ROLES OF SEX AND INSULIN ON MICROVASCULAR EXCHANGE FUNCTION

A Dissertation presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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AUGUST 2007

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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Virginia Huxley for her assistance, guidance, and patience during my years as a graduate student. She has been a significant factor in my development as both a researcher and teacher. I would also like to thank my committee members, Dr. Ronald Korthuis, Dr. Douglas Bowles, Dr. Christopher Hardin, and Dr. Freeman for their insights and suggestions that contributed to the improvements of my research. Thank you Dr. Freeman for your consideration and generosity through these years. Thanks Chris for all your help and advice, and for recruiting me to Missouri. I would like to thank the faculty in the Medical Pharmacology and Physiology department, in particular, Dr. Kerry McDonald and Dr. Michael Rovetto for their support. I also thank the members of the Huxley lab for all their support and contributions. I thank Steve Sieveking for his excellent technical assistance, but most of all for his continual encouragement and kindness. I thank Susan Bingaman for her assistance in the clearance experiments and for her moral support. I thank our post-docs Jianjie Wang and Olga Glinskii for their scientific input and insights, and for being great traveling companions. I also thank Dr. Steve Whitt, James Wenzel, and Scott Lucchese for their assistance with the venular leakage experiments.

I thank all of my friends and family who have supported me in this endeavor. Most of all, I thank God for bringing me through these years here in Missouri.

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ROLES OF SEX AND INSULIN ON

MICROVASCULAR EXCHANGE FUNCTION

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ABSTRACT

One of the major complications observed in the pre-diabetic stage of what becomes the disease, Type 2 diabetes, is microvascular dysfunction, involving alterations in endothelial function, also referred to as endothelium dysfunction (ED). A characteristic of ED is the alteration of the microvascular barrier, affecting the microvascular permeability to a wide variety of materials whose partitioning into the vascular and tissue compartments is required to sustain organ function. We measured the venular leakage and clearance of albumin in response to suffusion with high-dose insulin (10^{-7}M) in the autoperfused rat mesentery. We also compared the permeability responses of the mesenteric microvasculature of juvenile and adult male rats to determine whether maturation, *per se*, altered either basal properties or responses to insulin. Further, we assessed the influence of insulin on exchange function in sexually mature animals of to test whether the exchange responses differed between males and females.

Insulin suffusion lead to increased venular albumin leakage by 63% in adult male rats. Although we expected the venular permeability to reflect the overall clearance of proteins from the blood to the interstitial fluid, albumin and total protein clearance decreased with insulin treatment and hence, had an inverse relationship. In the juvenile male rats, venular albumin leakage was increased by 103% with insulin suffusion. The total protein clearance in the juvenile males was unchanged, while the albumin clearance increased with insulin suffusion, resulting in an uncoupling of the clearance responses between albumin and the other plasma proteins. Finally, as we expected, we did not observe any changes in the venular albumin leakage with insulin suffusion in the adult female rats. Insulin did decrease the total protein clearance, but not the albumin clearance, again reflecting an uncoupling of their clearance responses.

We demonstrated novel data that not only can insulin result in changes in macromolecule exchange in intact, autoperfused mesenteric microvasculature, but that these changes were dependant on the sex and the sexual maturity of the animal. We also determined that it is not appropriate to measure venular leakage response at the single vessel and expect it to reflect the overall protein clearance of the mesenteric network, or vice versa. The uncoupling of the relationship between albumin and total protein clearance response with insulin also provides clear evidence that albumin movement from the vessel and through the tissue is regulated via different mechanism/s compared to other plasma proteins.

Finally, in aggregate, this dissertation provides data demonstrating that it is not sufficient to study a physiological process in an animal model of a single sex and age and to then interpret the data as applicable to the circumstances of the species in general. The implication is that the treatment and progression of diseases need to be considered with the genomic and phenotypic backgrounds in mind.

CHAPTER 1: INTRODUCTION

Type 2 Diabetes Mellitus

Diabetes is a set of metabolic diseases characterized by high levels of plasma glucose secondary to errors in the production of insulin or the action of insulin, or a combination of both processes. Type 2 diabetes, previously referred to as adult onset diabetes, or non insulin-dependent diabetes mellitus (NIDDM), is the most common form of diabetes that affects millions of people. In the United States alone, according to the American Diabetes Association and the Centers for Disease Control (CDC), 20.8 million children and adults, roughly 7% of the population has the disease [1]. Of those, Type 2 accounts for 90-95% of all cases of diagnosed diabetes. Further, diabetes is deadly, being the sixth leading cause of death in the United States (2002). It is particularly noteworthy that being diabetic is thought to double the risk for death in people of the same age without the disease [1].

With respect to cardiovascular disease (CVD), diabetes is a major concern as mortality from heart disease is 2-4 times higher in adults with diabetes and the risk for stroke is similarly increased. Almost 75% of the adults with diabetes have blood pressures of ≥ 130/80 mmHg or are on antihypertensive medications [1]. Further, diabetes is the leading cause of renal failure and over half of the non-traumatic lower limb amputations occur in diabetics. Therefore, it is important to not only study the disease but to also study approaches for the treatment of the disease.

A second condition involving insulin dysfunction is insulin resistance or pre-diabetes (also known as metabolic syndrome), characterized by high circulating levels of insulin, or hyperinsulinemia, secondary to the target tissues (i.e. muscle, fat, and liver) not transporting glucose in response to insulin. Individuals with insulin resistance are generally asymptomatic and at a higher risk of developing type 2 diabetes. Studies have shown that the majority of pre-diabetes who do not make changes in life style, go on to develop type 2 diabetes within 10 years [2]. In addition, individuals with pre-diabetes are at higher risk of developing heart disease. One of the complications observed in the pre-diabetic stage is microangiopathy. Microangiopathy along with other microvascular dysfunctions involve alterations in the endothelium. In a recent review, it has been suggested that microcirculatory abnormalities may not only be secondary, but also causal to the development and/or exacerbation of insulin resistance and the metabolic syndrome [3].

As stated above, insulin, a hormone produced by pancreatic beta cells, and its actions on target tissues in fat, skeletal muscle, and liver are central to the disease of diabetes and the condition of insulin-resistance. Most of the current basic research is focused on insulin signaling, assuming that insulin gets to the receptors on the primary target organs without impedance. But insulin, secreted into the circulation from the islets of pancreas, must first cross the vascular wall in the microcirculation to reach its receptors on the surface of its target tissues. No one has investigated whether the barrier function of the microvascular wall changes in diabetic conditions such as hyperinsulinemia.

One manifestation of diabetes-related endothelial dysfunction is an alteration in the exchange barrier characterized by changes in the inherent property of porous barriers, resulting in changes in permeability different substances. In turn, the permeability functions of microvascular endothelium regulate the milieu of the extravascular or interstitial compartment surrounding the target tissues. In addition to influencing which solutes move to and from the cells, exchange barrier integrity is central to inflammatory responses as well as volume balance.

Reports on the effect of insulin on microvascular permeability are conflicting (as addressed below), likely reflecting the difficulty of quantifying and or controlling conditions in each of the compartments, vascular, interstitial, and lymphatic, separated by endothelial barriers. Methods used in our laboratory focus on microvascular exchange in intact *in situ* microvessels and will permit us to assess the responses to insulin directly.

The Microcirculation

The microcirculation, composed of arterioles, capillaries, and venules, regulates diverse functions. Of the cells comprising the microcirculation, the endothelium has been found to be central in the regulation of multiple vascular functions. Of these, control of blood flow is the most widely studied. Once the blood reaches the microvasculature, the equally important, and often overlooked, role of the endothelium is delivery of substrates and removal of wastes from metabolizing tissue. While textbooks often simplified the microvascular wall to a

static sieve, recent research shows that the microcirculation is dynamic and subject to much regulation and change depending on the physiological and pathophysiological conditions [4, 5].

Our understanding of whether insulin itself regulates the microvascular exchange function, the focus of this dissertation, will provide the information necessary for understanding insulin's role in health and its involvement in disease.

Several possible transport pathways exist for substances to cross the endothelium in the microvasculature, such as passive diffusion, pores, and vesicular (receptor-mediated) transport [6]. Permeability describes the ease at which a substance crosses the vascular wall. Microvascular permeability is subject to short-term regulation in response to diverse physiologic and pharmacologic conditions [7]. There are multiple stimuli that can alter microvascular permeability from basal conditions, including endogenous and exogenous vasodilators [8, 9], shear stress [10], hypoxia and hyperoxia [11], membrane potential changes [12], inflammatory humoral mediators [13, 14] and cellular mediators [5, 15].

Insulin Receptors on Microvascular Endothelium

Insulin is a peptide hormone that is released from the pancreas into the blood to be distributed to the peripheral tissues, such as adipose and skeletal muscle, where it binds to tissue surface receptors to regulate the uptake of

glucose from the blood following expression of glucose transporters. Since insulin must cross the microvascular wall to exert its action on its target tissues, 2 questions arise: what is the permeability of the wall to insulin and if it is altered in different physiological and pathological conditions. Insulin receptors (IRs) have been identified in endothelial cells (ECs) of microvessels [16]. Studies have shown that insulin binds IRs in the ECs and treatment with increasing insulin doses have shown to competitively inhibit labeled-insulin binding to IRs in ECs at high doses ($>10^{-7}$M) [17, 18]. Some biological effects have been associated with insulin in cultured microvessel ECs, including stimulation of glucose transport, amino acid transport, and glucose oxidation [19]. Most recently, IRs have been identified in the skeletal muscle microvascular endothelium [20]. Whether the insulin binding capacity of the ECs is involved in the translocation of the hormone and/or alteration of barrier function has not been studied.

Conflicting results for mode of insulin transport in microvasculature

The evidence as to how insulin crosses the microvascular wall is conflicting. Several studies indicate that insulin transport occurs via passive diffusion. Steil et al [21] have shown in vitro that IRs were not saturable with treatment with increasing concentrations insulin (10^{-12} to 10^{-10}M), indicating insulin is not subject to receptor-mediated transport. Brunner et al [22] showed in the capillaries of isolated perfused rat hearts that insulin flux across the endothelium is a bi-directional convective transport. Euglycemic clamps performed on dog hindlimb muscle showed that insulin transport from plasma

into muscle interstitial fluid (ISF) increased with pharmacological insulin concentration, indicating that insulin is freely passing through the capillaries [23]. In a similar experiment, the plasma/ISF ratio for insulin analog remained unchanged in the absence and presence of elevated insulin levels [24].

Contrarily, other studies have shown that insulin is transported across the microvascular wall via receptor-mediated pathways. In cultured rat fat pad capillaries, insulin transport was inhibited 88% in the presence of anti-IR antibody and unlabeled insulin (10^{-7}M) [25]. A similar inhibition of insulin transport at high (10^{-6} M) insulin was observed in cultured bovine aortic ECs [26] and in intact, beating hearts [17]. A study in dog hindlimbs showed that, inulin, a small carbohydrate, with similar size to insulin (inulin molecular weight (MW) = 5kDa ; insulin MW = 4.8kDa), reached an equilibrium between plasma and lymph, whereas steady-state plasma insulin was higher than lymph [27]. In the eel rete mirabile, insulin and albumin were found to associate with different sets of plasmalemmal vesicles in the capillary network [28]. In the same study, capillary permeability to inulin was significantly higher than insulin, and permeability to insulin was higher than that to albumin even though albumin is a larger molecule (MW = 63kDa). From these data, we predict the permeability to insulin to be different from inulin. Yet the aforementioned studies supporting passive diffusion suggest that the venular permeability to inulin and insulin are similar and less than albumin. The conflicts among the published studies may reflect differences in the methods for assessing insulin transport across the microvascular wall.

Insulin and Microvascular Permeability

Thus far, few studies have investigated, directly or indirectly, whether insulin can regulate microvascular permeability and if it can, by what signaling mechanism, and by which endothelial structures. Close inspection of this work reveals that most of the responses could result from factors other than insulin, *per se*.

It has been well documented that diabetes is associated with microvascular dysfunction, one of which is an alteration in permeability. As described above, the studies investigating the effect of insulin on microvascular permeability are conflicting. The majority of the studies investigating microvascular permeability to albumin were performed in type 1 diabetic rat models, where there is no insulin secretion by the pancreas. These rat models have a significantly higher microvascular permeability to albumin than normal rats [29-32]. In non-diabetic human subjects another marker of reduced barrier function, transcapillary escape rate of albumin, was increased with hyperinsulinemia [33]. A similar study in dogs showed that hyperinsulinemia increased peripheral and splanchnic interstitial distribution of inulin (a small fructose polymer of molecular weight similar to insulin) [34].

