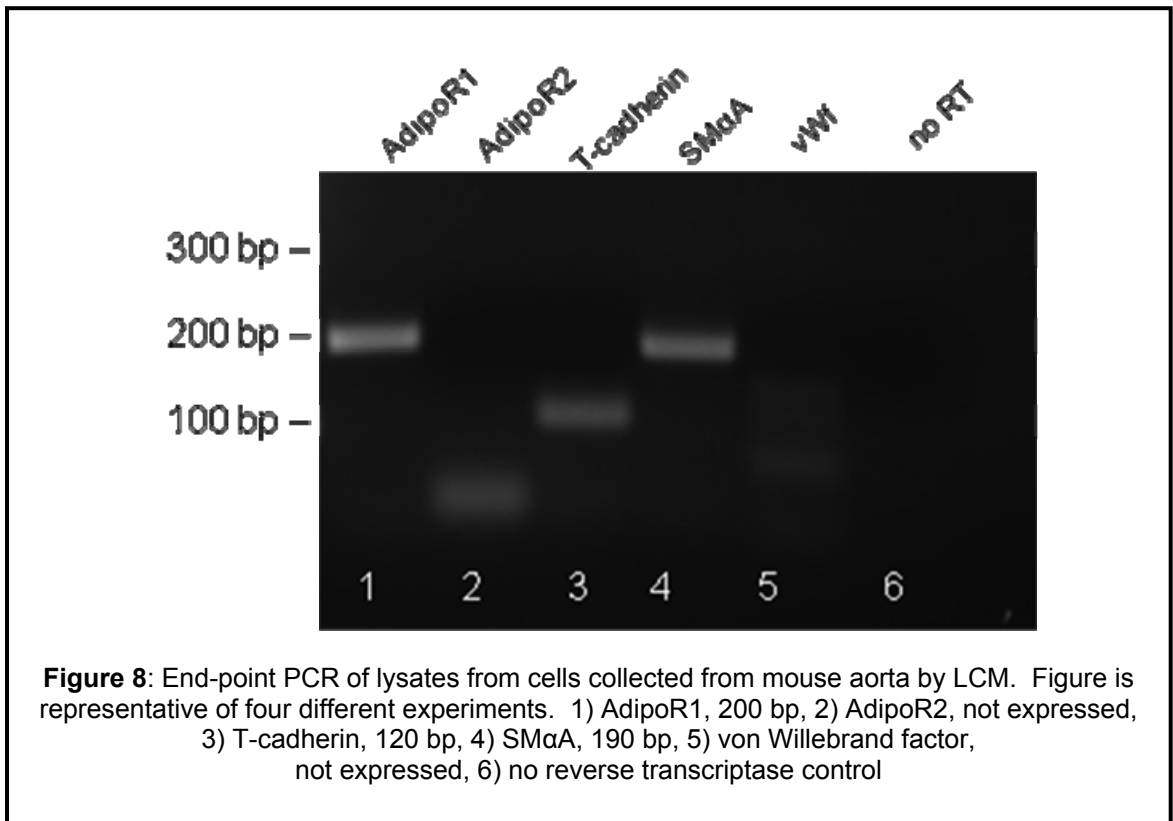
I next investigated AdipoR gene expression *in vivo* using RNA from homogenized porcine coronary artery lysates that was kindly donated by Doug Bowles, PhD. Vessel segments were snap-frozen in liquid nitrogen immediately after excision and pulverized to form a whole-vessel homogenate before RNA extraction. I subjected total RNA isolated from homogenates to reverse transcriptase and end-point PCR. PCR products of the predicted size were observed for each set of AdipoR primers, though the band observed for T-cadherin was faint. Negative control reactions lacking reverse transcriptase yielded no detectable PCR product (Figure 7). These results suggest that



AdipoRs are expressed in porcine coronary artery, and that the predominant AdipoRs expressed being AdipoR1 and AdipoR2. However, I was unable to

discern if the VSMC in particular expressed AdipoRs, as the entire vessel was homogenized during RNA preparation.

To investigate AdipoR RNA by VSMC *in vivo*, I used laser capture microdissection (LCM) to selectively extract cells from the tunica media layer of



murine aortas. Total cellular RNA from collected cells was subjected to reverse transcriptase and end-point PCR to examine AdipoR gene expression (Figure 8). Bright bands are visible in lane 2 around 200 bp, corresponding to AdipoR1 PCR product and in lane 4 at 105 bp, corresponding to T-cadherin PCR product. Lane 3 contains a band well below 100 bp and I believe this to be mis-primed product, not PCR product for AdipoR2, which would be seen around 120 bp. Also, there is some faint “laddering” of PCR product in lane 6, which corresponds to primers

specific for von Willebrand factor. I believe this to be mis-primed product as well and not von Willebrand factor PCR product, which would be seen around 135 bp. Strong signal of smooth muscle α -actin (lane 5) but not vonWillebrand factor (lane 6) suggests that the population of cells analyzed was primarily smooth muscle cells, with little to no endothelial cells, which have already been shown to express AdipoRs and may yield falsely positive results for AdipoR expression. Samples lacking reverse transcriptase showed no visible PCR product (example in lane 6 using the PCR primer set for smooth muscle α -actin, for other primer sets, data not shown), demonstrating the samples did not contain genomic DNA contamination. Overall, these data suggest that VSMC express AdipoR1 and T-cadherin *in vivo* in mouse aorta.

APN does not alter PAI-1 expression by HCAVSMC

To test the hypothesis that APN will reduce PAI-1 mRNA and protein in HCAVSMC, I treated cultured HCAVSMC with 10 μ g/ml recombinant human APN (rhAPN) or PBS control for up to 48 hours. Cells were lysed and analyzed using real-time PCR and Western blotting as described in Chapter 2.

In preliminary experiments, PAI-1 abundance was compared to smooth muscle α -actin, with the results suggesting a robust decrease in PAI-1 expression with rhAPN treatment (data shown in Appendix 6). However, further analyses³³ showed that smooth muscle α -actin expression changes significantly over time and with rhAPN treatment and thus yielded falsely positive results. An explanation and example of these analyses are shown in Appendix 7. Hence, I performed a survey of potential housekeeping genes using the Human Housekeeping Gene kit (Realtimeprimers.com) and found a more suitable housekeeping gene, RPL13a, that neither changed significantly over time nor with treatment in this system. I repeated the real-time PCR using RPL13a as the housekeeping gene and results revealed no change of PAI-1 gene expression in cells treated with 10 μ g/mL rhAPN vs. PBS control-treated cells (Figure 9,

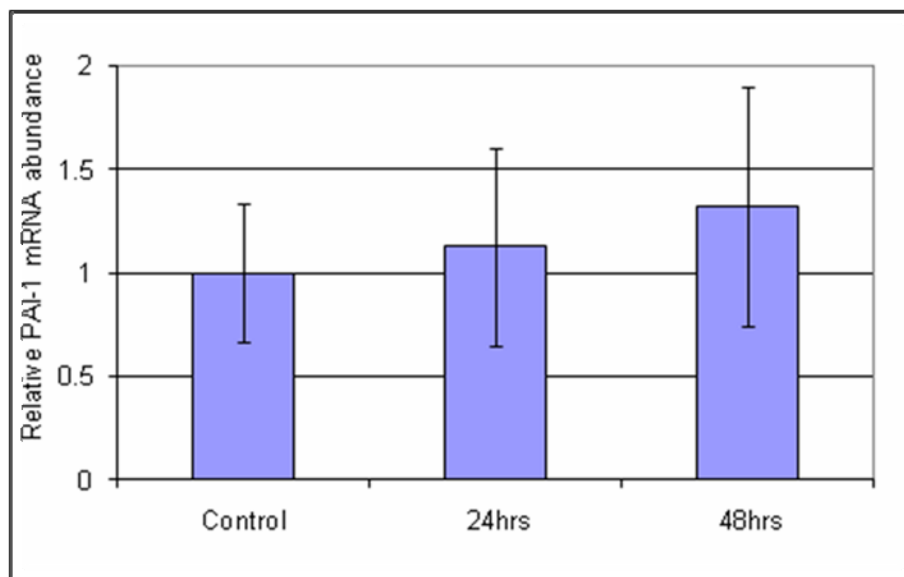
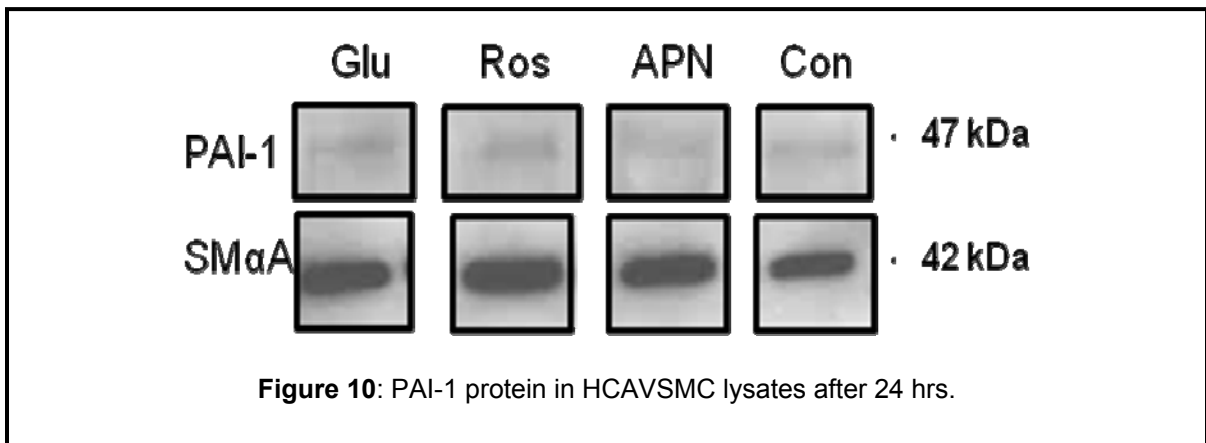


Figure 9: Effect of 10 μ g/ml rhAPN on PAI-1 mRNA expression in cultured HCAVSMC. N = 8, p = 0.41, t-test of 48 hr time vs. control, power = 10.2%

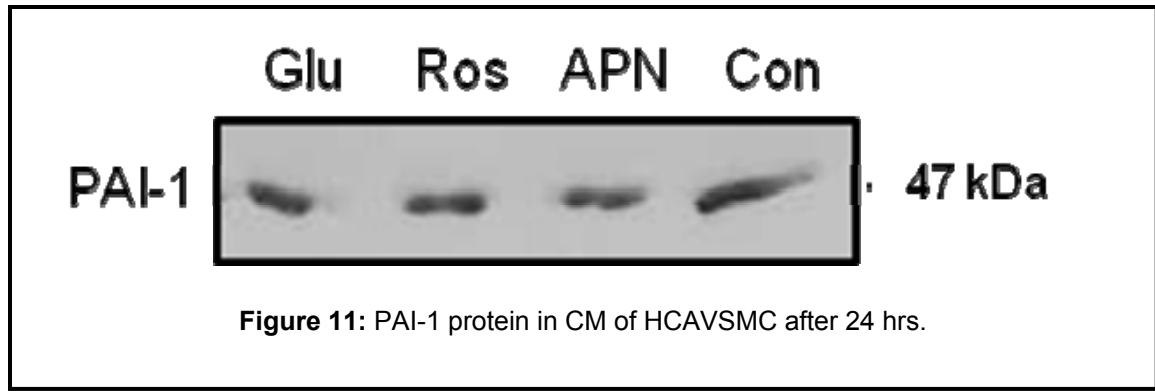
student's t-test comparing rhAPN-treated cells at 48 hours vs. Time 0 controls, $p = 0.41$, $n = 8$, power = 10.2%). A two-way ANOVA was also performed, with results confirming that there was no change in PAI-1 mRNA expression over time or with rhAPN treatment.

To examine whether a change in PAI-1 expression could be seen at the protein level, whole cell lysates and conditioned media from HCAVSMC were collected after 24 hrs of rhAPN (10 $\mu\text{g/ml}$) or PBS vehicle control treatment. Cells treated with glucose (27.5 mM) and rosuvastatin (10 μM) served as positive controls. High glucose in treatment media (5-35 mM) increases PAI-1



expression *in vitro* in cultured VSMC^{32,33}. HMG CoA reductase inhibitors (herein referred to as “statins”) reduce PAI-1 expression in vascular smooth muscle and endothelial cells *in vitro*^{36,37}. Rosuvastatin was chosen as a control over other statins because of its connection to APN in adipocytes—PAI-1 mRNA expression and protein secretion is decreased in cultured adipocytes treated with 10 μM rosuvastatin³⁸ as are adipocytes treated with 10 $\mu\text{g/ml}$ rhAPN³¹. Western analyses of cell lysates (Figure 10) and conditioned media (Figure 11) showed

no significant difference in PAI-1 protein expression between rhAPN-treated vs. control-treated cells.



Summary

- Adiponectin receptors (AdipoR) genes are expressed in cultured HCAVSMC, in porcine coronary artery sections, and in VSMC collected from murine aorta.
- The distribution of each receptor is different in each species and each location; HCAVSMC and porcine coronary artery segments express AdipoR1/R2 and T-cadherin genes while murine aortic VSMC express only AdipoR1 and T-cadherin genes.
- HCAVSMC express AdipoR proteins.
- HCAVSMC treated with 10 µg/ml rhAPN reflect no change in PAI-1 expression.

CHAPTER 4: DISCUSSION

This body of work tested the hypotheses that 1) AdipoRs are expressed by VSMC *in vitro* and *in vivo* and 2) APN will reduce PAI-1 expression by cultured HCAVSMC through a receptor-mediated mechanism. Strong signals using HCAVSMC lysates in reverse transcriptase end-point PCR reactions and Western blot analyses are consistent with expression of AdipoRs by HCAVSMC. AdipoR gene expression was also observed *in vivo* by porcine coronary artery homogenates and murine aortic VSMC extracted using LCM. Thus, my data support hypothesis 1. However, the observations of no statistically significant change in PAI-1 gene or protein expression in rhAPN-treated HCAVSMC do not support hypothesis 2. Therefore, I am unable to reject the null hypothesis that rhAPN-treated HCAVSMC and PBS vehicle control-treated HCAVSMC have equal PAI-1 expression.

There appear to be species and physiological location differences in expression of AdipoRs between pigs and mice: pigs appear to have robust AdipoR1 and AdipoR2 mRNA expression in coronary artery segments while mice appear to have more robust AdipoR1 and T-cadherin expression in aortic smooth muscle cells. As different isoforms of APN bind preferentially with each receptor, this could suggest that different signaling pathways may be utilized between the

species and depending on physiological location. Reduced expression of T-cadherin as compared to AdipoR1 and AdipoR2 in pigs suggests that APN signaling may be equal from globular, full-length, and higher-molecular-weight APN. However, the PCRs for AdipoR1 and AdipoR2 may have been more efficient than the PCR for T-cadherin in pigs, thus reflecting a fainter band.

Mice express AdipoR1 and T-cadherin but not AdipoR2 mRNA in aortic VSMC, therefore APN signaling in this species and cell type may be predominantly mediated through globular and high-molecular-weight APN, with little signaling from the low- or mid-molecular-weight APN isoforms.

I tested the hypothesis that APN will decrease PAI-1 expression by HCAVSMC in an effort to test the functionality of the AdipoRs expressed by HCAVSMC. APN reduces PAI-1 expression in cultured adipocytes³¹, but I was unable to discern a similar effect in cultured HCAVSMC. One possible explanation for this could be related to the different receptors expressed between the cell types. AdipoR1 and AdipoR2, which are expressed on both adipocytes and HCAVSMC, tend to use common downstream mediators, typically by activation of AMPK or members of the MAPK family such as ERK and p38⁷. T-cadherin is expressed by HCAVSMC but not by adipocytes and utilizes a completely different set of cellular signaling pathways from AdipoR1 and AdipoR2, usually through Ras and Rac¹⁶. Perhaps a decrease in PAI-1 expression is mediated by AdipoR1 and AdipoR2 in both HCAVSMC and adipocytes, but a robust opposite effect through T-cadherin occurs in HCAVSMC, thus returning a net no-change in PAI-1 gene expression.

Another possible explanation for this could be that the rhAPN dose was not high enough to see any effect on PAI-1 expression by VSMC. The dose used (10 µg/mL) is commonly used in the literature^{18,20,27,31} and was chosen for this fact and also that it fell well within physiologic levels of 3-30 µg/mL. The purchased rhAPN was verified to be active in this cell type by its effects on smooth muscle α -actin RNA expression in HCAVSMC (data shown in Appendix 8), but perhaps the dose was too low to have an effect on PAI-1 expression. A dose-response experiment would be appropriate to determine effective dose(s), if any, of rhAPN on VSMC expression of PAI-1 mRNA.

Overall, the statistical power of the experiments was very low, thus the chance of measuring a significant difference in this set of experiments was very low. Using the reported data, a sample size of 12 is needed to detect a statistically significant change. I used an N of 8, so experiments to increase the N to 12 are needed for the future.

I was unable to replicate the results of previously-published literature regarding changing PAI-1 expression by VSMC using D-glucose^{34,35} and rosuvastatin³⁸. The difference in results could be explained by the use of different populations of smooth muscle cells—I used HCAVSMC purchased from Casdade Biologics, but in the previously-published work VSMC were harvested from umbilical vein and artery, mammary artery, as well as directly harvested from human coronary arteries.

Perhaps the dosage of D-glucose and rosuvastatin used was incorrect for this experimental system. The doses used in these experiments corresponded to

those in the literature³⁴⁻³⁷ but perhaps were too low to return visible results in this system. Experiments using several different doses of D-glucose and rosuvastatin would be appropriate to determine a dose (if any) that alters PAI-1 mRNA and/or protein expression in this experimental system.

Should D-glucose fail to be an effective positive control, a candidate for an alternative could be PDGF-BB, which increases PAI-1 synthesis in smooth muscle cells through ERK and JNK^{39,40}. Rosuvastatin was chosen because it also reduces PAI-1 expression in adipocytes³⁸. Many statins reduce PAI-1 expression in VSMC, but not all³⁶. Perhaps rosuvastatin is one of the few statins that has a very weak or no effect on PAI-1 expression by VSMC. If this is the case, an alternative would be to use a different statin such as cerivastatin or simvastatin, which robustly decrease PAI-1 expression in VSMC^{36,37}.

CHAPTER 5: FUTURE DIRECTIONS

Determine APN's effect, if any, on other circulating coagulation factors *in vitro*.

APN is deposited in the subendothelial space after arterial injury²⁵ and I have shown that VSMC express AdipoRs. Therefore, it is reasonable to speculate that APN has receptor-mediated effects on VSMC. APN is anti-thrombotic, but the mechanism(s) of this effect is/are not well-understood⁹. Perhaps APN has receptor-mediated effects on VSMC influencing other circulating pro-thrombotic molecules besides PAI-1 such as tissue factor (TF) or C-reactive protein (CRP).

Globular APN can increase TF activity, mRNA expression, and protein expression by human umbilical vein endothelial cells (HUVECs), but this effect is not reflected using full-length APN⁴¹. If TF is chosen as a new target molecule, the effect of different molecular isoforms of APN should be taken into consideration.

Full-length APN reduces CRP mRNA and protein expression in human aortic endothelial cells (HAECs) under hyperglycemic conditions⁴². Also, secreted CRP levels are inversely proportional to circulating APN levels in male CAD patients, and CRP mRNA in subcutaneous WAT are inversely proportional to APN mRNA expression in healthy males⁴³. Furthermore, *Apn*^{-/-} mice have

elevated CRP mRNA expression in WAT as compared to wild-type controls^{9,43}, suggesting an APN-CRP connection.

Should APN alter the expression of other circulating coagulation factors *in vitro*, the next step would be to establish the receptor(s) and signaling molecule(s) involved. A survey of possible downstream mediators could be done using Western blotting with phospho-specific antibodies for known downstream mediators of APN, such as ERK, p38, AMPK, or Rho. Increased phosphorylation of a candidate signaling molecule would suggest that it is being used in APN-mediated signaling. Pharmacological blockade of candidate signaling molecules would also be an effective method of determining signaling pathways if the blockade resulted in abrogating treatment effects. To determine the receptor(s) involved in APN-mediated signaling, knockdown of AdipoRs using siRNA could be utilized; alternatively, using receptor-null cells would also be effective.

Determine whether the mRNA expression of AdipoRs *in vivo* is reflected at the protein level.

I established that murine aortic VSMC express AdipoR1 and T-cadherin mRNA *in vivo* (Chapter 3, Figure 8). However, gene expression does not necessarily reflect protein expression. Further experiments are warranted to examine if AdipoR1 and T-cadherin protein are expressed *in vivo*. Unfortunately, it is very difficult to use LCM as described in Chapter 2 to collect enough cells to use a conventional Western blot analysis to examine AdipoR expression in captured

cells. Immunohistochemistry would be an effective technique to use, so long as specific antibodies could be found—the commercially available antibodies for AdipoR1 and AdipoR2 tend to cross-react with each other as they share significant sequence homology.

Examine circulating PAI-1 as well as PAI-1 mRNA expression in aortic VSMC in wild-type and APN-deficient (*Apn*^{-/-}) mice.

This study did not show any significant difference in PAI-1 expression by HCAVSMC *in vitro*, however, this may not be reflected *in vivo*. Obese individuals have increased plasma PAI-1 levels, partly from increased PAI-1 mRNA and protein in the adipose tissue⁵ magnified by an increase in adipose tissue mass itself, but paradoxically low plasma APN concentration⁷. APN reduces PAI-1 expression in cultured adipocytes³⁷, but this effect was not studied on vascular smooth muscle cell PAI-1 expression.

Using the APN-deficient (*Apn*^{-/-}) mouse would be an effective means of determining the physiological role of APN in influencing the expression of circulating prothrombotic molecules *in vivo*. *Apn*^{-/-} mice on a regular chow diet are not obese, therefore any increase of circulating PAI-1 could not be attributed to increased adipose tissue mass and would support a role for APN reducing PAI-1 expression by cells besides adipocytes *in vivo*.

A sensitive PAI-1 ELISA could be used in measuring circulating PAI-1 protein in mouse plasma. I expect that circulating PAI-1 would be increased in

Apn^{-/-} mice based on the observation that APN reduces PAI-1 mRNA in cultured adipocytes. As described in Chapter 2, aortic VSMC PAI-1 gene expression could be examined with LCM and reverse transcriptase real-time PCR. I expect aortic PAI-1 mRNA expression to be increased in *Apn*^{-/-} mice, if not by a direct, receptor-mediated effect then by an indirect effect of binding growth factors that stimulate vascular PAI-1 expression such as PDGF-BB^{39,40}. APN binds PDGF-BB and blocks PDGF-BB-stimulated VSMC migration and proliferation *in vitro*^{26,27}, and this binding may also limit PDGF-BB-stimulated PAI-1 expression in VSMC.

Should a difference in PAI-1 expression be observed in wild-type and *Apn*^{-/-} mice, a potential role for APN could be verified by increasing the plasma APN concentration in the *Apn*^{-/-} mice to physiologic levels, either by short-term injections of recombinant protein or by longer-term infection of an adenovirus coding for APN of *Apn*^{-/-} mice. Reversal of changes noted would support a role of APN in vascular PAI-1 expression. Further experiments *in vitro* would be needed to examine the physiological mechanisms.

APPENDIX 1: DETAILED PROTOCOL FOR CELL CULTURE

You will need: Cells (Human coronary artery vascular smooth muscle cells, #C-017-5C)
Large Petri dishes (100 mm²) or other cell culture vessel (6-well plates, 12-well plates, etc)
Media 231 (Cascade #M-231-500)
Smooth Muscle Cell Growth Supplement (SMGS, Cascade #S-007-25)
Amphotericin B (Fungizone, Invitrogen)
Gentamicin (Abraxis Pharmaceuticals)
Phosphate-buffered Saline (Gibco)
D-glucose (Sigma)
Rosuvastatin (Toronto Research Chemicals)
rhAPN (R&D Biosystems)
Vacuum-filtered media bottles
15 mL conical tubes
Pipettes, tips, etc
Centrifuge

Prepare Media

1. Media must be warmed before use in a 37°C water bath for about 20-30 min. If you are making media to be used immediately, all components must be warmed prior to preparing the media. Otherwise, just thaw the Fungizone and SMGS.
2. Put on gloves. Spray gloved hands, turn on the blower and white light for the hood, and spray all surfaces of the hood with 75% ethanol and wipe them down with Kimwipes.
3. Spray vacuum-filtered media bottle pack, bottles of media, SMGS, gentamicin, and fungizone with 75% ethanol and wipe them down before placing them in the hood.
4. Pour off 50 mL of media to be kept for later to make treatment media.
5. Pour the remaining 450 mL media, the 25 mL SMGS, and 3 mL Fungizone into the top chamber of the vacuum-filtered media bottle. Use a syringe to add 600 uL gentamicin.
6. Attach the vacuum assembly, turn on the vacuum, and filter the media through to the bottom chamber.
7. When all of the media has moved through the filter to the bottom bottle, turn off the vacuum and remove the top chamber by unscrewing it from the bottom bottle.
8. LABEL the bottle of media with what is in it (+supp, +Ab, etc), your initials, and the DATE the media was made.