On the contrary, another study reported that brachial skeletal muscle capillaries in type 2 diabetic patients have decreased permeability-surface area for glucose during hyperinsulinemia [35]. With respect to water movement, the resting capillary filtration coefficient for type 1 diabetic patients was decreased compared to control subjects [36]. Fewer studies have focused on insulin and its

movement directly. In a rat model of the metabolic syndrome (exhibiting obesity, insulin resistance, hyperlipidemia, and hyperinsulinemia) transfer of insulin across the endothelium was delayed [37]. Obese patients, during induced hyperinsulinemia, also had a delayed delivery of insulin from the blood to the interstitial fluid relative to the lean subjects [38].

As these models exhibit different levels of insulin, and the pathologies of type 1 and type 2 diabetes are not identical, it is difficult to predict whether insulin, *per se*, alters barrier function.

Experiments described in this dissertation test directly whether insulin alters the flux, distribution or clearance of albumin in normal healthy animals. Our expectation was that insulin, *per se*, would influence albumin flux from venules or tissue clearance of albumin or plasma proteins.

Insulin and Endothelial Dysfunction

As we mentioned before, one of the major complications observed in the pre-diabetic stage is microvascular dysfunction, involving alterations in the endothelium, also referred to as endothelium dysfunction (ED). Since these complications occur at stages before hyperglycemia is fully developed [2], this may indicate a causal role of high insulin on the complications leading to full-blown diabetes, and not necessarily due to high plasma glucose levels. There are several studies implicating insulin is involved in either the development or progression of ED [39, 40].

Sexual Maturity Differences in Insulin Response

The difference in physiological response to insulin with sexual maturity has not been studied extensively. Of significance, it was shown that during puberty, adolescents have decreased insulin sensitivity [41-43]. Among adolescents, there were conflicting data as to the degree of changes in insulin sensitivity between girls and boys [44, 45]. Further studies are needed to discern the differences. These findings require further study, especially as the incidence of type 2 diabetes and obesity are increasing rapidly among children and adolescents [46-51].

We compared the permeability responses of the mesenteric microvasculature of juvenile and adult male animals to determine whether maturation, per se, altered either basal properties or responses to insulin.

Sexual Dimorphism in Insulin Response

Until recently, the differences in physiological and pathophysiological responses with respect to sex have attracted little interest, as it has been tacitly assumed that there were no significant differences in the physiology (except reproductive function) of males and females. The data however, suggest otherwise. Recent studies have shown that diabetic women have a higher risk for developing coronary heart disease and higher risk of death than diabetic men [52-54]. Endothelial-dependent vasodilation in premenopausal women is more robust than men, yet premenopausal women with type 2 diabetes lose that robust response and do not differ from diabetic men [55]. In fact, in recent

statistics by the American Diabetes Association has reported that in women with diabetes, deaths from heart disease have increased 23% over the past 30 years compared to a 27% decrease in women without diabetes. Moreover, deaths from heart disease in men with diabetes have decreased by 13% compared to a 36% decrease in men without diabetes [56]. Diabetic women also have a higher risk of developing heart disease than diabetic men [57]. Determining whether differences in microvascular response to insulin with sex exist is imperative in eliciting the etiology of the disease and determining the treatment of patients. Also, although there are more diabetes-related studies now being performed in females, they often lack a direct comparison with males, and comparison between studies by different laboratories are difficult due to the differences in the design and models of the studies [58].

In this dissertation a further goal is to assess the influence of insulin on exchange function in healthy, non-diabetic sexually mature animals of both sexes to test the hypothesis that basal exchange and the exchange response to insulin differ with sex.

Significance of Study

The outcomes of the work on insulin's action with respect to regulating microvascular exchange and the different responses to sex and age, will facilitate the design and implementation of better treatment for insulin-associated diseases and dysfunctions. The fundamental questions of how insulin affects the movement of proteins between blood and tissue and whether insulin can alter

barrier function, need to be answered to understand the pathophysiology of insulin resistance and pharmacokinetics of insulin action. Answers to these questions will also provide the requisite framework for subsequent studies focusing on signaling pathways, junctional proteins, cytoskeletal structures and other factors that regulate insulin-induced changes in permeability, thus opening a door for new areas of research.

Given that type 2 diabetes is among the most prevalent and fastest growing disorders in the U.S. [56], and is a significant risk factor for developing cardiovascular disease (the number one cause of death in the U.S.), progress in this area of research will leave positive repercussions in the road to discovering new therapeutic interventions that will impact directly the quality of life of the millions of people with diabetic disorders.

CHAPTER 2: METHODS

Animals:

Description of animals:

Sprague-Dawley rats were chosen for a variety of reasons. First, these animals are readily available and second, many of the baseline characteristics to be studied in this research are familiar. Rats of both sexes were acquired from Hilltop Labs (Scottsdale, PA). The animals were housed, 2 rats per cage, in temperature and light controlled rooms in an ALAAC approved facility where temperature and light/dark cycles are controlled with *ab libitum* access to food (Purina 5008 Formulab chow) and water. The animals were acclimatized to the housing facilities after arrival for a minimum of one week before being used for experiments.

Classification of animals:

Laboratory rats on a 12:12 light: dark cycle become reproductively active at 50 ± 10 days [59, 60]. Therefore the studies included juvenile (<60 days) and mature (>9 wks) rats. Hormone status of multiple rats studied was determined thus verify reproductive/hormone status.

Sexually mature, adult male (AM) rats were used at >9 weeks and ranged in weight from 334–511 g.

Sexually mature adult female (AF) rats were used at >7 weeks and ranged in weight from 190–368 g.

Juvenile (sexually immature) male (JM) rats were used at <8 weeks old and ranged 227–352 g.

Surgical Preparation:

Prior to experimentation the animals were anesthetized by intraperitoneal (IP) injection with 125 mg/kg thiobutabarbital (Inactin®; Sigma, St. Louis, MO). Additional anesthesia was administered during the course of the experiment as needed either IP (25 mg/kg) or intravenously (IV) (12.5 mg/kg). The fur was shaved from the surgical site: the anterior abdomen and neck with a clipper (Oster, WI). Blunt dissection with use of surgical tweezers (INOX, Germany) was used at the two sites following an anterior incision was made in the midline and exposure of the trachea. A tracheotomy was performed using a polyethylene tube (~2.5cm long PE240 tubing, Clay Adams, NJ) to facilitate the breathing. The internal carotid artery and external jugular vein were catheterized with polyethylene tubing (PE-50, Clay Adams, NJ) for monitoring mean arterial pressure (MAP), and for injecting dye-labeled solution, respectively. The venous catheter was also used to administer supplemental anesthetic to the animal as needed throughout the experiment. The arterial catheter was flushed with 0.1 ml of heparinized saline (50U heparin/ml) prior to use. The skin on the neck incision was sutured (4-0 silk suture, USSC Sutures, CT). A midline incision was performed and a loop of small bowel was identified and quickly covered with wet gauze.

Once the surgery was completed, the animal was moved to the microscope table and connected to a small animal ventilator (Cwe, Inc., Ardmore, PA) to ensure constant, regulated respiration (Ventilation rate = 70 breaths/min and tidal volume = 9 ml/kg) and isotonic saline, 1 ml/hour was administered via the arterial catheter with a syringe pump (Razel, Stamford, CT) to replace volume losses during the experiment and prevent blockage or collapse of the artery. The animal was placed on a custom made plexiglass tray with a glass heating coil to maintain the body temperature at 37°C in a right lateral decubitus position. The gauze was removed from the midline incision in the abdomen, and mesentery was exteriorized using cotton swabs and intestine carefully draped over a polished quartz pillar.

The mesentery was superfused continuously at a rate of 5 ml/min (Polystaltic pump, Buchler instruments, NJ) with bicarbonate buffer solution (BBS) at 37°C. MAP was monitored continuously during the experiment with a pressure transducer (Abbott Critical Care Systems, North Chicago, IL) using a clinical BP monitor (Hewlett Packard, Germany). The mesentery preparation was viewed under the Diaphot 200 inverted microscope (Nikon, Japan) and was allowed to equilibrate for ~30 minutes prior to data gathering.

The superfusate was collected continuously through a suction tube every 2.5 minutes as it flowed off the mesentery. The concentrations of BSA-A594 and total protein in the collected superfusate were measured from the fluorescence intensity of each aliquot (BSA594: EX/EM = 575/608, total protein: EX/EM 280/360; SpectraMax M2, Molecular Devices).

Solutions:

Bicarbonate Buffer Solution (BBS) for suffusion of mesentery:

132 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$ and 2.0 mM $CaCl_2$ at pH 7.4, 37°C. The osmolarity of the solution was calculated to be ~292 mOsm.

2 Liters of BBS were prepared from a 20X stock solution on experiment day. The BBS was placed in a heated water bath and was bubbled with a gas mixture containing 5% CO_2, 5% O_2, and 90% N_2 [61]. The pH was measured during equilibration with the gas mixture and maintained at 7.4 ± 0.05. HCl (1 M stock solution) was added with a dropper to the solution as needed to maintain the pH.

Fluorescently labeled albumin

Proteins were labeled by methods described in numerous publications, most recently in [62-65]. Bovine serum albumin (BSA; Sigma, St. Louis, MO) was labeled with the fluorescent dye Alexa Fluor 594™ (Invitrogen: Molecular Probes, Carlsbad, CA). Alexa Fluor 594™ and BSA in 0.05 M borate, pH 8.3 (3.3:1; molar/molar starting concentration 0.125 M albumin) were mixed and reacted for 30 minutes at room temperature. Free dye was removed by centrifugation (4,000 g; Vivaspin 20, 30,000 NMWL, Sartorius) followed by removal of free dye with D-Salt Polyacrylamide Desalting Columns (Pierce, Rockford, IL). The column eluate was reconcentrated at 4,000 g, assayed for protein concentration (Bio-Rad, Hercules, CA), and diluted to ~20 mg/ml with

Krebs. The final perfusate (BSA-594) used for leak index measurement at ~20 mg/ml final concentration consisted of conjugated BSA and unlabeled dialyzed BSA (mixed 1:9; weight/weight) in Krebs solution. Physicochemical characteristics (charge, mobility, molecular size, tendency to polymerize) are unchanged by 1:1 labeling of albumin with Alexa Fluor 594 fluorescent dye.

For each solution protein content (by absorption spectroscopy), osmotic pressure (freezing point depression), oncotic pressure, and pH are determined. Alexa 594 was chosen as our fluorescent reporters. This decision was made to achieve a balance of minimal influence of the dyes on the barrier properties, maximal similarity between the labeled and native materials [66], maximal brightness permitting use of low probe concentrations, minimal fade of fluorescent signal at physiological pH and temperature, and maximal separation of the emission and excitation wavelengths. To evaluate these assumptions, physicochemical characterizations (i.e. native and SDS PAGE gel electrophoresis, isoelectric focusing techniques; low pressure column chromatography, fluorescence spectroscopy, and osmotic and oncotic pressure measurements by freezing point depression and membrane techniques) were conducted [66].

Statistics:

All statistical analyses were performed using Excel® or Prism® software. Statistical analyses for the venular leakage and clearance measurements were performed using unpaired two-tailed *t*-tests and one-way ANOVA with Newman-

Keuls multiple comparison for the post test for normally distributed variables. Results were expressed as mean $\pm$ standard error of mean (SEM) and a probability $p<0.05$ was considered significant. Nonparametric analyses were performed where appropriate with either Mann-Whitney test or Kruskal-Wallis test. Kinetic analyses were performed with two-way ANOVA and rates and half-lives of the curves determined by non-linear curve fitting on Prizm software. Power analysis was performed to determine the requisite sample sizes for an α = 0.05 and a power (1-β) = 0.8. To detect a 40% change from basal leak index with a treatment, 3 animals were required in each group.