Prepare and Grow Cells

1. Cells are ordered from Cascade Biologics. They are received at passage 1. Use these cells or any of the tubes of cells that have been previously frozen at passage 3.
2. Tubes of cells should be plated at a density of 2.5×10^3 cells/cm² according to the manufacturer's protocol. This means that one tube will seed about 2 100 mm² Petri dishes.
3. Warm your media in the water bath.
4. Cells take very little time to thaw. Generally, if you place them in the water bath after your media is warm but before you spray the work surfaces and media bottles with ethanol they will be thawed by the time you are ready for them.
5. Add ~10 mL media to the Petri dish(es) and pipette the appropriate volume of cells into the media. Swirl the plate very gently to disperse cells.
6. Cover the dishes and place them in the incubator. Check the incubator pan on the bottom shelf to be sure there is water in it. It should be about half-full. Also, check the CO² hoses and tank to be certain there is enough going to the incubator.
7. Allow the cells to grow to 80% confluence before passaging. To pass, aspirate media and rinse cells once with sterile PBS. Add ~3ml TyrpLE trypsin to the cells and incubate about 10 minutes in the incubator.
8. View cells under the microscope to ensure that they have detached from the plate. Rinse cells down with media or PBS and transfer mixture to a sterile 15 mL conical tube.
9. Centrifuge the cell mixture at 1000 x g for 5 minutes.
10. Aspirate the supernatant, being careful to not disturb the cell pellet.
11. Reconstitute the pellet in an appropriate volume of media and plate cells at the desired concentration in the vessel of your choice.

Treating Cells

You will need: the 50 mL of Media 231 you poured off when making growth media
Antibiotics if you wish
Whatever you are treating cells with
50-mL vacuum filter tube

1. If you will be serum-starving cells prior to treating them, prepare treatment media (described below). When cells are appropriately confluent (according to your experimental conditions, you may want 80-100% confluent, post-confluent, etc), aspirate growth media, rinse with sterile PBS, and add serum-free media.
2. Prepare treatment media: combine media and antibiotics ONLY into the 50-mL conical tube and screw the vacuum filter 50 ml conical tube onto the top.

3. Attach the vacuum attachment, flip the tube assembly over, and allow the media to filter through to the bottom (sterile) tube. You now have a serum-free media suitable for treatment with a variety of drugs.
4. LABEL the media with what's in it, your initials, and the DATE.
5. Determine how much media you will need to treat all cells in your experimental group. Aliquot out treatment media needed, giving yourself a little extra just in case.
6. Add treatment/drug.
7. Aspirate growth media or serum-free media from cells (collect CM if you want it for experiments) and rinse with sterile PBS gently three times.
8. Add treatment media + drug, incubate for experimental time period, and lyse as needed. See RNA Extraction protocol if future PCR is desired.

APPENDIX 2: DETAILED PROTOCOL FOR RNA

EXTRACTION

You will need: CLEAN/sterile Petri dish(es)
Sterile PBS (preferably cold)
Sterile razor or scalpel blade
Ice
TRIzol or TRI reagent (Invitrogen or Sigma)
Centrifuge capable of 12,000 x g or more in the cold (2-8°C)
CLEAN 1.5 or 2 ml bullet tubes, RNase/DNase free
Hand-held homogenizer or another way to homogenize samples (CLEAN)
Chloroform
3M Sodium Acetate
100% (200 proof) EtOH
Glycogen (molecular biology grade, from Roche)
Reconstituting buffer (RNase/DNase free water, TE buffer, EB buffer, etc)
CLEAN Pipettes and barrier pipette tips

*Note: RNA is VERY FRAGILE! Change gloves often or clean gloves with RNase Away or EtOH and keep the lab space where you work as RNase/DNase free as possible! Wipe down the table and any instruments you will use. Use ONLY autoclaved tubes and RNase/DNase free barrier pipette tips. Always use chloroform in the fume hood.

From fresh tissue:

*Note: RNA will start degrading as soon as the tissue is excised. Perform these steps quickly but well—TRIzol will stabilize the RNA and degrade RNase activity.

1. Sacrifice a mouse and extract tissue desired (liver, skeletal muscle, aorta, fat, etc). If you are extracting a vessel, remove the peri-vascular fat as best you can before excising.
2. Quickly clean tissue as best you can in ice-cold PBS. I use a small Petri dish with a small amount of PBS in it and just swish it around for about 30 seconds 2 or 3 times.
3. Use a razor blade or scalpel to chop tissue into small pieces. I usually do this in the Petri dish lid as it is a sterile surface. This will make a tissue “mush” which is much easier to extract RNA from.
4. Somehow scoop the mush into a CLEAN 1.5 or 2 ml bullet tube. You can use a pipette or the edge of the scalpel blade, depending on what works.

5. Place tube on ice.
6. Add 250 uL TRIzol or TRI reagent to the mush on ice. Let it sit while other samples are being chopped (CLEAN THE BLADE or get a fresh blade between samples) or immediately use a hand-held homogenizer to mix the samples for 1-2 minutes.
7. Allow the samples to sit on ice while all are being homogenized.
8. Add 750 uL TRIzol/TRI reagent to each sample, bringing the total volume to 1 mL.
9. Centrifuge samples at 12,000 x g for 10 minutes at 2-8°C. In all samples but fat, the insoluble material will collect as a pellet at the bottom of the tube. Remove the pink supernate and keep. Discard the pellet. In fat samples, fat will collect at the top of the tube in a buffy layer. Carefully remove this layer and discard, keeping the pink liquid underneath.
10. Incubate samples at room temp for 5 minutes.
11. Continue to Phase Separation

From adhered (monolayer) cells:

1. Rinse cells three times with PBS, aspirating completely between rinses.
2. Add appropriate volume TRIzol/TRI reagent to cells. For a 6-well plate, use 1 mL TRIzol per well. For 12-well use 500 uL, for a big plate use 3 mL, for a flask use 4-5 mL.
3. Swirl the TRIzol around to ensure complete coverage and incubate at least 3 minutes at room temp.
4. After incubating, swirl the plate again and tip towards you so that the TRIzol pools in the bottom of the well. Pipette the sample up and down several times and move to a clean tube.
5. Proceed to Phase Separation.

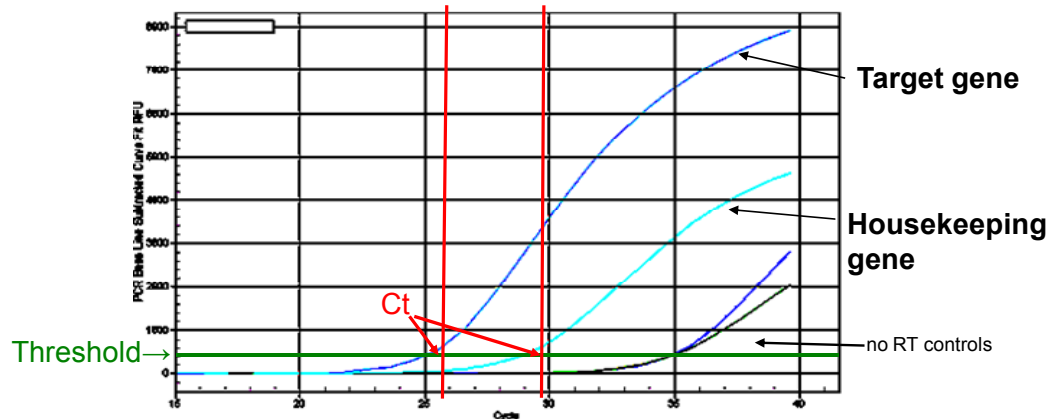
Phase Separation

1. Add 200 uL chloroform to each sample, hand shake vigorously for 15 seconds, and incubate at room temp 3 minutes.
2. Centrifuge samples for 15 minutes at 12,000 x g. Samples will separate into a clear, aqueous top layer and a pink bottom layer. Sometimes a white interphase will be seen between the layers. Be very careful with centrifuged samples—they re-mix easily!
3. VERY CAREFULLY remove the clear, aqueous phase using a pipette and transfer to a clean **2 mL** tube. This is usually ~500 uL (for 1 mL TRIzol) and contains the RNA. Avoid collecting the white interphase which contains DNA or the pink lower phase which contains proteins. Re-centrifuge any samples that get re-mixed.
4. To the aqueous phase, add 10% vol/vol 3M sodium acetate (for 500 uL aqueous phase, add 50 uL NaAc). Pipette up and down a few times to mix.

5. Add 2-3 volumes 100% EtOH (for 500 uL aqueous phase, add 1.5 mL or almost fill the rest of the tube with EtOH).
6. Add 1% total vol Glycogen (if you started with 500 uL aq phase and now have ~2mL, add 20 uL Glycogen).
7. Carefully close tubes and hand shake to mix. You will know you did well if you see yellowish or whitish strings of genetic material floating in the tubes. LABEL samples and freeze overnight in -20°C or -80°C. Several hours in -80°C is also usually sufficient.
8. After freezing, centrifuge samples at max speed (preferably 14,000 or 16,000 x g) for at least 30 minutes at 2-8°C.
9. Remove tubes and carefully invert once or twice to ensure pellet is firmly adhered to the bottom of the tube.
10. If pellets are secure, discard supernate by decanting the tube. Watch for non-adhered pellets, though, and remove the supernate by careful pipetting if needed.
11. Allow pellet to dry at room temp. This usually takes 20-60 min.
12. Reconstitute pellet in desired buffer, usually RNase DNase free water or TE buffer. Elution buffer from Qiagen kits works well, too.
13. Freeze samples until use.

APPENDIX 3: QUANTITATION OF REAL-TIME PCR DATA

USING THE $2^{(-\Delta\Delta Ct)}$ METHOD



- $Ct_{(\text{target})} - Ct_{(\text{HK})} = \Delta Ct_{(\text{sample})}$
- $\Delta Ct_{(\text{sample A})} - \Delta Ct_{(\text{sample B})} = \Delta\Delta Ct$
- $2^{(-\Delta\Delta Ct)} = \text{fold change in gene expression}$

Figure 12: Example of raw real-time PCR data and assignment of values for the $2^{-\Delta\Delta Ct}$ equation.

For the experiments described in this thesis:

- $Ct_{(\text{PAI-1})} - Ct_{(\text{RPL13a})} = \Delta Ct_{(\text{sample})}$
- $\Delta Ct_{(\text{sample})} - \Delta Ct_{(\text{Time 0})} = \Delta\Delta Ct$

APPENDIX 4: PRIMER SEQUENCES AND PCR

PRODUCT SIZES

Primer Sets (3'→5')

			Product size (bp)
<i>Mouse</i>			
AdipoR1	f	ACGTTGGAGAGTCATCCCGTAT	200
	r	GCATCGTCAAGATTCCCAGAAAGAG	
AdipoR2	f	TCCCAGGAAGATGAAGGGTTTAT	120
	r	TGAGGGATCACTCGCCATCGGCC	
T-cadherin	f	TTGAGATCCACACCAACCCACAGA	105
	r	CTCCACTTTGATCAGCAGGGTGT	
Adiponectin	f	GCAAGCTCTCCTGTTCTCTTAATC	211
	r	TGCATCTCCTTTCTCTCCCTTCTC	
SMAA	f	TCCGATAGAACACGGCATCATCACTCAACT	192
	r	TACATGGCGGGGACATTGAAGGTCT	
vWf	f	TGTGTGGTGGCCATTGGGAGACCT	137
	r	GGCATCTTCCTGCATGTCTTC	
<i>Human</i>			
AdipoR1	f	TTCTTCCTCATGGCTGTGATGT	172
	r	CCTGAAGGTTGGAGACTCCATAG	
AdipoR2	f	ATAGGGCAGATAGGCTGGTTGA	159
	r	AAAAGCTCCAGCAACCACAAAG	
T-cadherin	f	TCTGTTTACAAGGACCCAGCAGGT	185
	r	TCCAGGGTTATCAGCAAAGTCCCA	
PAI-1	f	GCACGGTGGCCTCCTCATCCACAG	110
	r	CTGTGGGGTTGTGCCGGACCACAA	
SMAA	f	TGACAATGGCTCTGGGCTCTGTAA	142
	r	TTCGTCACCCACGTAGCTGTCTTT	
vWf	f	GGTAAAATTATGAAAATTCCAGG	138
	r	CTGGCAGTAGTGGATATCCACCTC	
RPL13a	f	CCTGGAGGAGAAGAGGAAGAGA	125
	r	TTGAGGACCTCTGTGTATTTGTCAA	
<i>Pig</i>			
T-cadherin	f	*all primers and products same as <i>Human</i> except CAGATATTCCATTTACAAGGAC	185

APPENDIX 5: DETAILED PROTOCOL FOR LCM

Sample preparation and mounting

You will need: Sample, cleaned and prepared

Liquid nitrogen

OCT tissue mounting media

CLEAN plastic or aluminum foil cryomolds

Screw-top cryotubes

CLEAN forceps, needles, scalpel bladed as needed

Gloves

1. Snap-freeze cleaned sample with liquid nitrogen. Do not add PBS or other buffer to the sample—just freeze the tissue. Use screw-top cryotubes designed for this purpose. Bullet tubes will not work. Sample may be kept in -80°C or in liquid nitrogen until use. (see other protocols for sample collection)
2. Very briefly warm the sample so that it can be extracted from the tube. Try to avoid thawing the sample completely, just enough to remove it from the side of the tube.
3. Fill the cryomold $\frac{1}{2}$ - $\frac{3}{4}$ full with OCT and place in -80°C freezer briefly. The objective is to get the OCT to become more viscous, not solid, so that the sample may be manipulated within the media.
4. Place sample (<10 mm, preferably around 5 mm long, TINY) in the OCT in an orientation that is conducive to sectioning. For example, with mouse aorta, orient the tissue so that the samples will make an “O” when cross-sections are cut from the top of the mold. You may put in as many sections of tissue per cryomold as you please—I usually put in 2-3 sections of aorta per cryomold so that I get 2-3 cross-sections each time I cut the sample. During this time, it is easiest to use a needle to manipulate the tissue, if needed, rather than forceps.
5. Place the cryomold and tissue back in the -80°C freezer to allow the OCT to solidify. Manipulate tissue as needed during this process.
6. Once the cryomolds have solidified, LABEL them and either send them to Cherie with Histology for sectioning (usually takes the better part of the week to get them back) or use the cryostat in the LSC to section samples. Samples in OCT may be kept at -80°C until use.

Using the cryostat

You will need: Gloves

Slides (positively-charged, plain glass, or Excell)

OCT

Dry ice

Small slide box(es) (up to 25 slides)

Cryostat blades, chucks, etc as needed

*Note: before you use this machine, get trained by a member of Dr Will's or Dr Cornelison's lab. This is an expensive, NICE piece of equipment and must be kept CLEAN and in good working order!

*Note: I recommend having a "sacrificial sample" available for optimization. This can be anything, but it is best if it is the same tissue you will be using for samples, ie, if you will be using mouse aorta, sacrifice a junk mouse for your first try. It will save you a LOT of headache, not to mention your samples.

*Note: many of the labs in the LSC doing cryosectioning will share a box of cryostat blades and other disposables. Be sure you either bring your own blades, OCT, slides, etc, or have a prior standing agreement with Dr Will's or Dr Cornelison's lab to share these items. I ALWAYS bring my own slides in a sort of "care package" with gloves and OCT—these are used the fastest and I don't want to be without enough for my need that day.

1. Be sure to sign up ahead of time for the cryostat on the third floor of the Life Sciences Center in Dr Mark Will's lab.
2. The stat will be kept around -10°C by default. You will need to arrive early enough or block off enough time to get the temperature low enough for the tissue you wish to section. For mouse aorta, I use -28°C for the chuck temperature and -30°C for the general temperature. There is no one temperature that works for all tissues—you will have to optimize this by trial and error.
3. Carry your samples over on DRY ICE. Ice packs are OK, but not ideal.
4. Allow the cryostat to cool to desired temperature and get set up. I like to put my slide box in the stat to keep finished slides frozen, lay out a few slides in easy reach, have a box of gloves handy, change the cryostat blade, and warm up and RNase the chucks if needed. Again, be sure to get trained on this machine before you start adjusting it!
5. When the cryostat is ready to go, take out the desired OCT-mounted sample and allow it to warm up slightly so that it can be easily freed from the cryomold.
6. Attach the sample to the chuck with OCT and allow it to harden into place inside the cryostat. Up to 4 samples can be held frozen in the cryostat on chucks, but be sure you remember which sample is which! BE CAREFUL you don't get them mixed up!
7. Move the chuck mount back as far as it will go and secure the chuck to the chuck mount. Be sure you tighten both adjusters on the chuck mount.
8. Move the chuck mount forward so that the sample is nearly touching the blade. Using the hand wheel, make a few trial cuts and adjust the anti-roll plate as needed. If the sections are rolling, turn the knob at the bottom of the plate clockwise. If sections are shredding up against the plate, turn the knob counter-clockwise. These adjustments are usually very minute.
9. Be sure you are slicing sections at the appropriate thickness. For mouse aorta, I use $6\ \mu\text{m}$ and for pig coronary vessel I use either 6 or $4\ \mu\text{m}$. Sections this thin are difficult to manage—they will roll, shred, and otherwise be un-useable in many

different ways. Be patient and take the time to get all the adjustments necessary to have smooth sectioning. It is worth it to preserve as much of your sample as possible!

10. Use the hand wheel to section tissue. Frequency and velocity of turning the hand wheel is subjective; it just takes practice. Sometimes a section of tissue will adhere to the edge of the section before it, forming a “ribbon” of sections. This is the easiest way to get good slides, but it is not always possible depending on the sample.
11. Carefully pull the anti-roll plate away from the section(s). For singles, use a paintbrush to VERY CAREFULLY flatten and adjust the section such that it can adhere to the slide. Sample ribbons usually will not need as much manipulation, but you will need to get the ribbon oriented so that it will fit on the slide.
12. Place a ROOM TEMPERATURE slide “face-down” on top of the frozen sections. They will adhere to the warmer slide and usually the tissue sample will become visible as the OCT surrounding it becomes clear. Look at the slide or use a microscope to examine placement and quality of the section(s). Make adjustments as needed (temperature, orientation, etc) and don't forget to LABEL the slides.
13. When a slide is finished (I usually put 6-8 sections per slide), place it in the slide box in the cryostat to keep them frozen.

Slide staining and dehydration

You will need: RNase Away or other solution that will eliminate RNase
DEPC-treated water, both for staining and for dilution of other staining solutions
10X PBS, used only for this purpose
95% and 100% EtOH
Hematoxylin (Sigma) and Eosin (VWR)
Xylenes
Desiccating cabinet or container with Drierite
Fume hood
Timer

*Note: RNA will be degrading from the moment you thaw the slides—act quickly but carefully.

*Note: use RNase free technique!! It is VITAL that you not contaminate the slides. Have a space devoted entirely to staining if possible that is away from other lab activities and can be kept as CLEAN as possible. Wipe down or spray EVERYTHING with RNase away, change gloves often, etc!!

*Note: prepare solutions as you need them but always replace any solution that has been sitting out for more than a day uncovered. Use DEPC-treated water to dilute the 95% or 100% EtOH to 70% EtOH and also to dilute the 10x PBS to 1x.

*Note: I have found that it works best to stain only a few sides at a time, collect those slides, and then stain more, collect more, etc. This keeps the slides fresh and as dehydrated as possible and yields the best results.

1. Allow slides to thaw at room temperature briefly, maybe 2-5 minutes.
2. Arrange slides in slide holder so that the labels will not get splashed with ethanol.
3. Dip slides in 70% EtOH for 30 sec
4. Move to DEPC-water for 30 sec
5. Stain in Hematoxylin for 1 minute
6. Rinse in DEPC-water 30 sec
7. Rinse in 1X PBS 15 sec
8. Rinse in DEPC-water 30 sec
9. Dehydrate in 70% EtOH for 1 minute
10. Move to 95% EtOH for 30 sec
11. Dip TWICE slowly in Eosin, then move to 95% EtOH for 15 sec
12. Move to another container of 95% EtOH for 30 sec
13. Dehydrate in 100% EtOH for 30 sec
14. Dehydrate in xylenes for ~3 minutes.
15. After staining and dehydration, allow slides to dry under a fume hood for 5-10 minutes until the xylenes have evaporated. Then move them to a desiccating cabinet with Drierite under the slides until they are to be used.

Collecting cells from stained and dehydrated samples

You will need: CapSure caps (I use CapSure Macro) from Arcturus
Extraction buffer from the PicoPure RNA extraction kit (or similar)
0.5 ml tubes, RNase free!
Gloves
Pipette with RNase-free barrier tips
RNase away or similar RNase-removing cleaner
Dry ice
Incubator set at 42°C
Centrifuge

*Note: you will need to get trained by someone at the Molecular Cytology Core on the machine to be a qualified user. During the training and for the first couple of times using the machine, I suggest having “sacrificial samples” to use so that you do not waste your good stuff.

*Note: every LCM machine is different. This protocol is particularly designed for the LCM in the Molecular Cytology Core in the Life Sciences Center.

*Note: you will need to be extra-careful to use RNase-free technique during this time. It can take awhile to collect all the cells, but be careful to not introduce RNases from your hands, food, or otherwise. The cells you collect here and the RNA you extract from them will be your sample for all future experiments. Do a good job!! Wipe down the counters

and spaces you will be using with RNase away, change gloves often, do not eat or drink anything near the scope, etc.

*Note: You can bring the entire PicoPure RNA kit with you if you like, but I usually just bring the buffer and the tubes. It keeps the rest of the kit cleaner.

1. Turn the machine on, log in, and open the Arcturus software for the scope.
2. Click “Present Stage” and load the slides and caps.
3. On the 2x objective, locate your sample and adjust the settings as needed.
4. Collect a tiled image of the slide, retaining the settings you’ve just created.
5. Focus on the part of the sample you wish to capture—you may need to change the objective to 4x or 10x. Load a cap and bring it to the field. Do a few test fires of the laser and adjust the laser intensity and duration as needed.
6. Draw the area to be captured using the mouse or the stylus. Remember to use the laser spot size appropriate for the sample. I use the smallest spot size (7.5 μm) for mouse tissue.
7. Collect the cells. Be sure that the laser is not burning the tissue as it is collecting. If the tissue is getting singed, decrease the intensity and duration of the laser. Caps can be moved to different slides to make the most of them.
8. Pipette the appropriate amount of extraction buffer into a tube—I use 40 μl EB. LABEL the tube.
9. Offload the cap, and attach it to the microcentrifuge tube, inverting it so the EB covers the top of the cap.
10. Incubate tube and cap assembly at 42°C for 30 minutes.
11. After incubation, centrifuge the tube and cap assembly for 2 minutes at 8,000 x g.
12. Remove the cap from the tube, LABEL the tube if necessary, and freeze the lysate.
13. Samples are now ready to be transported back to the lab for RNA extraction using the PicoPure RNA extraction kit from Arcturus. Use the manufacturer’s protocol for best results.

APPENDIX 6: RELATIVE PAI-1 EXPRESSION IN HCAVSMC USING SM α A AS HOUSEKEEPING GENE

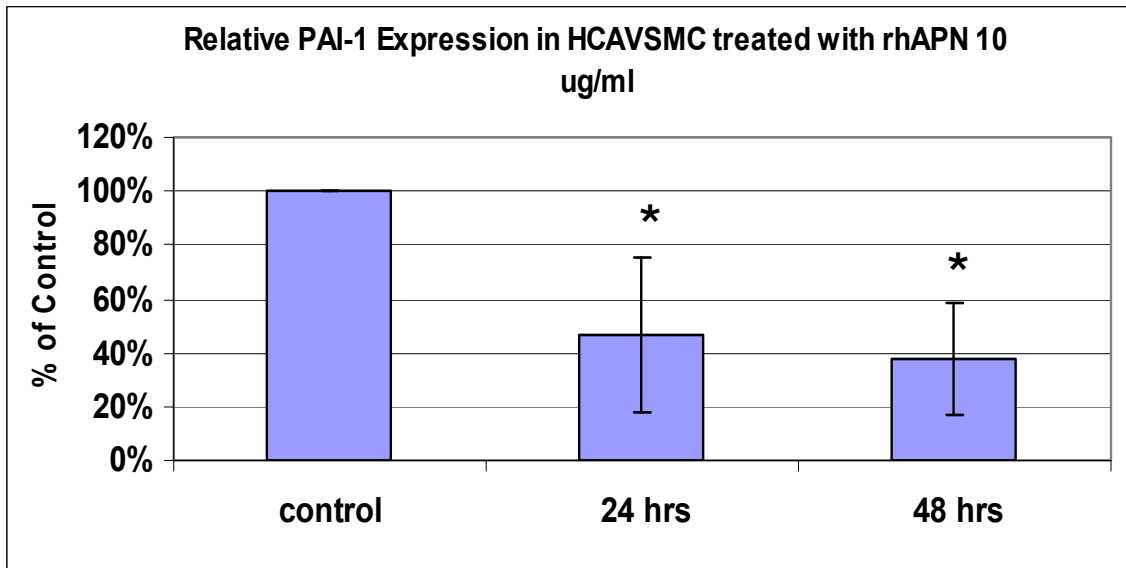


Figure 13: Relative PAI-1 expression in HCAVSMC using SM α A as housekeeping gene.