Experimental Methods:

Venular Albumin Leak Measurements:

The following protocol has been described extensively by Rumbaut et al [67, 68]. The exposed rat mesentery was suffused continuously with bicarbonate-buffered solution (BBS) bubbled with N_2/CO_2 gas mixture. The mesentery was placed over a Nikon Diaphot 200 inverted microscope (Nikon, Japan) and viewed with 25X objective lens (Leitz, Germany) and images captured on videotape. The exposed mesentery excluding the area of evaluation was covered with gauze soaked in BBS to prevent drying. A Plunix CA-7CN camera (Plunix America, Sunnyvale, CA) was used to capture bright field image and fluorescence observed using a PTI IC-100 intensified charge-coupled device (CCD) camera (Photon Technology International, Monmouth Junction, NJ) using a filter cube for the red spectra (EX/EM 595/615nm). Postcapillary venules were

selected and videotaped throughout the experiment. The experiment proceeded if the animal's MAP was >75 mmHg, the body temperature within 35.5-38.5°C, the selected postcapillary venule had steady, and robust flow (detailed description of vessel selection below). BSA-594 (8 mg/kg) was injected intravenously (IV) and fluorescence intensity was recorded on videotape every 10 minutes for 30 minutes of BBS suffusion and 75 minutes suffusion of BBS plus a particular dose of insulin (10^{-7}M)

Calculation of Venular Leakage (Leak Index):

The fluorescence intensity was measured from the video screen using NIH Image video analysis software. An index of albumin leakage was calculated as fluorescence intensity in a 5 x 20 μm window over a postcapillary venule (I_v) relative to fluorescence intensity of the adjoining tissue (I_t): (I_v - background)/(I_t - background) (**Figure 1**). (Background fluorescence was measured over the same window area at the darkest visible area of the screen in the first fluorescence reading).

Vessel Classification and Selection:

Postcapillary venules from the rat mesentery were used for the venular leakage experiments. Postcapillary venules were identified visually by flow direction as lying downstream of capillary venules and having convergent flow at both inflow and outflow. Venules with strong flow and diameter ranging 20 - 40 μm with less than 3 adhering leukocytes over a 100 μm length of the vessel were

selected. Venules near the intestinal wall, fat, or lymphatic vessels were avoided. One venule was chosen per animal for each experiment.

Matrix Metalloproteinase Activity Analysis:

The superfusate collected from time 80-110 minutes from adult males and females of the experiment described above (venular leakage and clearance) were pooled and concentrated over VivaSpin20 centrifugal filters (3,000 MWCO filter) at 3220 x g for about 2 hours to reduce the volume to <0.5 ml. The samples were assayed for protein concentration (Bio-Rad, Hercules, CA), aliquoted (15μl) and stored at -80°C. Novex® 10% Zymogram gelatin gel (Invitrogen, Carlsbad, CA) with the sensitivity of 10^{-6} units of collagenase. After dilution with zymogram Novex® Tris-Glycine SDS Sample Buffer (2X), samples were loaded at 15 μg/well. The samples were run on the gel for electrophoresis. After development, gels were stained with Pierce™ Imperial Protein Stain. After drying, the gels were scanned with Un-Scan-It™ (Silk Scientific, Orem, UT) was used to analyze the scanned images.

Western Gel of Degraded Proteins:

The superfusate from mesentery of adult and juvenile male rats were collected during leakage/clearance experiments described above over 80 -110 minutes were pooled and concentrated (3,000 MWCO filter). Samples of 10.7 μg of protein in 8 μl per well were run on a gel composed of NuPage Bis-Tris 4-12%, with MES Buffer (Invitrogen, Carlsbad, CA) for electrophoresis. The gel was

stained with SilverQuest™ silver stain kit (Invitrogen, Carlsbad, CA). The gel was slightly overdeveloped to visualize less abundant small proteins.

Radioimmunoassay for serum levels of testosterone, estradiol, progesterone, and insulin:

Immediately prior to euthanasia blood was collected via the arterial cathether line with a syringe, placed in heparinized tubes, and centrifuged at 4,000 rpm for 15 min. The supernatant was decanted and frozen at -80°C. Serum testosterone, 17 β–estradiol, and progesterone were determined by radioimmunoassay with commercially available kits (Diagnostic Products, Los Angeles, CA). Each assay, usually run in duplicate required 50-200 µL depending on the assay. Assay sensitivities were 0.4 ng/ml and 8 pg/ml for testosterone and estradiol, respectively.

Glucose Measurements:

Blood from the anesthetized rat during the experiment was taken from the tail by cutting off the tip (~2mm). Blood glucose was measured using a Precision® glucose monitoring system (Abbot Laboratories, Abbot Park, IL).

Experimental Protocol:

Subsequent to surgery, as described above, the rat mesentery was suffused with bicarbonate buffer solution (BBS) for 30 minutes. During this time a venule to use for the experiment was identified. Usually the venule was

located in a mesenteric window 2-3 panes from the cecum in the adult animals, and typically >3 panes for the juvenile animals. At the end of the equilibration period, 8mg/kg (on average, 0.1-0.2 ml depending on body weight) of Alexa-594 BSA (20 mg/ml, in saline) was infused via arterial catheter that had been placed in the carotid artery during initial surgery. The initial measurement for leak index was taken within 1-2 minutes of the bolus infusion. For the determination of the "Control" measures of leak index were then taken every 10 minutes for 30 minutes. After 5 minutes (35 minutes following labeled protein injection) half of the animals continued to receive BBS (Control animals) while half had the suffusate switched to BBS containing high dose insulin (10^{-7}M) (Test, high dose Insulin). In either case, leak index measures were taken every 10 minutes, ending at 75 minutes (after switching to insulin suffusate) (**Figure 1**).

Throughout the protocol for the measures of leak index the suffusate coming off of the mesentery was collected continuously (4-5 ml/2.5 minutes) in a fraction collector and evaluated for clearance analysis.

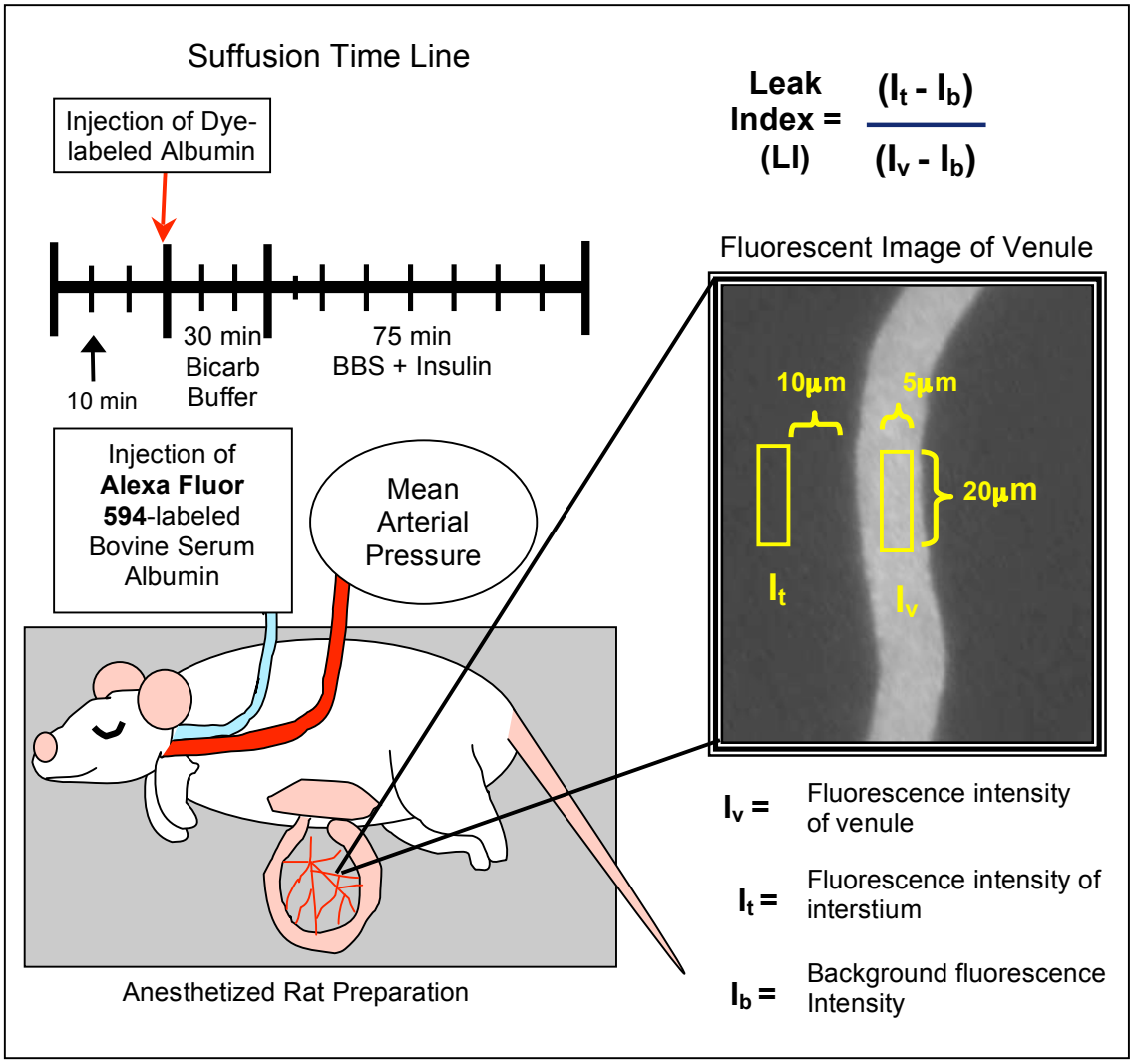


Figure 1: Schematic diagram of experimental set-up and calculation of leak index.

CHAPTER 3: INSULIN AND ADULT MALE MICROVASCULAR EXCHANGE FUNCTION

Rationale and Hypothesis:

Whether insulin can influence the movement between and net distribution of proteins in the blood and tissue compartments or whether insulin alters barrier function have yet to be discerned. This lack of information reflects conflicting data from previous studies (See **Chapter 1**). Study of the direct and indirect actions of insulin on the microvascular barrier need to be performed to understand the pathophysiology of insulin resistance and related diseases such as type 2 diabetes and provide the rational basis for subsequent treatment of the disease.

Among the hallmark complications observed in severe (end-stage) type 2 diabetic patients are microvascular dysfunctions, often leading to retinopathy, neuropathy, and nephropathy. One of the characteristics defining microvascular dysfunction is the alteration of the microvascular barrier, affecting the permeability to a wide variety of materials whose partitioning into the vascular and tissue compartments is required to sustain organ function. Previous studies investigating the effect of insulin on microvascular permeability have been conflicting (See **Chapter 1**). Thus this dissertation provides an evaluation of the response of the microvascular exchange function to insulin using both *in situ* and *ex vivo* approaches.

Albumin is a favorable marker for us to use to evaluate permeability, since it makes up ~60% of the plasma proteins and binds and transports substances across those compartments. Investigating microvessel permeability to albumin and its clearance from the mesenteric tissue can provide information regarding changes in permeability occurring in the system.

High physiological concentrations of insulin (4.4×10^{-10} M) have been shown to have negative effects on endothelial function with respect to flow regulation [39, 40]. With the understanding that the microvascular barrier is compromised under hyperinsulinemic conditions [39, 40], we hypothesized that treatment of the mesenteric microvasculature with a pharmacologic (but clinical relevant) dose of insulin (10^{-7}M) will result in an increase in permeability to albumin and plasma proteins. This concentration is also within the range of insulin concentration considered “high-dose” in other studies which elicited physiological responses in rats [69, 70].

Measuring Venular leakage of dye-labeled albumin:

Given that exchange across venules are considered to be the primary site of macromolecule movement, the favorable model for studying the effects of overall microvasculature exchange function was venular leak in the mesentery. Given that this tissue is thin and easily accessible we can measure venular leakage of albumin in venules of known anatomical location in living, autoperfused tissue. Further, the knowledge of venular leak would provide a measure of the behavior of the tissue as a whole.

We hypothesized that high dose insulin would increase the permeability in the venules to albumin, since as stated above, high physiologic doses insulin has been shown to have harmful effects on the microvascular endothelium [39, 40].

Measuring clearance of plasma protein and dye-labeled albumin:

From the same preparation in which venular leakage was measured we can also quantify the clearance of proteins and albumin, providing separate information about the global macromolecule exchange characteristics in intact tissue. In our case the intact tissue was the mesentery that is thought to be the primary site for exchange during peritoneal dialysis (PD).

We hypothesized that the albumin clearance would follow venular leak index and would, in turn, match (as a constant fraction) plasma protein clearance.

Investigating tissue matrix density:

Protein, after its efflux from the venules and into the extracellular (interstitial) fluid, can take several paths. It can pass through to the suffusate, bind and become sequestered in the interstitial matrix, be degraded by the proteases in the interstitium , or be taken up by the lymphatics. The clearance data provided a measure of what material passes. We investigated whether its passage through the tissue matrix was being increased by measuring the activity of one of the major enzymes that breaks down collagen, one of the major constituents of the matrix. Thus the more activity enzymatic activity, the more

tissue matrix has been broken down, increasing protein mobility in a less constrained space. In addition, if we observe increases in enzymatic activity, the more likely that there were alterations in the forces for fluid and macromolecule flux through changes in interstitial osmotic pressure and maintenance of a gradient for protein flux.

From the suffusate collected from the same preparation described above, we measured the activities of the matrix metalloproteinases (MMPs). We decided to measure MMP-2 and -9 activity levels since their plasma levels have been shown to be elevated in type 2 diabetics [71]. MMP-2 is also one of three (including MMP-1 and -3) predominant MMPs that are secreted by endothelial cells [72]. We also measured MMP-9, the major MMP species secreted by macrophages [73].

Therefore, our hypothesis was that venular permeability and clearance would increase in the presence of insulin; along with that we also expected MMP-2 and -9 levels and activity to increase.

Investigating protein degradation:

As mentioned above, another path that the protein can take is to be degraded by the proteases present in the tissue and interstitial fluid. Thus if the protein is degraded and taken up via endocytosis by cells in the tissue or lost to lymph, we will no longer detect it as “protein” in the interstitial fluid.

Since we expect insulin to increase protein clearance, we hypothesize that just as MMP activity might rise in the pro-inflammatory condition of high insulin,

so too other proteases may be active resulting in increased levels of smaller peptides and lower amounts of intact plasma proteins. To test this hypothesis the suffusate solutions were probed by gel electrophoresis.

Results:

Influence of Insulin on AM Venular Albumin Leakage:

As we predicted, suffusion of venules with high dose insulin (10^{-7}M) resulted in a 62% increase in venular albumin leakage (N=16; 0.55 ± 0.03) compared to suffusion with BBS alone (N=7; 0.34 ± 0.06; $p<0.01$) (**Figure 2**). The decline in albumin leak index observed over 75 minutes in the control animals was abolished in the insulin-suffused animals (**Figure 3**).

Influence of Insulin on AM Total Protein Clearance:

It was expected that the venular leak index data would precede and mirror the clearance of albumin and other plasma proteins from the mesenteric microvasculature. We were in the position to determine whether this was true by measuring albumin and protein content in the suffusate over the time of the experiment (clearance) to assess overall microvascular exchange function. We also determined the extent to which changes in clearance of proteins relate to the venular leakage. Contrary to what we observed with respect to venular leakage, insulin-suffusion (N=6; 2598 ± 271 ng/ml) significantly lowered the total protein clearance by 39% from BBS-suffusion (N=6; 4273 ± 345 ng/ml; $p<0.01$) (**Figure 4**). The rate of decline of protein was greater in the insulin-suffused rats (k=0.06

± 0.02 ng/ml*min^{-1}, $t_{1/2}$=11.9 min^{-1}) relative to the control group (k=0.01 ± 0.03 ng/ml*min^{-1}, $t_{1/2}$=67.6 min^{-1}; p<0.01). Insulin significantly (p<0.01) and in a time-dependent manner (p<0.01) decreased total albumin clearance over the 75-minute course of the experiment (**Figure 5**). Thus insulin induced opposite responses with respect to venular leakage and protein clearance.

Influence of Insulin on AM Albumin Clearance:

Albumin clearance in animals suffused with Insulin was 41% lower (N=6; 93 ± 6 ng/ml) than control animals (N=6; 157 ± 19 ng/ml; p<0.01) (**Figure 6**). The insulin-treated group had a higher rate constant (k) and lower half-life (k=0.24 ± 0.19 ng/ml*min^{-1}, $t_{1/2}$=2.9 min^{-1}) compared to the control group (k=0.004 ± 0.003 ng/ml*min^{-1}, $t_{1/2}$=158.9 min^{-1}; p=0.01). Albumin clearance, in a similar manner to what we observed in the total protein clearance, was reduced significantly (p<0.01) in a time-dependent (p=0.05) manner by 10^{-7} M Insulin (**Figure 7**).

Thus the protein and albumin clearance responses to insulin matched, but had opposite responses from the venular albumin leak index.

Influence of Insulin on Tissue Matrix Density via Protease Activity:

We hypothesized that an increased clearance of albumin from the mesenteric tissue would be due to a reduction in matrix density secondary to activity of one of the proteases which breakdown the tissue matrix - matrix metalloproteinases (MMPs). Surprisingly we observed a reduction in protein and

albumin clearance with insulin treatment and we further observed an increase in MMP-2 activity in insulin-treated animals (N=6, 66.7 ± 2.0 **%** Pixel Total; $p<0.05$) compared to controls (N=3, 60.1 ± 1.2 % Pixel Total) (**Figure 8A**). Also contrary to our expectation, MMP-9 activity did not differ with insulin-treatment from controls (N=6, 6.3 ± 3.0 % Pixel Total vs. N=3, 12.6 ± 1.7 % Pixel Total, respectively) (**Figure 8B**).

Influence of Insulin on Protein Degradation:

Since protein clearance was reduced by insulin, we expected to see increased small peptides (<66 kDa) in the suffusate relative to the control animals. Instead there was a significantly reduced level of low molecular weight staining material with addition of insulin to the mesentery suffusate (**Figure 8**).

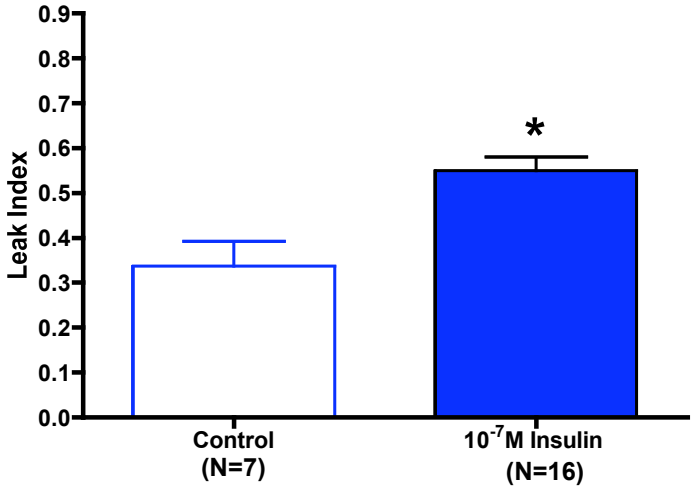


Figure 2: Average venular leakage of albumin from *in situ,* autoperfused adult male rat mesentery superfused for 75 mins with either (bicarbonate buffered solution) BBS (Control; open bar) or BBS plus insulin (10^{-7}M Insulin; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. Venules suffused with insulin had a significantly higher venular leakage compared with BBS suffused groups ($p<0.05$). Data are means ± SEM.

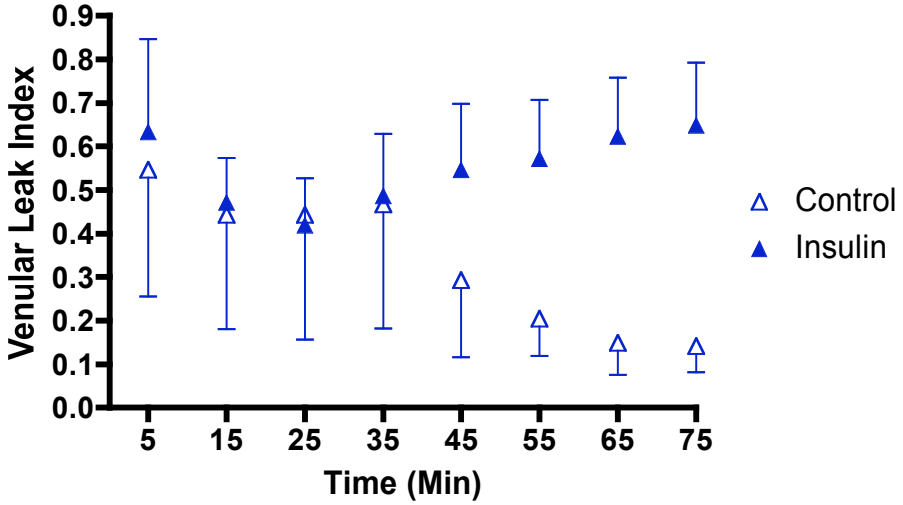


Figure 3: Differences in venular leak index with insulin treatment over time relative to control in adult male rats. Venular albumin leakage in adult male rats suffused with (10^{-7}M, filled triangles) or without (open triangles) insulin. Insulin suffusion abolished the decrease in leakage over time observed in the control group after 35 mins. Data are means ± SEM.

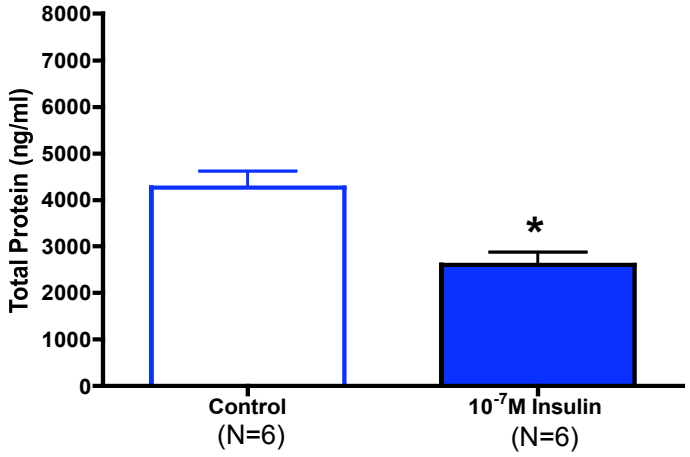


Figure 4: Average total protein clearance from of *in situ* autoperfused mesenteries of adult male rats suffused for 75 mins with either bicarbonate buffered solution (BBS, Control; open bar) or insulin (10^{-7}M; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. Total protein clearance was significantly lower with insulin suffusion compared to BBS suffusion (*p<0.01). Data are means ± SEM.

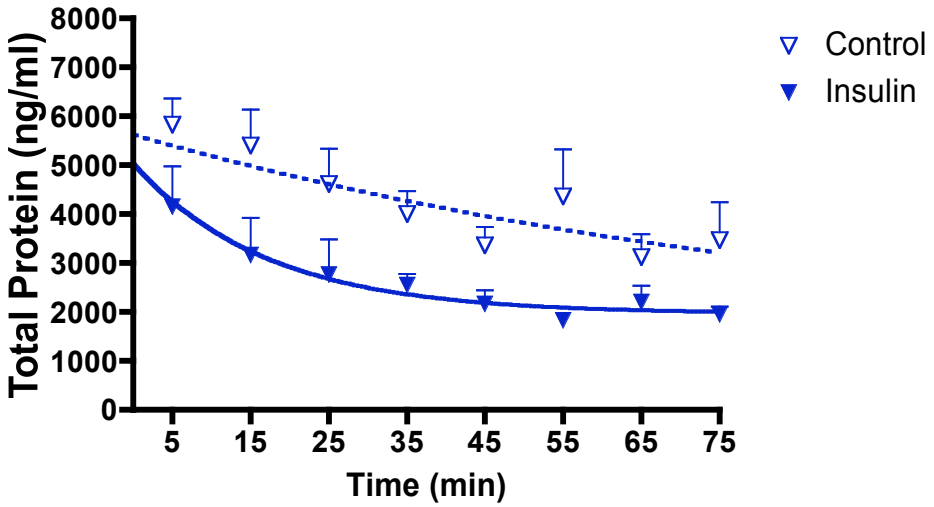


Figure 5: Kinetics of total protein clearance in control and insulin-treated adult male rats. Non-linear fitted curves of total protein clearance in adult male rats suffused with either bicarbonate buffered solution (BBS, Control: dotted line, empty triangles) or insulin (10^{-7}M solid line, filled triangles). The rate of decline in protein removed into the suffusate was faster in the insulin-treated group relative to the BBS suffused group (p<0.01). Data are means ± SEM.