HCAVSMC were treated with either 10 μ g/mL rhAPN or PBS vehicle control for 24-48 hours. Cells were then lysed and RNA extracted from the lysates and used in reverse transcriptase real-time PCR to measure relative PAI-1 expression using smooth muscle α -actin as a housekeeping gene. After 24 or 48 hours, rhAPN-treated cells showed significantly decreased PAI-1 mRNA vs PBS vehicle control-treated cells. Results are expressed +/- standard error and the asterisk (*) notes data that are significantly different ($p < 0.05$) from control. N=8.

APPENDIX 7: PURSUIT OF A BETTER HOUSEKEEPING

GENE

According to Livak and Schmittgen³³ a gene is deemed a “good” housekeeper if its expression does not change under the condition(s) of the experimental system.

Method for determining “good” housekeeping gene:

- Perform real-time PCR using prospective housekeeping gene(s)
- Express data as $2^{(-Ct)}$
- Use t-test to determine if candidate HK varies under experimental conditions.
 - Time (over 24 hours)
 - Treatment with rhAPN
- Housekeeping gene good if no significant ($p>0.05$) change over time or with treatment

Example using **Smooth Muscle α -Actin** (n=4, duplicates of each sample)

HCAVSMC were serum-starved overnight, then treated with 10 μ g/mL rhAPN or PBS vehicle control for 24 hours. Time 0 refers to cells that were lysed before treatment but after overnight serum-starving. After 24 hours, cells were lysed and lysates subjected to reverse transcriptase real-time PCR.

Time 0		Con 24		APN 24	
Ct	$2^{(-Ct)}$	Ct	$2^{(-Ct)}$	Ct	$2^{(-Ct)}$
25.13	2.72343E-08	23.02	1.18E-07	21.65	3.04E-07
25.09	2.8E-08	23.37	9.22E-08	21.65	3.04E-07
24.91	3.17207E-08	23.39	9.1E-08	21.6	3.15E-07
25.84	1.66489E-08	23.31	9.62E-08	21.71	2.92E-07
20.47	6.88519E-07	27.65	4.75E-09	28.39	2.84E-09
21.05	4.60594E-07	27.23	6.35E-09	23.63	7.7E-08
20.61	6.24844E-07	24.58	3.99E-08	24.63	3.85E-08
20.97	4.86857E-07	24.51	4.19E-08	23.34	9.42E-08
t-test for time (time 0 and CON 24)			0.061455		
t-test for treatment (APN 24 vs CON 24)			0.048175		

APPENDIX 8: RELATIVE SM α A EXPRESSION IN HCAVSMC TREATED WITH rhAPN

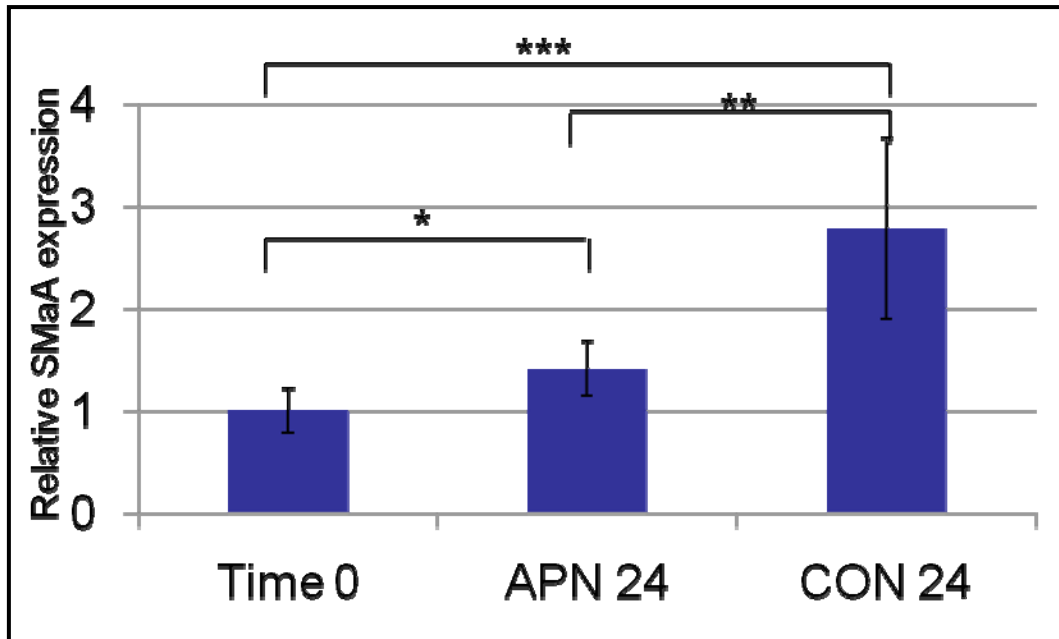


Figure 14: Relative SM α A expression in HCAVSMC treated with 10 μ g/mL rhAPN or PBS vehicle control for 24 hours.

HCAVSMC were treated with either 10 μ g/mL rhAPN or PBS vehicle control. After 24 hours, cells were lysed and RNA was extracted from the lysates. Reverse transcriptase real-time PCR data are reported above, showing relative smooth muscle α -actin expression (y-axis) normalized to RPL13a in rhAPN-treated cells (APN24) or PBS vehicle control-treated cells (CON24). Time 0 are samples that had been serum-starved overnight and lysed. Data are shown +/- standard error.

n = 2, * p = 0.03, ** p = 0.02, *** p = 0.005.

APPENDIX 9: EXPRESSION OF ADIPONECTIN RECEPTORS BY VASCULAR SMOOTH MUSCLE CELLS

Introduction

Adiponectin (APN) is a ~30 kDa adipokine secreted almost exclusively from adipose tissue. Originally studied as an insulin sensitizer, APN increases fatty acid oxidation in skeletal muscle and reduces liver gluconeogenesis¹⁰⁻¹². In mouse models of obesity and type 2 diabetes mellitus, APN treatment ameliorates hyperglycemia and hyperinsulinemia induced by a high-fat diet¹⁰. APN is present in the plasma in high levels (3-30 µg/mL)⁶. APN exists in four different forms in the plasma: globular APN, low molecular weight (LMW) trimers, mid-molecular weight (MMW) hexamers, and high molecular weight (HMW) homomultimers⁷.

APN has strong effects on the vasculature. APN reduces TNF- α -induced vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, and E-selectin expression in human aortic endothelial cells (HAECs) as well as TNF- α -induced monocyte adhesion to HAEC, presumably through activation of NF- κ B²⁰. APN increases nitric oxide (NO) production by

upregulating endothelial nitric oxide synthase (eNOS) activity in human umbilical vein endothelial cells (HUVEC)¹⁸.

APN binds its receptors (AdipoRs), AdipoR1, AdipoR2, and T-cadherin and exerts many of its effects on several cell types. AdipoR1 is abundantly expressed in skeletal muscle and moderately in other tissues while AdipoR2 is expressed predominantly in the liver¹⁵. All three receptors are expressed by endothelial cells, but the expression of AdipoRs by other vascular cells is not well-reported. Here, we test the hypothesis that AdipoRs are expressed *in vitro* by human coronary artery VSMC (HCAVSMC) and *in vivo* in porcine coronary artery and murine aortic VSMC.

In vitro studies have focused primarily on indirect effects of APN on smooth muscle cells such as binding platelet-derived growth factor (PDGF)-BB and endothelial growth factor (EGF) as possible mechanisms to reduce DNA synthesis and migration of VSMC²⁷. APN is deposited in the subendothelial space after arterial injury²⁵, so it is possible that APN has additional receptor-mediated effects on VSMC. APN reduces PAI-1 mRNA and protein expression in cultured adipocytes³¹, so in this study we test the second hypothesis that APN reduces PAI-1 expression in HCAVSMC through a receptor-mediated mechanism.

Methods

Cell culture. HCAVSMC were purchased and grown in Medium 231 with Smooth Muscle Growth Supplement (Cascade Biologics) with added Fungizone (Amphotericin-B, Invitrogen) and gentamicin (Abraxis Pharmaceuticals). Cells were passaged at around 80% confluency using TrypLE (Gibco) up to and including passage 6. In some studies, HCAVSMC at 100% confluency were serum-starved overnight and then treated with 27.5 mM D-glucose (Sigma), 10 μ M rosuvastatin (Toronto Research Chemicals), 10 μ g/mL human recombinant APN (rhAPN, R&D Biosystems), or PBS vehicle control for 24 or 48 hours.

Polymerase Chain Reaction (PCR). Confluent HCAVSMC were lysed with TRIzol reagent (Invitrogen). After chloroform phase separation, total cellular RNA was precipitated with 3M sodium acetate, 100% ethanol, and glycogen (Roche). RNA was quantified using a NanoDrop 1000 (Thermo Scientific). cDNA was reverse transcribed from RNA (2 μ g) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and random hexamer primers. Negative control reactions lacking reverse transcriptase were included in each experiment. Primer pairs spanned an intron to distinguish products derived from cDNA vs. contaminating genomic DNA. Samples of cDNA were used in subsequent real time and end-point PCR reactions with cycles of initial hold at 95°C for 3 minutes followed by 35 cycles of 95°C for 30 seconds and annealing temperature for 20

seconds. Aliquots of PCR product (10 μ L) were subjected to electrophoresis through 2% agarose gels. Bands were visualized using a FluorChem 8000 imager. A melt curve was conducted after real-time PCR to assess purity of product. Results were calculated using the $2^{(-\Delta\Delta Ct)}$ method described by Livak and Schmittgen^{32,33}.

Briefly, fold change in gene expression = $2^{(-\Delta\Delta Ct)}$

where $\Delta\Delta Ct = \Delta Ct_{(treated\ group)} - \Delta Ct_{(control\ group)}$

and $\Delta Ct = Ct_{(target\ gene)} - Ct_{(housekeeping\ gene)}$

A list of PCR primers and product sizes for these experiments is presented in Appendix 4. Power analysis and sample size calculations were performed using software from DSS Research.

Western blotting. HCAVSMC were lysed with RIPA buffer (Pierce) containing protease inhibitors (Complete EDTA-free, Roche). Lysates were centrifuged to pellet insoluble material and supernates were stored on ice. Samples of protein lysates (up to 50 μ g) were electrophoresed through 10% agarose gels. Proteins were transferred to nitrocellulose membranes and blocked with 2.5% nonfat milk or 2% BSA and probed with appropriate primary and HRP- or biotin-tagged secondary antibody (all from Santa Cruz). Membranes were developed with ECL substrate (Pierce) and imaged on x-ray film.

Animals. 8-week old male wild-type (WT) mice on a C57BL/6J genetic background were purchased from Charles River Laboratories. Mice were kept in groups of <5 littermates in polycarbonate cages with a 12-hour light cycle and provided *ad libitum* normal chow diet.

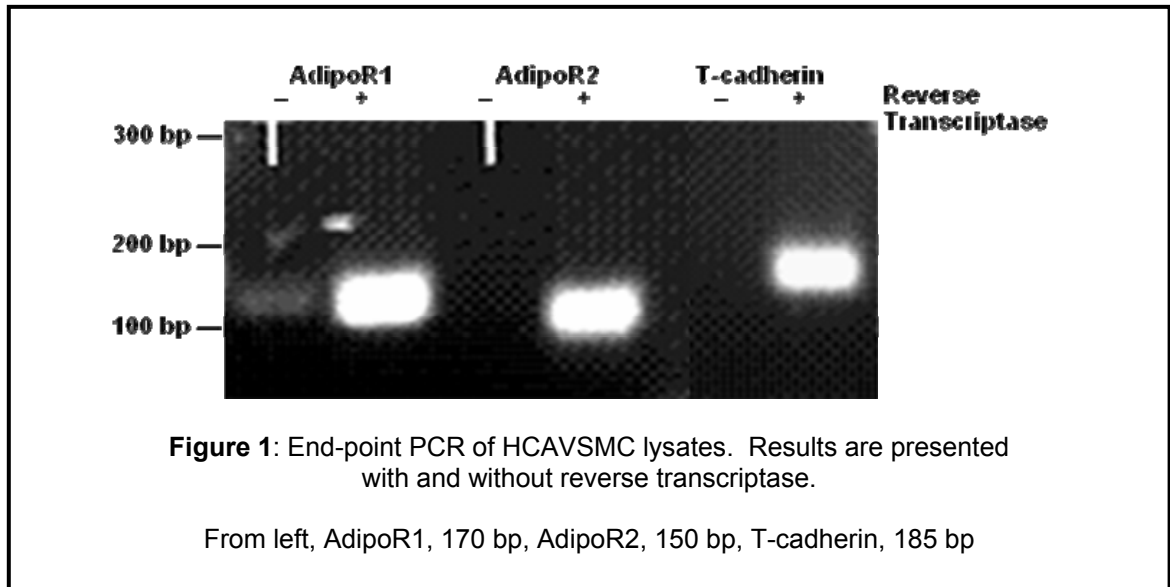
Surgical procedures. 10-16-week old animals were anesthetized by inhalation of a mixture of room air and 4% isoflurane. The thoracic aortas were harvested from the aortic arch to the diaphragm through an abdominal incision that included opening the rib cage. Aortas were cut into <1-cm sections, and snap-frozen in liquid nitrogen for laser capture microdissection (LCM).

Tissue preparation and laser capture microdissection (LCM). Frozen vessel segments were cryo-sectioned into 6 μ m cross-sections on a Leica Cryostat, stained with HistoGene stain (Arcturus), and dehydrated in increasing concentrations of ethanol with a final dehydration in xylenes. Slides were dried in a fume hood and kept in a desiccator with Drierite (Fisher) until use. Slides were used within 1 hour of staining and dehydrating to reduce RNA degradation and decrease rehydration of the slides. The vascular media was visualized and laser microdissection was performed using a Molecular Devices Arcturus system located in the Molecular Cytology Core at the University of Missouri. Media cells were collected using CapSure Macro LCM caps (Arcturus). RNA from freshly-captured cells was extracted using the PicoPure RNA Isolation Kit (Arcturus). PCR was performed as described above.

Results

AdipoRs are expressed by VSMC in vitro and in vivo

To examine the expression of AdipoRs by VSMC, lysates of cultured HCAVSMC were prepared and subjected to reverse transcriptase and end-point PCR. Robust bands of predicted sizes were seen for each set of AdipoR primers. Very faint or no bands were seen in reactions lacking reverse transcriptase, showing very little to no genomic DNA contamination (Figure 1). Sequencing of PCR products showed that the products were identical to corresponding AdipoR1, AdipoR2, and T-cadherin genomic sequences. These results suggest the gene for each APN receptor is expressed in cultured HCAVSMC.



To examine the expression of each AdipoR at the protein level, HCAVSMC lysates were subjected to SDS-PAGE/Western blotting using

antibodies specific for each receptor. Cell lysates prepared from mouse skeletal muscle and liver were used as positive controls as each of the polyclonal primary antibodies used is known to cross-react with murine and human AdipoR1, AdipoR2, or T-cadherin, respectively. Strong Western blot signals were observed for each receptor in HCAVSMC lysates, consistent with expression of all three receptors in cultured HCAVSMC (Figure 2).

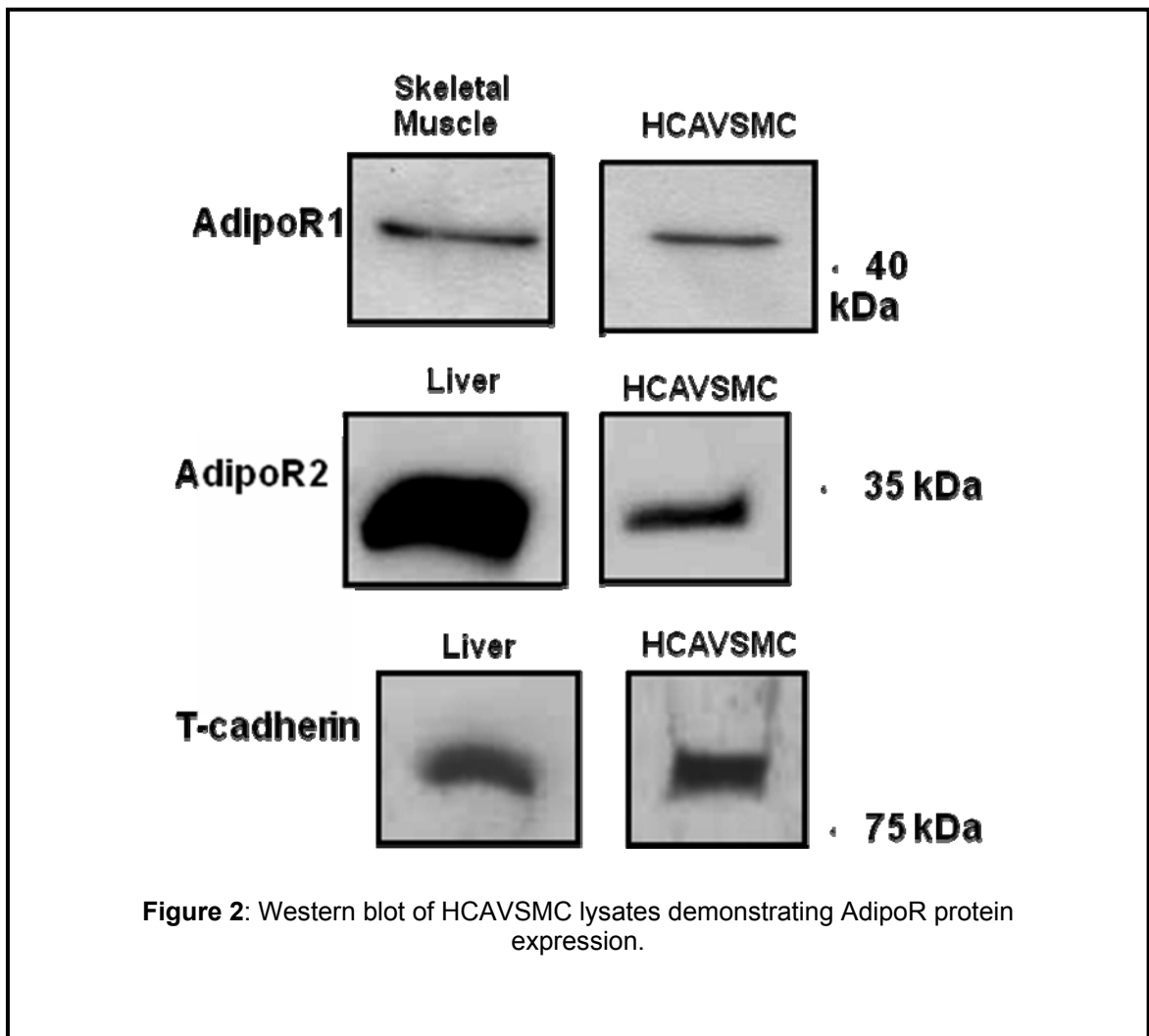
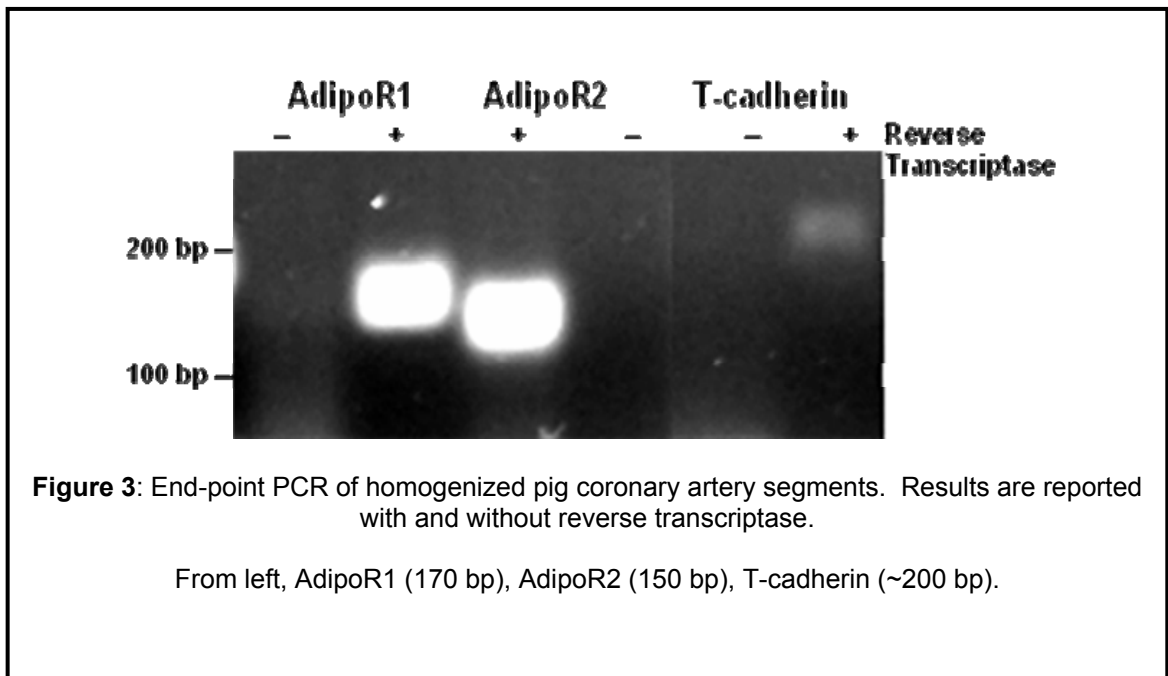


Figure 2: Western blot of HCAVSMC lysates demonstrating AdipoR protein expression.

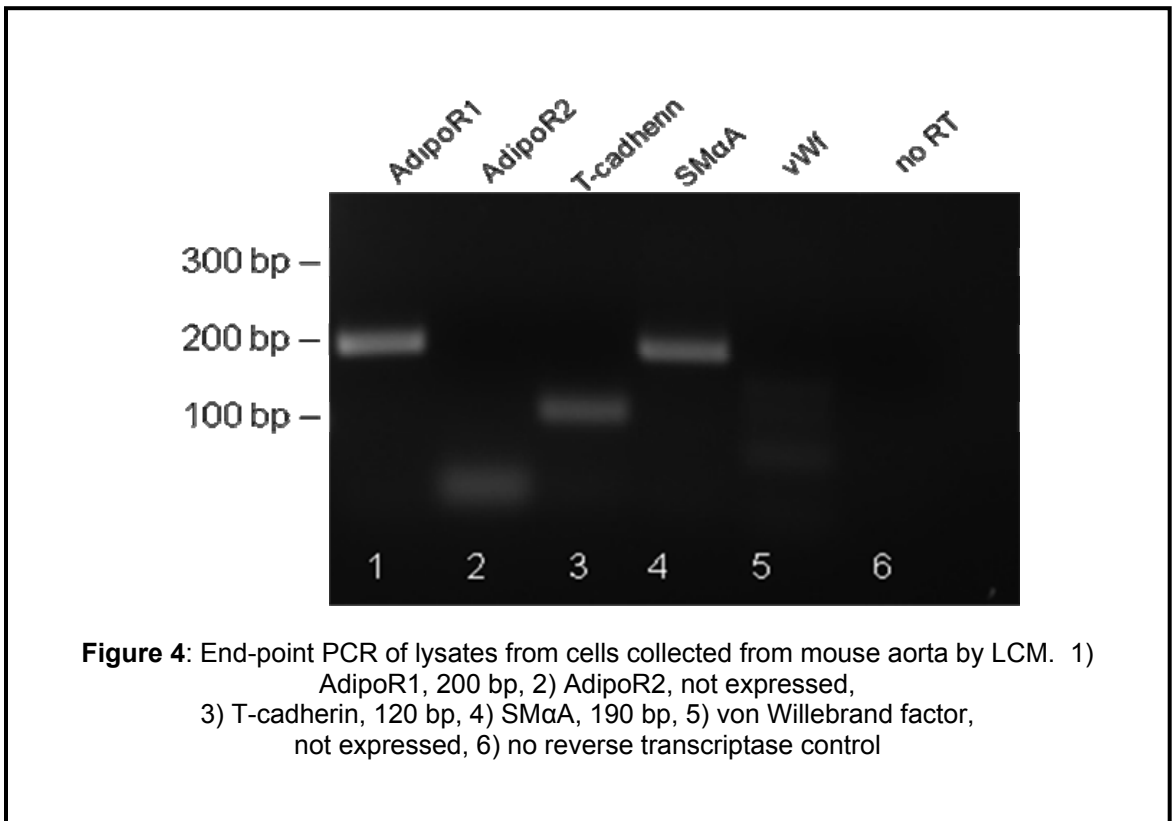
RNA from homogenized pig coronary artery lysates was kindly donated by Doug Bowles, PhD, for investigation of AdipoR gene expression *in vivo*. Vessel segments were snap-frozen in liquid nitrogen immediately after excision and pulverized to form a whole-vessel homogenate before RNA extraction. We subjected total RNA isolated from homogenates to reverse transcriptase and end-point PCR. PCR products of the predicted size were observed for each set of AdipoR primers, though the band observed for T-cadherin was faint. Negative control reactions lacking reverse transcriptase yielded no detectable PCR product (Figure 3). These results suggest that AdipoRs are expressed in porcine



coronary artery, and that the predominant AdipoRs expressed being AdipoR1 and AdipoR2. However, it is possible that reduced efficiency of T-cadherin PCR as compared to AdipoR1 and AdipoR2 PCRs could account for the observed

results. We were unable to discern if the VSMC specifically expressed AdipoRs, however, as the entire vessel was homogenized during RNA preparation.