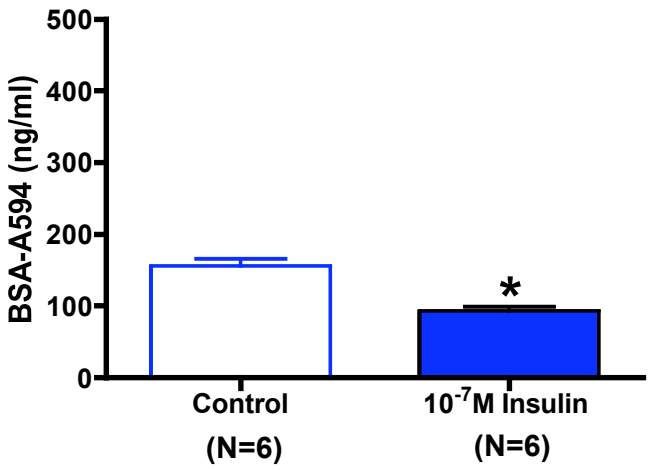


Figure 6: Average clearance of Alexa Fluor 594-labeled bovine serum albumin (BSA–A549) from *in situ* autoperfused mesenteries of adult male rats suffused for 75 mins with either bicarbonate buffered solution (BBS, Control; open bar) or insulin (10^{-7}M; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. BSA clearance was significantly lower with insulin suffusion compared to BBS suffusion (*$p<0.01$). Data are means ± SEM.

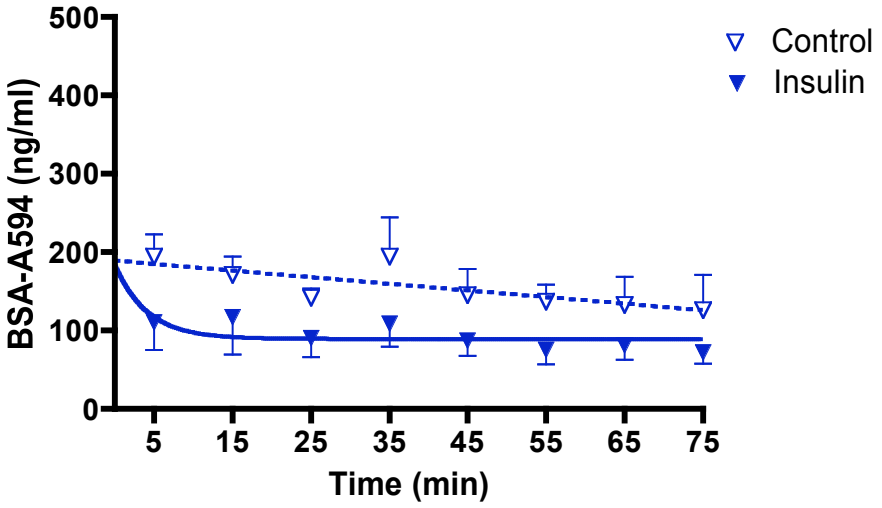
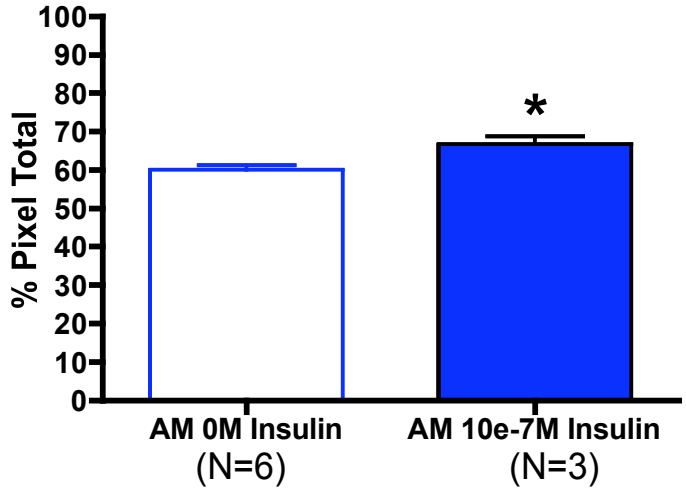


Figure 7: Differences in the kinetics of Alexa Fluor 594-labeled bovine serum albumin (BSA–A549) clearance from mesenteries of adult male rats with insulin treatment. Non-linear fitted curves of BSA clearance in adult male rats suffused with either bicarbonate buffered solution (BBS, Control: dotted line, empty triangles) or insulin (10^{-7} M solid line, filled triangles). The rate of decline in BSA removed into the suffusate was faster in the insulin-treated group relative to the BBS suffused group ($p<0.01$). Data are means ± SEM.

A)



B)

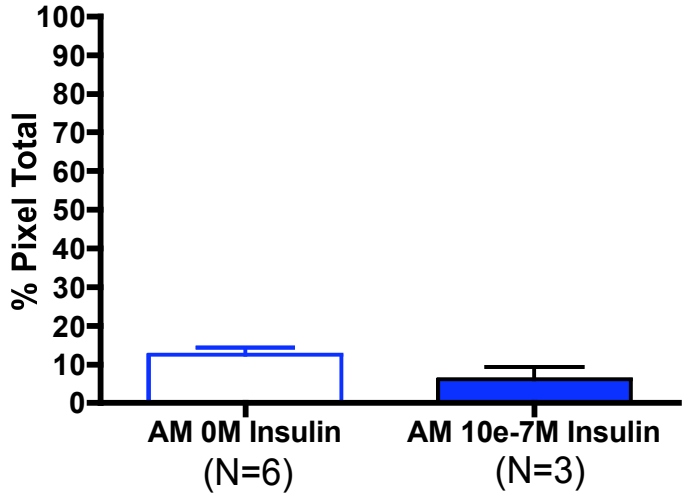


Figure 8: Influence of insulin on the activity of mesenteric MMP-2 (A) and MMP-9 (B) from the suffusate collected from adult male rats. Insulin increased the MMP-2 activity (*p=0.02), whereas it was without effect on the MMP-9 activity.

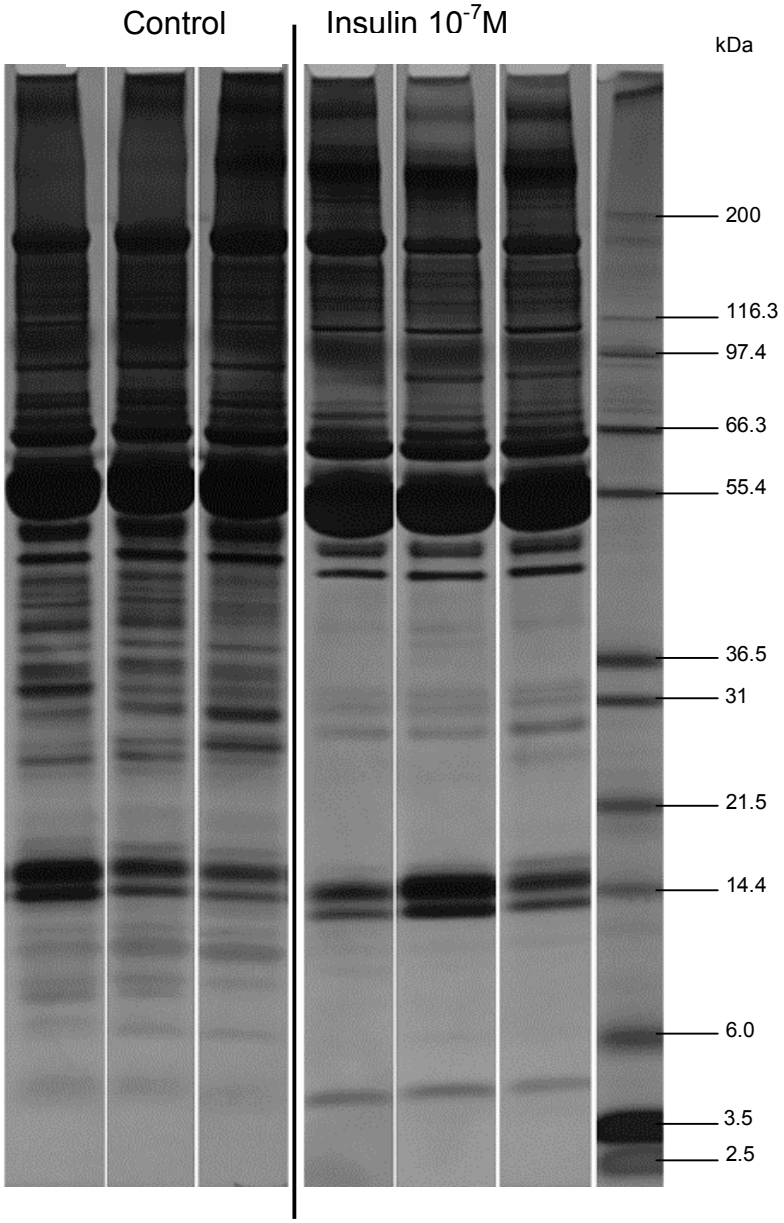


Figure 9: SDS gel electrophoresis of proteins and protein fragments of >2.5k MW from rat mesentery suffusate in representative samples of control and insulin treated (10^{-7}M) adult males. Treatment with insulin resulted in decreased levels of small proteins observed in the control suffusate.

CHAPTER 4: INSULIN AND JUVENILE MALE MICROVASCULAR EXCHANGE FUNCTION

Rationale and Hypothesis:

In recent years, the incidence of insulin resistance, obesity, and of type 2 diabetes in children and adolescents has increased significantly [46-48, 51]. Obese children often develop hyperinsulinemia along with severe insulin resistance [74]. Adolescents also have a higher risk for developing insulin resistance due to the increased insulin production and decreased insulin sensitivity during puberty [42, 43, 75]. Since hyperinsulinemia is hallmark symptom of insulin resistance and of the early stages of type 2 diabetes, there is an increased urgency to understand the effects of insulin in children and adolescents and determine whether the responses differ from those observed in adults. Additionally, understanding these differences in children and adolescents is crucial in knowing how to intervene and/or prevent the development of the diseases of glucose metabolism in this population at an early stage.

Investigating the microvascular exchange responses in juvenile, sexually immature subjects will also help us determine whether the changes observed in the adult male mesenteric venular albumin leakage and clearance with insulin were due to the presence of testosterone. Previous studies have suggested that testosterone plays a role in the development of insulin resistance [76]. Previous studies have shown that administration of testosterone to ovariectimized young female rats contributed to insulin resistance [77]. In our following experiments,

we chose to use juvenile male rats that were less than 60 days when testicular descent occurs, indicating the start of puberty [60].

We hypothesized that the changes we observed with insulin treatment in the adult male rats with respect to microvascular exchange, were due to the presence of testosterone and that those changes would be abolished in the juvenile male rats. Thus we did not expect to see any differences in Leak Index, albumin, protein clearance, or MMP responses to insulin suffusion in the juvenile male rats in the experiments conducted below.

Results:

Influence of Insulin on JM Venular Albumin Leakage:

Whereas we did not expect to see any leak index to change from control levels in the presence of insulin treatment, venular albumin leakage increased by 106% from control (N=15, 0.37 ± 0.03 vs. N=6, 0.18 ± 0.02, for insulin and control, respectively; $p<0.01$) following suffusion of the mesenteric microvasculature with high insulin dose 10^{-7}M (**Figure 10**). Venular albumin leakage in juvenile male rats with insulin suffusion was greater over time compared to the control (**Figure 11**).

Influence of Insulin on JM Total Protein Clearance:

As predicted, insulin suffusion was without effect on total protein clearance from mesenteric microvasculature (N=6, 3779 ± 313 ng/ml) compared to the control (N=6, 3573 ± 322 ng/ml) (**Figure 12**). The rate of loss of total protein

clearance, while significant ($p<0.05$), did not differ between groups (Insulin: $k=0.03 \pm 0.02$ ng·ml^{-1}*min^{-1}, $t_{1/2}=26.2$ min^{-1} vs. Control: $k=0.04 \pm 0.04$ ng·ml^{-1}*min^{-1}, $t_{1/2}=18.3$ min^{-1}) (**Figure 13**).

Influence of Insulin on JM Albumin Clearance:

Similarly to what we observed with respect to venular albumin leakage, insulin increased albumin clearance from mesenteric microvasculature (N=6, 228 ± 17 ng/ml) relative to the control values (N=6, 156 ± 12 ng/ml; $p<0.01$). Overall this was a 46% increase (**Figure 14**). While BSA clearance remained high over time in the insulin-treated group, it was constant in the control animals (**Figure 15**).