To investigate whether VSMC *in vivo* express AdipoR RNA, we used laser capture microdissection (LCM) to selectively extract cells from the tunica media



layer of murine aortas. Total cellular RNA from collected cells was subjected to reverse transcriptase and end-point PCR to examine AdipoR gene expression (Figure 4). Bright bands are visible in lane 2 around 200 bp, corresponding to AdipoR1 PCR product and in lane 4 at 105 bp, corresponding to T-cadherin PCR product. Lane 3 contains a band well below 100 bp and we believe this to be mis-primed product, not PCR product for AdipoR2, which would be around 120 bp. Also, there is some faint random “laddering” in lane 6, which contains PCR

product for von Willebrand factor. We believe this to be mis-primed product as well and not von Willebrand factor PCR product, which would be at ~135 bp. Strong signal of smooth muscle α -actin (lane 5) but not vonWillebrand factor (lane 6) suggests that the population of cells analyzed was primarily smooth muscle cells, with little to no endothelial cells, which have already been shown to express AdipoRs and may yield falsely positive results for AdipoR expression. Samples lacking reverse transcriptase showed no visible PCR product (example in lane 6 using PCR primers for smooth muscle α -actin, for other primers, data not shown), demonstrating the samples did not contain genomic DNA contamination. Overall, these data suggest that VSMC express AdipoR1 and T-cadherin *in vivo* in mouse aorta.

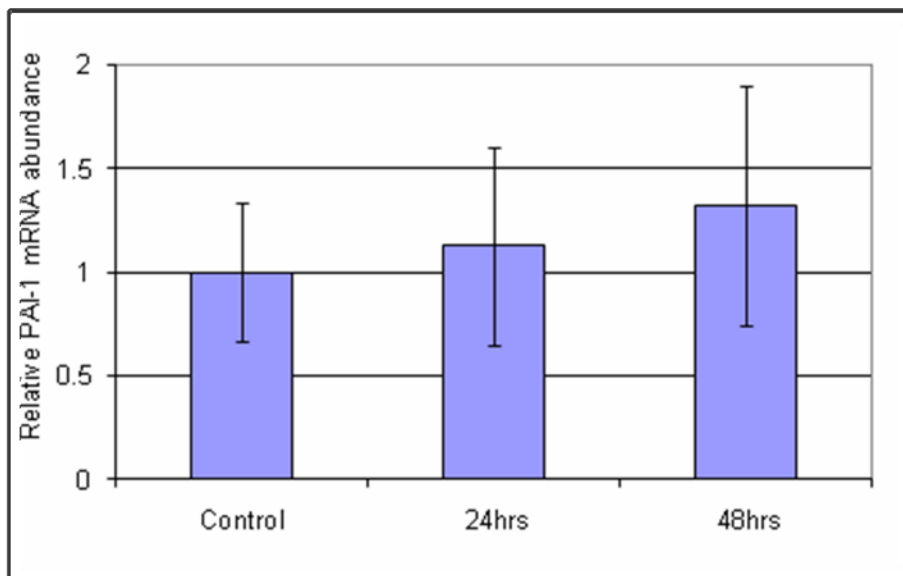
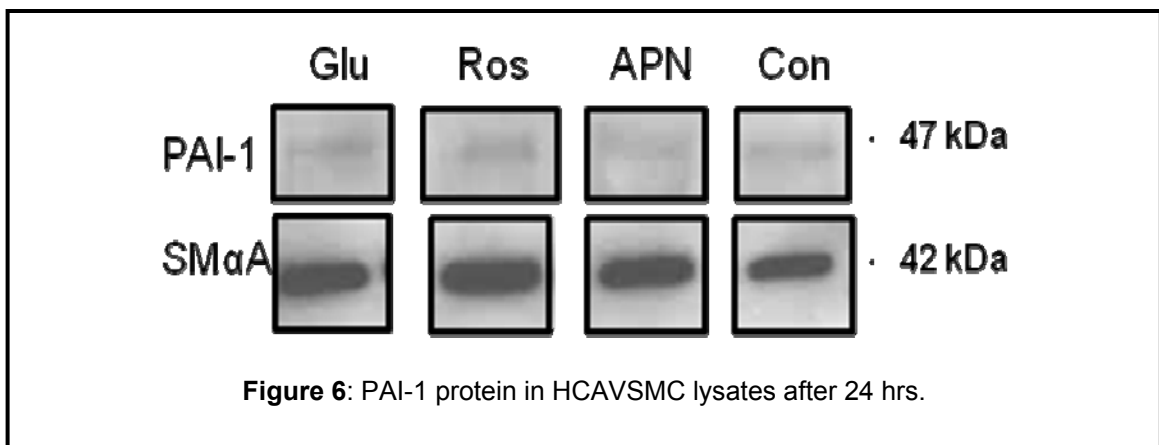


Figure 5: Effect of 10 μ g/ml rhAPN on PAI-1 mRNA expression in cultured HCAVSMC. N = 8, p = 0.41, t-test of 48 hr time vs. control, power = 10.2%

APN does not alter PAI-1 expression by HCAVSMC

To test the hypothesis that APN will reduce PAI-1 mRNA and protein in HCAVSMC, we treated cultured HCAVSMC with 10 µg/ml recombinant human APN (rhAPN) or PBS control for up to 48 hours. Cells were lysed and analyzed using real-time PCR and Western blotting. Relative PAI-1 mRNA abundance from real-time PCR data was estimated using the $(2^{-\Delta\Delta C_t})$ equation as described by Livak and Schmittgen^{32,33}.

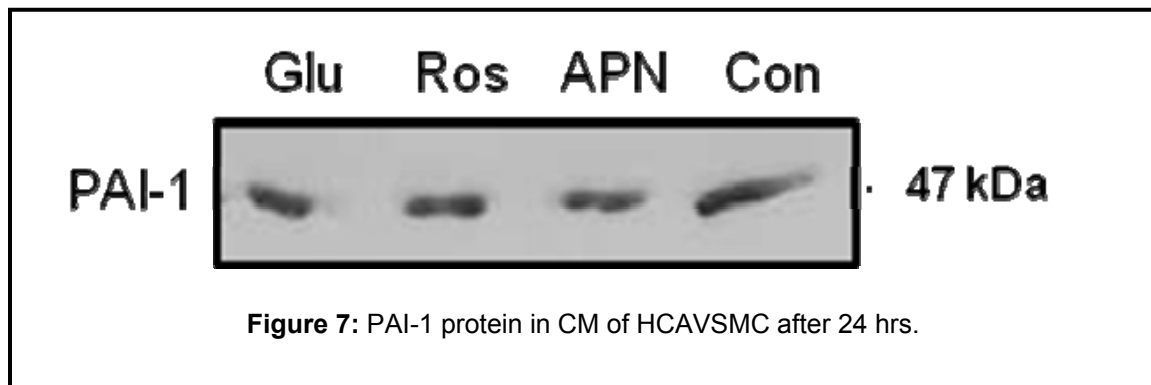
In preliminary experiments, PAI-1 abundance was compared to smooth muscle α -actin, with the results suggesting a robust decrease in PAI-1 expression with rhAPN treatment (data shown in Appendix 6). However, further analyses³³ showed that smooth muscle α -actin expression changes significantly over time and with rhAPN treatment and thus yielded falsely positive results. An



explanation and example of these analyses are shown in Appendix 7. Hence, we performed a survey of potential housekeeping genes using the Human Housekeeping Gene kit (Realtimeprimers.com) and found a more suitable

housekeeping gene, RPL13a, that neither changed significantly over time nor with treatment in this system. We repeated the real-time PCR using RPL13a as the housekeeping gene and results revealed no significant difference in PAI-1 gene expression in cells treated with 10 µg/mL rhAPN vs. PBS control-treated cells. A trend towards an increase in PAI-1 mRNA expression was seen, however, this trend was not significant (power = 10.2%, Figure 5).

To examine whether a change in PAI-1 expression could be seen at the protein level, whole cell lysates and conditioned media from HCAVSMC were



collected after 24 hrs of rhAPN (10 µg/ml) or PBS control treatment. Cells treated with glucose (27.5 mM) and rosuvastatin (10 µM) served as positive controls. High glucose in treatment media (5-35 mM) increases PAI-1 expression *in vitro* in cultured VSMC^{32,33}. HMG CoA reductase inhibitors (herein referred to as “statins”) reduce PAI-1 expression in vascular smooth muscle and endothelial cells *in vitro*^{36,37}. Rosuvastatin was chosen as a control over other statins because of its connection to APN in adipocytes—PAI-1 mRNA expression and protein secretion is decreased in cultured adipocytes treated with 10 µM rosuvastatin³⁸ as are adipocytes treated with 10 µg/ml rhAPN³¹. Western

analyses of cell lysates (Figure 6) and conditioned media (Figure 7) showed no significant difference in PAI-1 protein expression between rhAPN-treated vs. control-treated cells.

Discussion

We have demonstrated that VSMC express AdipoR mRNA *in vitro* and *in vivo* as evidenced by bright bands visible in end-point PCR gels of lysates prepared from cultured HCAVSMC, RNA from sections of porcine coronary artery, and lysates of VSMC collected from murine aorta. There appear to be species and physiological location differences in mRNA expression between pigs and mice: pigs appear to have robust AdipoR1 and AdipoR2 mRNA expression in coronary artery segments while mice appear to have more robust AdipoR1 and T-cadherin expression in aortic smooth muscle cells. As different isoforms of APN bind preferentially with each receptor, this could suggest that different signaling pathways may be utilized between the species. Reduced expression of T-cadherin as compared to AdipoR1 and AdipoR2 in pigs suggests that APN signaling may be equal from globular, full-length, and higher-molecular-weight APN. Mice express AdipoR1 and T-cadherin but not AdipoR2 mRNA in aortic VSMC, therefore APN signaling in this species and cell type may be predominantly mediated through globular and high-molecular-weight APN, with little signaling from the low- or mid-molecular-weight APN isoforms.

We tested the hypothesis that APN will decrease PAI-1 expression by HCAVSMC in an effort to test the functionality of the AdipoRs found to be expressed by HCAVSMC. We were unable to demonstrate a statistically significant difference in PAI-1 mRNA expression in cultured HCAVSMC in this set of experiments, however, the statistical power of the experiments was very low, thus the chance of measuring a significant difference in this set of experiments was very low. Using the reported data, a sample size of 12 is needed to detect a statistically significant change. We used an N of 8, so experiments to increase the N to 12 are needed for the future.

One possible explanation for the differences seen in mRNA and secreted PAI-1 expression in HCAVSMC (non-significant increase in PAI-1 gene expression) and adipocytes (significant decrease in PAI-1 gene expression) could be related to the different receptors expressed between the cell types. AdipoR1 and AdipoR2, which are expressed on both adipocytes and HCAVSMC, tend to use common downstream mediators, typically by activation of AMPK or members of the MAPK family such as ERK and p38⁷. T-cadherin is expressed by HCAVSMC but not by adipocytes and utilizes a completely different set of cellular signaling pathways from AdipoR1 and AdipoR2, usually through Ras and Rac¹⁶. Perhaps a decrease in PAI-1 expression is mediated by AdipoR1 and AdipoR2 in both HCAVSMC and adipocytes, but a robust opposite effect through T-cadherin occurs in HCAVSMC, thus returning a net no-change in PAI-1 gene expression.

Another possible explanation for this is the rhAPN dose was not high enough to see any effect on PAI-1 expression by VSMC. The dose used (10 µg/mL) is commonly used in the literature^{18,20,27,31} and was chosen for this fact and also that it fell well within physiologic levels of 3-30 µg/mL. The purchased rhAPN was verified to be active in this cell type by its effects on smooth muscle α-actin RNA expression in HCAVSMC (data shown in Appendix 8), but perhaps the dose was too low to have an effect on PAI-1 expression. A dose-response experiment would be appropriate to determine effective dose(s), if any, of rhAPN on VSMC expression of PAI-1 mRNA.

We were unable to replicate the results of previously-published literature regarding changing PAI-1 expression using D-glucose^{34,35} and rosuvastatin³⁸. The difference in results could be explained by the use of different populations of smooth muscle cells—we used HCAVSMC purchased from Casdade Biologics, but in the previously-published work VSMC were harvested from umbilical vein and artery, mammary artery, as well as directly harvested from human coronary arteries. Perhaps the dosage of D-glucose and rosuvastatin used was incorrect for this experimental system. The doses used in these experiments corresponded to those in the literature³⁴⁻³⁷ but perhaps were too low to show desired results in this system. Experiments using several different doses of D-glucose and rosuvastatin would be appropriate to determine a dose (if any) that alters PAI-1 mRNA and/or protein expression in this experimental system.

Summary

- Adiponectin receptors (AdipoRs) are expressed in cultured HCAVSMC, in pig coronary artery sections, and in VSMC collected from mouse aorta.
- The distribution of each receptor is different in each species; HCAVSMC and pig coronary artery segments express AdipoR1/R2 and T-cadherin genes while mice appear to express only AdipoR1 and T-cadherin genes in aortic VSMC.
- HCAVSMC express AdipoR proteins.
- HCAVSMC treated with 10 µg/ml rhAPN show no change in PAI-1 gene expression, protein expression in cell lysates, or protein secretion to the conditioned media.

LITERATURE CITED

1. American Heart Association Statistics and Stroke Statistics Subcommittee. Heart Disease and Stroke Statistics—2008 Update: A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2008; 117:e25-e146.
2. Frigolet Vazquez-Vela ME, Torres N and AR Tovar. White Adipose Tissue as Endocrine Organ and Its Role in Obesity. *Arch of Med Res* 2008; 39:715-728.
3. Ronti T, Lupattelli G and E Mannarino. The endocrine function of adipose tissue: an update. *Clin Endocrinology* 2006; 64:355-365.
4. Galic S, Oakhill JS and GR Steinberg. Adipose tissue as an endocrine organ. *Mol and Cellular Endocrinology* 2009 Article in press.
5. Rutkowski JM, Davis KE and PE Scherer. Mechanisms of obesity and related pathologies: The macro- and microcirculation of adipose tissue. *FEBS J* 2009; 276:5738-5746.
6. Ekmekci H and OB Ekmekci. The Role of Adiponectin in Atherosclerosis and Thrombosis. *Clin Appl Thrombosis/Hemostasis* 2006; 12(2):163-168.
7. Kadowaki T and T Yamauchi. Adiponectin and Adiponectin Receptors. *Endocrine Rev* 2005; 26(3):439-451.
8. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuoyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitmen ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S Tomita M, Frogue P and T Kadowaki. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipotrophy and obesity. *Nat Med* 2001; 7(8):941-946.
9. Kato H, Kashiwagi H, Shiriga M, Tadokoro S, Kamae K, Ujie H, Honda S, Miyata S, Ijiri Y, Yamamoto J, Maeda N, Funahashi T, Kurata Y, Shimomura I, Tomiyama Y and Y Kanakura. Adiponectin Acts as an Endogenous Antithrombotic Factor. *Arterioscler Thromb Vasc Biol* 2006; 26:224-230.
10. Freubis J, Tsao T-S, Javorschi S, Ebbets-Reed D, Erickson MRS, Yen FT, Bihain BE and HF Lodish. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Nat Acad Sci USA* 2001; 98(4):2005-2010.
11. Combs TP, Berg AH, Obici S, Scherer PE and L Rossetti. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J Clin Invest* 2001; 108(12):1875-1881.

12. Chang L-C, Huang K-C, Wu Y-W, Kao H-I, Chen C-L, Lai L-P, Hwang J-J and W-S Yang. The Clinical Implications of Blood Adiponectin in Cardiometabolic Disorders. *J Formos Med Assoc* 2009; 108(5):353-366.
13. Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Froguel P, Nagai R, Kimura S, Kadowaki T and T Noda. Disruption of Adiponectin Causes Insulin resistance and Neointimal Formation. *JBC* 2002; 277(29):25863-25866.
14. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R and T Kadowaki. Cloning of adiponectin receptors that mediate antidiabetic effects. *Nat Lett* 2003; 423:762-769.
15. Goldstein BJ and R Scalia. Adiponectin: A Novel Adipokine Linking Adipocytes and Vascular Function. *J Clin Endoc & Met* 2004; 89:2563-2568.
16. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao T-S and HF Lodish. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp31/adiponectin. *Proc Nat Acad Sci* 2004; 101(28):10308-10313.
17. Takeuchi T, Adachi Y, Ohtsuki Y and M Furihata. Adiponectin receptors, with special focus on the role of the third receptor, T-cadherin, in vascular disease. *Med Mol Morphol* 2007; 40:115-120.
18. Hattori Y, Nakano Y, Hattori S, Tomizowa A, Inukai K, and K Kasai. High molecular weight adiponectin activates AMPK and suppresses cytokine-induced NF- κ B activation in vascular endothelial cells. *FEBS Lett* 2008; 582:1719-1724.
19. Hattori Y, Suzuki M, Hattori S and K Kasai. Globular adiponectin upregulates nitric oxide production in vascular endothelial cells. *Diabetologia* 2003; 46:1543-1549.
20. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T and Y Matsuzawa. Novel Modulator for Endothelial Adhesion Molecules: Adipocyte-Derived Plasma Protein Adiponectin. *Circulation* 1999; 100:2473-2476.
21. Ouedraogo R, Gong Y, Berzins B, Wu X, Mahadev K, Hough K, Chan L, Goldstein BJ and R Scalia. Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo. *J Clin Invest* 2007; 117(6):1718-1726.
22. Ouchi N, Kihara S, Arita Y, Nishida M, Masuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T and Y Matsuzawa. Adipocyte-Derived Plasma

- Protein, Adiponectin, Suppresses Lipid Accumulation and Class A Scavenger Receptor Expression in Human Monocyte-Derived Macrophages. *Circ* 2001; 103:1057-1063.
23. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Teresaka N, Inaba T, Funahashi T and Y Matsuzawa. Adiponectin Reduces Atherosclerosis in Apolipoprotein E-Deficient Mice. *Circulation* 2002; 106:2767-2770.
 24. Tian L, Luo N, Klein RL, Chung BH, Garvey WT and Y Fu. Adiponectin reduces lipid accumulation in macrophage foam cells. *Atherosclerosis* 2009; 202(1):152-161.
 25. Zhu W, Cheng KKY, Vanhoutte PM, Lam KSL and A Xu. Vascular effects of Adiponectin: molecular mechanisms and potential therapeutic intervention. *Clin Sci* 2008; 114:361-374.
 26. Wang Y, Lam KSL, Xu JY, Lu G, Xu LY, Cooper GJS and A Xu. Adiponectin Inhibits Cell Proliferation by Interacting with Several Growth Factors in an Oligomerization-dependent Manner. *JBC* 2005; 280(18):18341-18347.
 27. Arita Y, Kihara S, Ouchi N, Maeda Y, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T and Y Matsuzawa. Adipocyte-Derived Plasma Protein Adiponectin Acts as a Platelet-Derived Growth Factor-BB-Binding Protein and Regulates Growth-Factor-Induced Common Post-Receptor Signal in Vascular Smooth Muscle Cell. *Circulation* 2002; 105:2893-2898.
 28. Matsuda M, Shimomura I, Sata M, Arita Y, Nishida M, Maeda N, Kumada M, Okamoto Y, Nagaretani H, Nishizawa H, Kishida K, Komuro R, Ouchi N, Kihara S, Nagai R, Funahashi T and Y Matsuzawa. Role of Adiponectin in Preventing Vascular Stenosis: The Missing Link of Adipo-Vascular Axis. *JBC* 2002; 277(40):37487-37491.
 29. Eitzman DT, Westrick RJ, Nabel EG and D Ginsburg. Plasminogen activator inhibitor-1 and vitronectin promote vascular thrombosis in mice. *Blood* 2000; 95:577-580.
 30. Kawasaki T, Dewerchin M, Lijnen HR, Vermylen J and MF Hoylaerts. Vascular release of plasminogen activator inhibitor-1 impairs fibrinolysis during acute arterial thrombosis in mice. *Blood* 2000; 96:153-160.
 31. Hoo RLC, Chow WS, Yau MH, Xu A, Tso AWK, Tse HF, Fong CHY, Tam S, Chan L and KSL Lam. Adiponectin Mediates the Suppressive Effect of Rosiglitazone on Plasminogen Activator Inhibitor-1 Production. *Arterioscler Thromb Vasc Biol* 2007; 27:2777-2782.

32. Livak KJ and TD Schmittgen. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C_t}$ Method. *Methods* 2001; 25:402-408.
33. Schmittgen TD and KJ Livak. Analyzing real-time PCR data by the comparative Ct method. *Nature Protocols* 2008; 3(6):1101-1108.
34. Ahn JD, Morishita R, Kaneda Y, Lee KU, Park JY, Jeon YJ, Song HS and IK Lee. Transcription factor decoy for activator protein-1 (AP-1) inhibits high glucose- and angiotensin II-induced type 1 plasminogen activator inhibitor (PAI-1) gene expression in cultured human vascular smooth muscle cells. *Diabetologia* 2001; 44:713-720.
35. Pandolfi A, Iacoviello L, Capani F, Vitacolonna E, Donati MB and A Consoli. Glucose and insulin independently reduce the fibrinolytic potential of human vascular smooth muscle cells in culture. *Diabetologia* 1996; 39:1425-1431.
36. Wiesbauer F, Kaun C, Zorn G, Maurer G, Huber K and J Wojta. HMG CoA reductase inhibitors affect the fibrinolytic system of human vascular cells *in vitro*: a comparative study using different statins. *Br J Pharmacology* 2002; 135: 284-292.
37. Bourcier T and P Libby. HMG CoA Reductase Inhibitors Reduce Plasminogen Activator Inhibitor-1 Expression by Human Vascular Smooth Muscle and Endothelial Cells. *Arterioscler Thromb Vasc Biol* 2000; 20:556-562.
38. Laumen H, Skurk T and H Hauner. The HMG CoA reductase inhibitor rosuvastatin inhibits plasminogen activator inhibitor-1 expression and secretion in human adipocytes. *Atherosclerosis* 2008; 196:565-573.
39. Reilly CF and RC McFall. Platelet-derived Growth Factor and Transforming Growth Factor- β Regulate Plasminogen Activator Inhibitor-1 Synthesis in Vascular Smooth Muscle Cells. *JBC* 1991; 266(15):9419-9427.
40. Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, Miyazaki H and Iwao. Role of JNK, p38, and ERK in Platelet-Derived Growth Factor-Induced Vascular Proliferation, Migration, and Gene Expression. *Arterioscler Thromb Vasc Biol* 2003; 23:795-801.
41. Bobbert P, Antoniak S, Schultheiss H-P and U Rauch. Globular adiponectin but not full-length adiponectin induces increased procoagulability in human endothelial cells. *J Molec Cell Cardiol* 2008; 44:388-394.
42. Devaraj S, Torok N, Dasu MR, Samols D and I Jialal. Adiponectin Decreases C-reactive Protein Synthesis and Secretion From Endothelial Cells. *Arterioscler Thromb Vasc Biol* 2008; 28:1368-1374.
43. Ouchi N, Kihara S, Funahashi T, Nakamura T, Nishida M, Kamuda M, Okamoto Y, Ohashi K, Nagaretani H, Kishida K, Nishizawa H, Maeda N,

- Kobayashi H, Hiraoka H and Y Matsuzawa. Reciprocal Association of C-Reactive Protein With Adiponectin in Blood Stream and Adipose Tissue. *Circulation* 2003; 107(5):671-674.
44. Kadowaki t, Yamauchi T, Kubota N, Hara K, Ueki K and K Tobe. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* 2006; 116(7):1784-1792.

VITA

Meredith Jean Stevenson was born to parents Brian Eugene and Doris Irene Stevenson on January 29, 1982. She graduated from Park Hill High School in Kansas City, MO, in 2000. In high school she was an excellent student athlete, earning First Place in the national high school powerlifting competition in 2000. She sang in the All-State Missouri choir in 2000, and was captain of her high school soccer team. Meredith moved to Columbia, MO, in 2000 to attend the University of Missouri and graduated with her Bachelor's of Science in Animal Science in December, 2004, while working full-time. She entered the Medical Pharmacology and Physiology program in August of 2005. She is an avid hiker and traveler and enjoys the company of her two dogs, Sydney and Cheyenne.