Influence of Insulin on JM Protein Degradation:

Since albumin clearance was increased with insulin suffusion, we expected to see decreased suffusate protein degradation in the insulin-treated JM compared to control animals. But protein degradation appeared to be slightly increased with insulin suffusion, as evidenced by the low molecular weight staining material (<21.5k MW) (**Figure 16**).

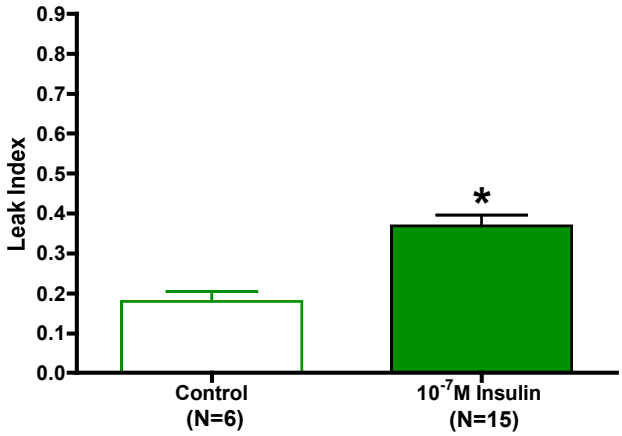


Figure 10: Average venular albumin leakage of *in situ* autoperfused juvenile male rat mesentery suffused for 75 min with either bicarbonate buffered solution (BBS,Control; open bar) or suffusate insulin (10^{-7}M; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. Venules suffused with insulin had a significantly higher venular leakage compared with BBS suffused groups ($*p<0.01$). Data are Mean ± SEM.

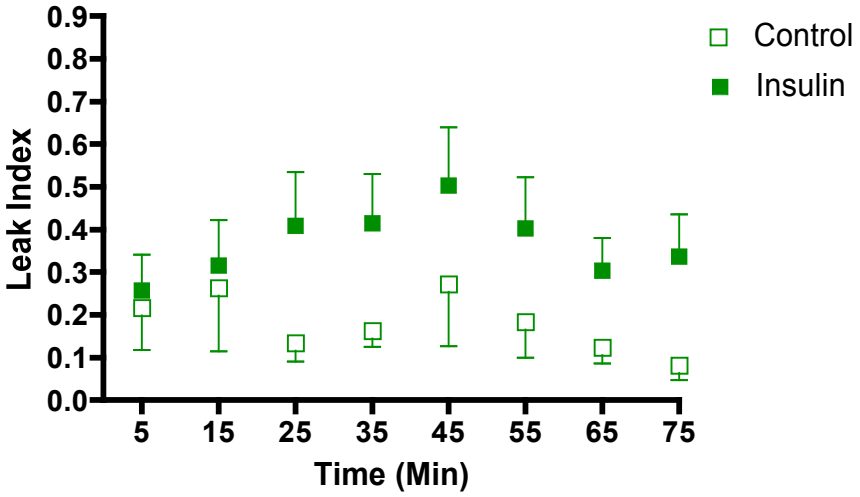


Figure 11: Differences in leak index with insulin treatment over time in juvenile male rats. Venular albumin leakage in juvenile male rats suffused with bicarbonate buffered solution (BBS) (empty squares) resulted in a sustained decrease over 75 mins, whereas those suffused with insulin (filled squares) had increased albumin leakage sustained over 75 mins. Data are Mean ± SEM.

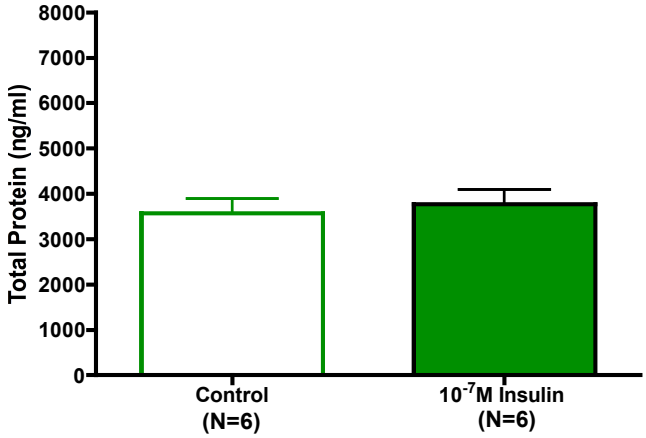


Figure 12: Average total protein clearance from of *in situ* autoperfused juvenile male rat mesentery suffused for 75 mins with either bicarbonate buffered solution (BBS, Control; open bar) or suffusate insulin (10^{-7}M; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. Insulin suffusion was without effect on the total protein clearance. Data are Mean ±SEM.

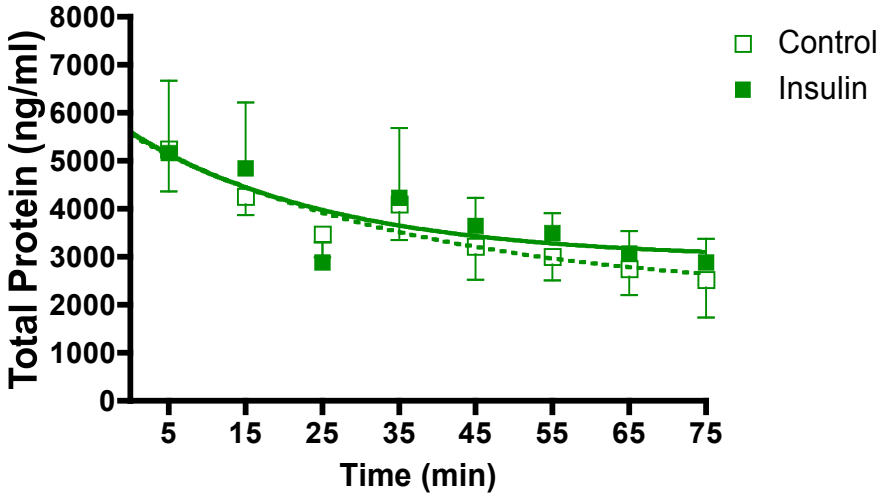


Figure 13: The kinetics of total protein clearance from the mesentery of juvenile male rats did not differ between suffusion with either bicarbonate buffered solution (BBS , Control: dotted line, empty triangles) or insulin (solid line, filled triangles). Data are Mean ±SEM.

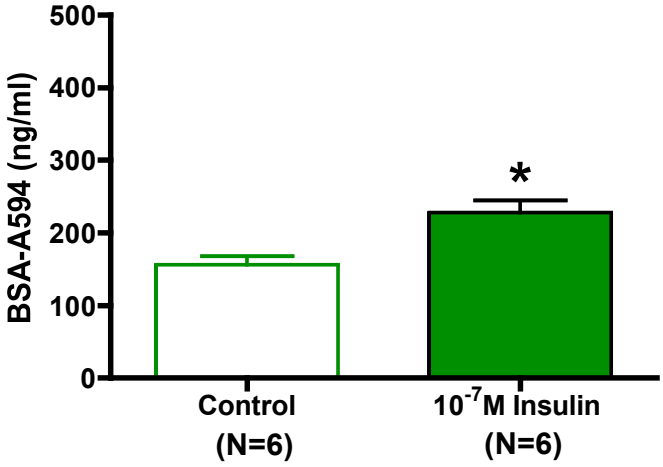


Figure 14: Average Alexa Fluor 594–labeled bovine serum albumin (BSA) clearance from *in situ* autoperfused juvenile male rat mesentery suffused for 75 min with either bicarbonate buffered solution (BBS, Control; open bar) or suffusate insulin (10^{-7}M; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. BSA clearance was significantly higher with insulin suffusion compared to BBS suffusion (*$p<0.01$). Data are Mean ±SEM.

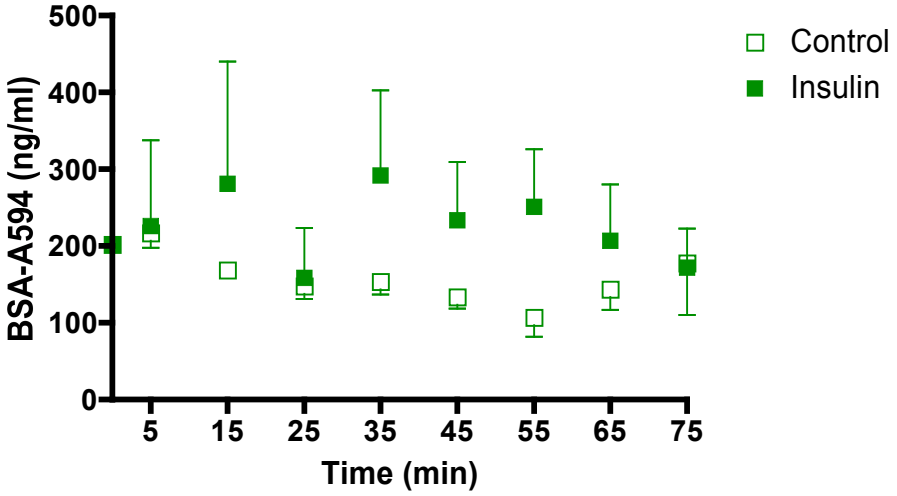


Figure 15: Differences in Bovine Serum Albumin (BSA) clearance kinetics with insulin treatment in juvenile males. BSA-A594 clearance in juvenile male rats suffused with bicarbonate buffer solution (BBS) (empty squares) resulted in a lower clearance than those suffused with insulin (filled squares). Data are Mean ± SEM.

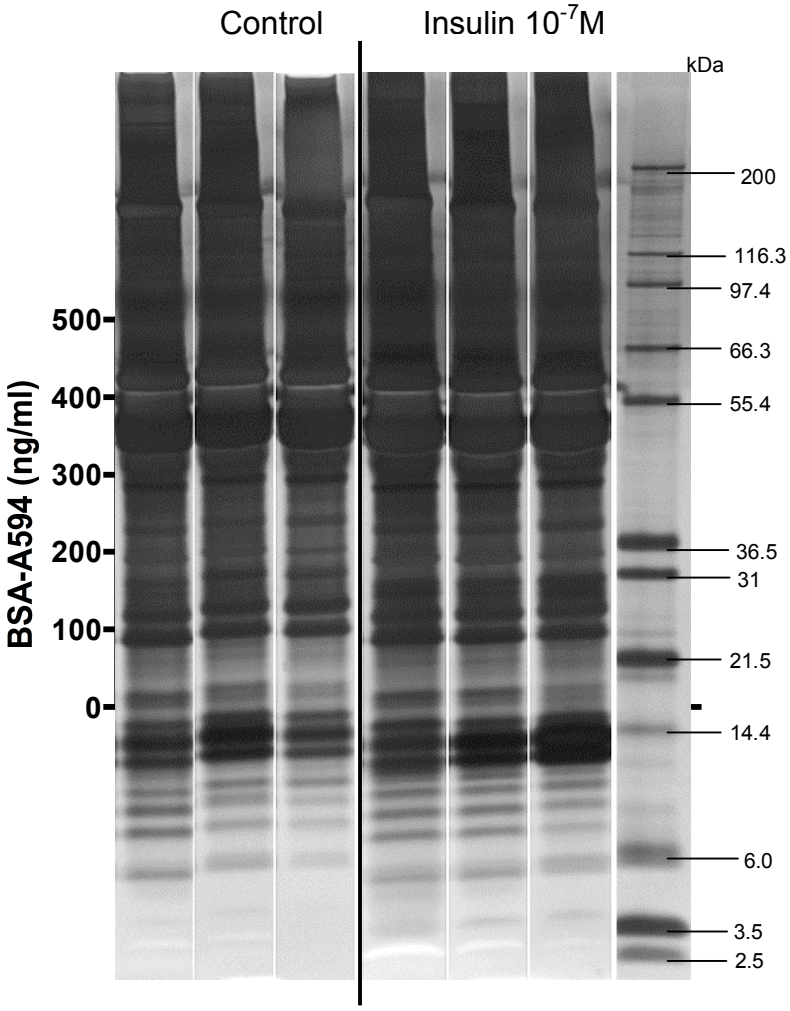


Figure 16: Analysis by SDS gel electrophoresis of the proteins and protein fragments of >2.5k MW from rat mesentery suffusate in representative samples of control and insulin treated (10^{-7}M) juvenile males. Treatment with insulin slightly increased the levels of small proteins(<21.5k MW) from the control suffusate.

CHAPTER 5: INSULIN AND ADULT FEMALE MICROVASCULAR EXCHANGE FUNCTION

Rationale and Hypothesis:

Previously, sex differences with respect to vascular tone and blood vessel responses to multiple stimuli have been reported [78-81], yet about microvascular permeability properties or responses to the stimuli with respect to sex, there is little study. In our laboratory we have shown sex differences in the microvascular permeability responses to factors such as exercise [62, 82], adenosine, a vasodilatory factor [83, 84], and microvessel location (arteriole vs. venule) [83]. Although there have been studies to evaluate the effect of insulin on microvascular permeability [33-35, 37, 38], most were performed on males. Therefore, prior to this study there has been no evaluation of the question whether sex is a player in the microvascular permeability response to insulin.

There are reasons to believe that indeed males and females may not have the same responses. The reproductive hormone, estradiol, while present in males at very low levels (as an androgen breakdown product), at the higher levels found in females has been shown to have protective effects on vessel wall physiology [85], including the endothelium [86-89]. One study showed that intact female rats were protected from the negative effects of fructose feeding whereas the ovariectomized females were not [90]. Further, estrogen replacement therapy has also been shown to improve insulin sensitivity in postmenopausal women [91-93].

We expect that the female animals would be protected by having high estradiol levels from the effects of high dose insulin. Consequently, we would not observe any changes in mesenteric microvascular permeability to albumin and proteins in response to high dose insulin.

Results:

Influence of Insulin on AF Venular Albumin Leakage:

Consistent with our hypothesis, venular albumin leakage did not change with insulin treatment (N=15, 0.33 ± 0.01) compared to control (N=6, 0.29 ± 0.02) (**Figure 17**). Venular leakage for both control and insulin-treated animals were fairly constant throughout the duration of the experiment and were not significantly different from each other (**Figure 18**).

Influence of Insulin on AF Total Protein Clearance:

Contrary to our hypothesis, in the females, insulin treatment decreased total protein clearance by 23% (N=6, 3746 ± 416 ng/ml) in the mesenteric microvasculature compared to control (N=7, 4894 ± 396 ng/ml; $p<0.01$) (**Figure 19**). Total protein clearance decreased at a significantly higher rate in the insulin-treated animals ($k= 0.05 \pm 0.03$ ng/ml*min^{-1}, $t_{1/2}= 13.9$ min^{-1}) than in control animals ($k= 8.6 \pm 140 \times 10^{-8}$ ng/ml*min^{-1}; $t_{1/2}=8.1\ 10^{6}$ min^{-1}; $p<0.01$) (**Figure 20**). The change in total protein clearance was also time-dependent ($p<0.01$).

Influence of Insulin on AF Albumin Clearance:

Of particular interest, consistent with the hypothesis but contrary to the total protein data, albumin clearance did not change with insulin treatment (N=7, 150 ± 17 ng/ml) compared to control (N=6, 157 ± 11 ng/ml) (**Figure 21**). Albumin clearance for both control (k= 6.7± 500 $x10^{-8}$ ng/ml*min^{-1}; $t_{1/2}$= 1.0 $x10^{7}$ min^{-1}) and insulin-suffused (k=0.04 ± 0.4 ng/ml*min^{-1}; $t_{1/2}$=19.0 min^{-1}) animals remained constant for the duration of the experiment (**Figure 22**).

Influence of Insulin on Tissue Matrix Density via Protease Activity:

As we anticipated, MMP-2 activity did not change with insulin treatment (N=5, 56.9 ± 4.3 %Pixel Total) compared to that observed in control animals (N=4, 60.7 ± 1.7 %Pixel Total) (**Figure 23A**). We also did not find any significant changes in MMP-9 activity with insulin treatment (N=5, 23.9 ± 5.2 %Pixel Total) compared to control (N=4, 16.3 ± 2.1%Pixel Total) (**Figure 23B**).

Influence of Insulin on JM Protein Degradation:

Since albumin clearance was unchanged with insulin suffusion, we did not expect to see changes in suffusate protein degradation in the insulin-treated animals compared to control animals. But protein degradation appeared to be slightly decreased with insulin suffusion, as evidenced by the low molecular weight staining material (<21.5k MW) (**Figure 24**).

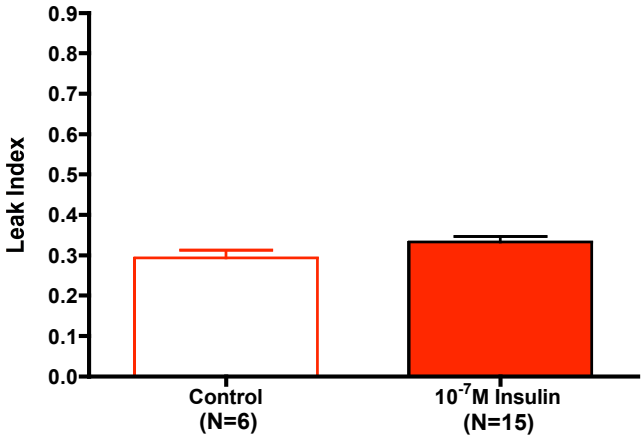


Figure 17: Average albumin leakage of *in situ* autoperfused adult female rat mesenteric venules superfused for 75 min with either bicarbonate buffered solution (BBS, Control; open bar) or suffusate insulin (10^{-7}M; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. Venules suffused with insulin were not different compared with BBS suffused groups. Data are Mean ±SEM.

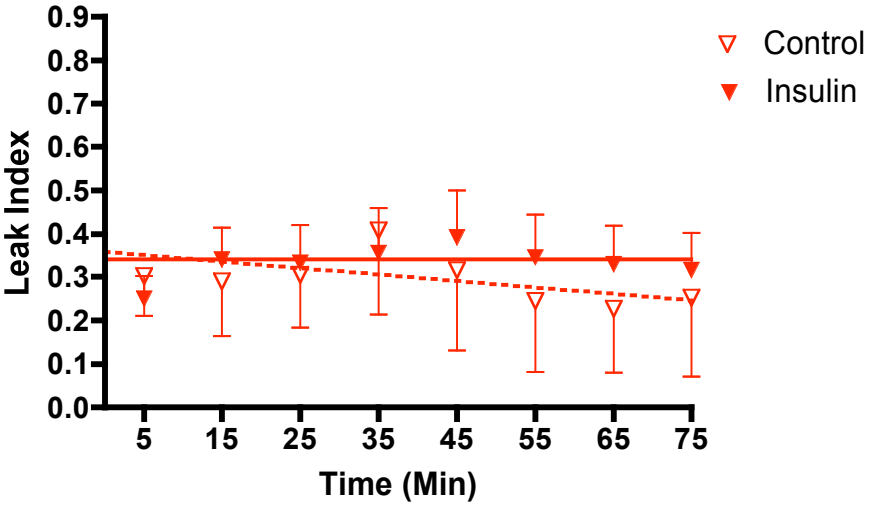


Figure 18: Linear regression analysis of venular leak index in adult females with (solid line; filled triangles) or without (dotted line; empty triangles) with insulin treatment over time, demonstrating the lack of change in control and of response to insulin. Data are Mean ± SEM.

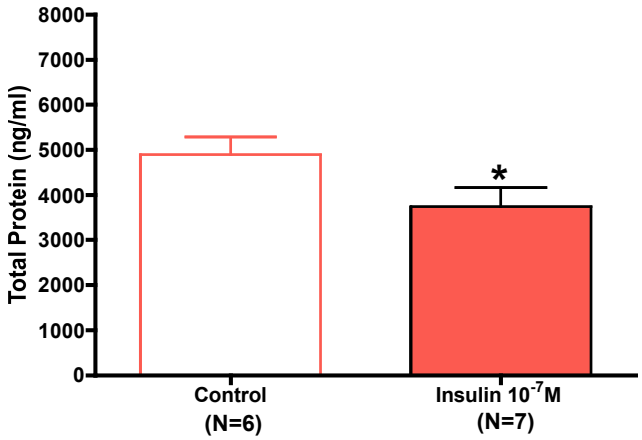


Figure 19: Average total protein clearance from of *in situ* autoperfused adult female rat mesentery suffused for 75 min with either bicarbonate buffered solution (BBS, Control; open bar) or insulin (10^{-7}M ; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. Total protein clearance was significantly lower with insulin suffusion compared to BBS suffusion (*p<0.01). Data are Mean ± SEM.

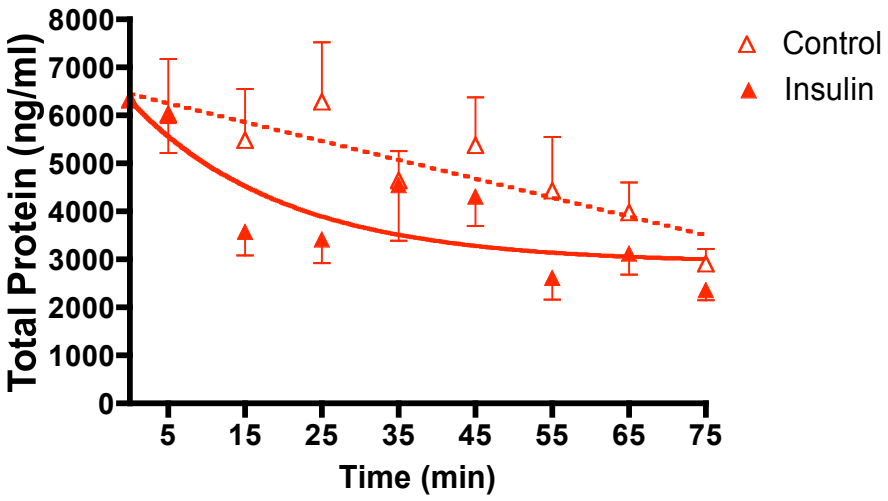


Figure 20: Differences in total protein clearance from mesenteries from female rats with insulin treatment over time. Non-linear fitted curves of total protein clearance in adult female rats suffused with either bicarbonate buffered solution (BBS, Control: dotted line, empty triangles) or insulin (solid line, filled triangles) are shown. The insulin suffused group had a faster rate of decline than the BBS suffused group (p<0.05). Data are Mean ± SEM.

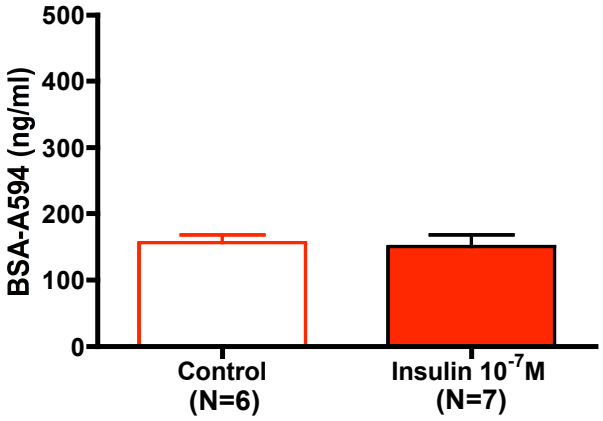


Figure 21: Average Alexa Fluor 594–labeled bovine serum albumin (BSA) clearance from mesenteries of *in situ* autoperfused adult female rats superfused for 75 mins with either bicarbonate buffered solution (BBS, Control; open bar) or insulin (10^{-7}M; filled bar). One animal was used per experiment, and is indicated as N in parenthesis for each group. BSA clearance was not different with insulin suffusion compared to BBS suffusion. Data are Mean ± SEM.

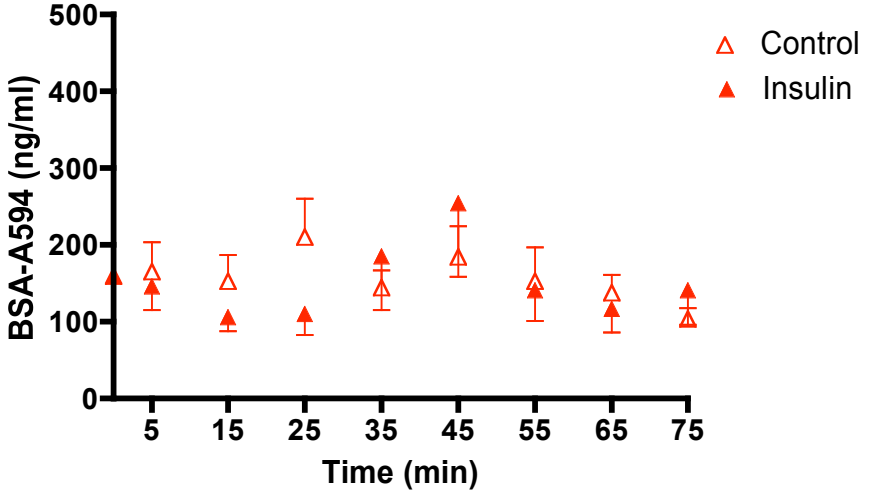


Figure 22: Kinetics of Bovine Serum Albumin (BSA) clearance from adult female mesentery was unchanged by insulin. BSA clearance in adult female rats suffused with either BBS (Control: empty triangles) or insulin (filled triangles) showed that they were not different.

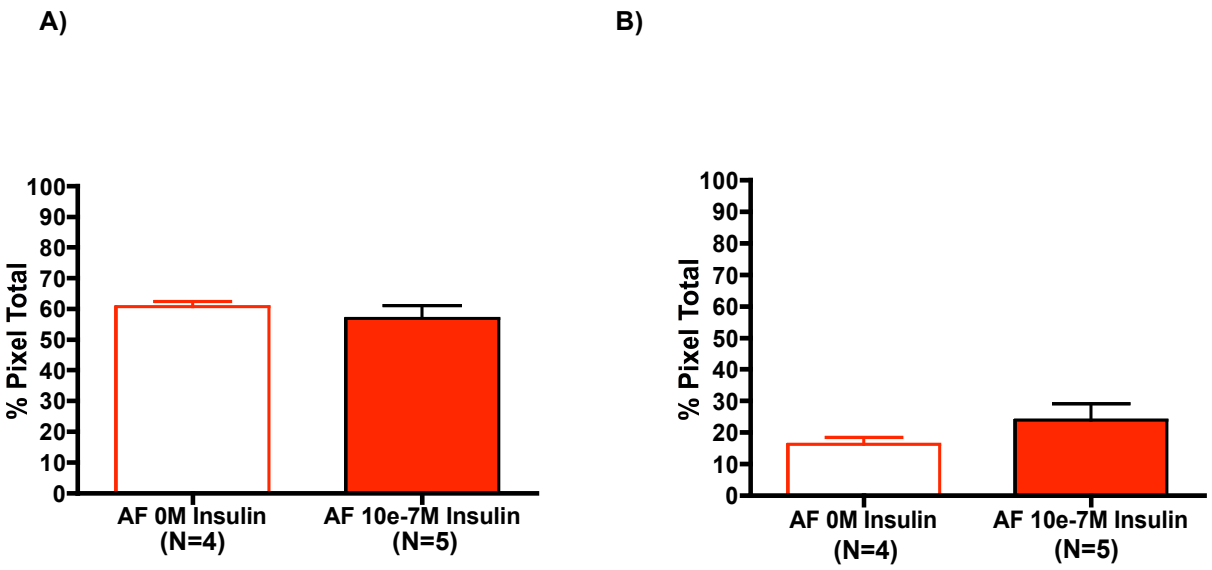


Figure 23: Influence of insulin on the MMP-2 (A) and MMP-9 (B) activities in mesentery suffusate from adult female rats. Insulin did not significantly change either MMP-2 or MMP-9 activity. Data are Mean ±SEM.

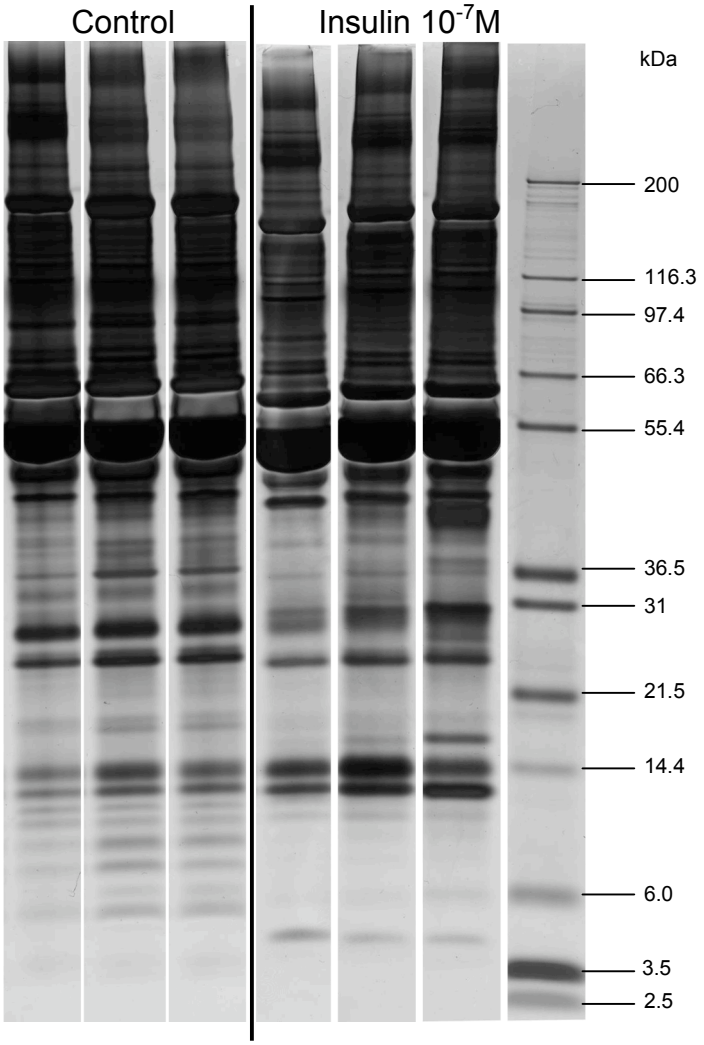


Figure 24: Analysis by SDS gel electrophoresis of the proteins and protein fragments of >2.5k MW from rat mesentery suffusate in representative samples of control and insulin treated (10^{-7}M) adult females. Treatment with insulin slightly decreased the levels of small proteins (<21.5k MW) from the control suffusate.

CHAPTER 6: DISCUSSION OF RESULTS

Adult Males:

Venular Albumin Leakage

Our data showing that insulin suffusion leads to increased venular albumin leakage (**Figure 2**) is consistent with some studies concluding that hyperinsulinemia leads to increased microvascular permeability [39, 40]. In many cases of the metabolic syndrome (MS), where hyperinsulinemia is often present, increased rates of transcapillary escape of albumin are also present [94]. Our kinetic studies showed that in adult males, insulin prevented the gradual decrease in venular leakage observed in the control animals (**Figure 3**). Thus under control conditions, the venules become "tighter" over time with suffusion of physiological solution. This may represent a protective mechanism of the venules adapting to exposure to suffusate and surgery to exteriorize the mesentery in the experimental protocol. Other studies using a similar *in situ* rat mesentery preparation (in adult males) have shown that superfusion with saline solution leads to increased tissue hydration and depletion of albumin from the mesenteric tissue [95].

The reduction in venular albumin leak may also indicate a compensatory mechanism by the venules to prevent further depletion of the albumin by making itself less permeable to albumin. If this were true, there would have to be a mechanism whereby the endothelium, or components in the vessel wall "sense" albumin concentration. In fact, changes in albumin content will lead to a

reduction in oncotic pressure external to the venule as well as an increase in the gradient for albumin between the blood and tissue compartments. A “simple” response would be for the endothelial cells to swell slightly, thereby reducing spaces between the cells and limiting albumin “traffic” out of the vasculature. The exact mechanism of this is unclear.

Thus based on our results, high dose insulin disrupts this compensatory mechanism, resulting in the increase in venular permeability. The mechanism of how insulin suffusion can lead to increase in microvascular permeability is unclear, but is discussed further in the General Discussion section (**Chapter 9**) of this dissertation.

Total Protein and Albumin Clearance

The mean clearance of all proteins was, unexpectedly, decreased with insulin treatment in the adult male rat mesentery (**Figure 4**) in a time-dependent manner (**Figure 5**). The common assumption is that venular permeability reflects the overall clearance of proteins from the blood to the interstitial fluid. Albumin clearance also decreased with insulin treatment (**Figure 6**) and reflected the total protein clearance in its response to insulin in a time-dependent manner (**Figure 7**). Thus insulin treatment resulted in inverse responses between venular leakage and clearances of albumin and proteins.

The decreased protein clearances with high insulin conflict with the previous studies showing increased incidences of microalbuminuria (MA), which can be indicative of increase in general clearance of albumin, in prediabetic

patients [96]. Nondiabetic subjects with MA also have multiple abnormalities in the CVD risk factors such as insulin, TGs, and BP [97]. Metabolic syndrome was associated with a 2-fold prevalence of MA [98]. In nondiabetic elderly subjects, MA was associated with endothelial dysfunction, evidenced in reduced forearm vasodilation [99]. One reason for apparently opposite results with respect to albumin and protein clearance relative to MA could be that our experiments were conducted in the mesentery whereas MA reflects a dysfunction with respect to loss of albumin from the glomerular capillaries, thus damage to the microvascular exchange barrier in the kidney. It is also possible that another feature of metabolic syndrome, hypertension and elevated capillary pressures, are responsible for the appearance of albumin in the urine of these patients by convective mechanisms rather than a loss of barrier function, per se.

One implication is that insulin is inducing an edematic effect, allowing the increased albumin that is leaking out from the venules to be retained in the interstitium. Some clinical studies suggest that insulin has an edematic effect in insulin resistant patients [100].

The opposing responses in adult males relative to the individual venule versus the overall clearance may account for the discrepancies observed across the permeability response studies to insulin. What individual laboratories refer to as “a measure of microvascular permeability” differ sufficiently (i.e. capillary hydraulic conductivity, protein reflection coefficient, leak index, transcapillary escape rate, lymph-to-plasma ratio, permeability surface area (PS) product in a variety of models from *ex vivo* to *in vitro*), and may only address a small aspect

of what is happening with the overall permeability, either at the individual microvessel level or the overall clearance level. In fact, majority of the *in vivo* studies reported measured the overall clearance in the whole body [34, 35, 38] and did not address what was occurring at the level of individual microvessels. The approach utilized in our lab, and this study in particular, is one of the few available methods that allow us to evaluate the permeability of the individual vessel as well as the overall protein clearance in the same tissue preparation.

Increased MMP-2 Activity and Decreased Protein degradation with Insulin

The increase in MMP-2 activity indicates that insulin treatment stimulated one of the two proteases resident in vascular tissue that can, in turn, lead to changes in protein flux in the compartment immediately adjacent to the microvessels in the mesenteric tissue.

With the increase in MMP activity the expectation was that there would be an increased breakdown of the tissue matrix, allowing for more mobility for the proteins, and increasing efflux into the suffusate. Instead, our results showed that protein clearance decreased and less protein was being cleared from the mesentery. Further more, the gel electrophoresis data (**Figure 9**) illustrate that the proteins in suffusate range in size from 4.5 kD to well over 500 kD in the control suffusate. What is interesting was that following insulin suffusion, the population of smaller proteins and peptides (4.5-40 kD) was markedly reduced.

Another explanation for the decreased appearance of small peptides may be that it is not necessarily due to decreased protein degradation, but simply to

the fact that there are less proteins available to be degraded in the interstitial fluid. This corresponds with our results of decreased albumin clearance across the mesenteric tissue observed in **Figure 4** and **5**. This indicates that the proteins that are coming across the venular wall from the blood to tissues are being transported across the tissue faster, to be able to be taken up by the lymphatics. To fully determine whether the proteins were being broken down, we would need to measure the activities of various proteases that are present in the interstitial fluid.

The elevation of MMP-2 activity in the face of a reduction in protein appearing in the suffusate along with a decrease in protein degradation would suggest that in the presence of insulin more intact proteins are retained in the interstitial fluid. Thus it can be implied that the lymphatic vessels are taking up the intact proteins. Thus it can be implied that the lymphatic vessels are taking up the intact proteins. Several studies point to the notion that there is an asymmetry in the transport of large proteins across the microvascular wall in the mesenteric cavity, and that substances greater than or equal to 39 kD transport from the peritoneal cavity to the plasma via the peritoneal lymphatics [101, 102]. Thus there may be increased mesentery lymphatic “pumping” to take up the increased efflux of large proteins from the blood to the interstitial fluid as a compensatory mechanism.

With these results in mind, we conclude that the changes in albumin movement between the vascular and suffusate is multifactorial and the consequences of insulin’s actions are observed on not just the single microvessel

exchange function, but also on the overall tissue clearance of albumin and proteins, as well as changes in the MMP activity and degradation of proteins (**Figure 25**).

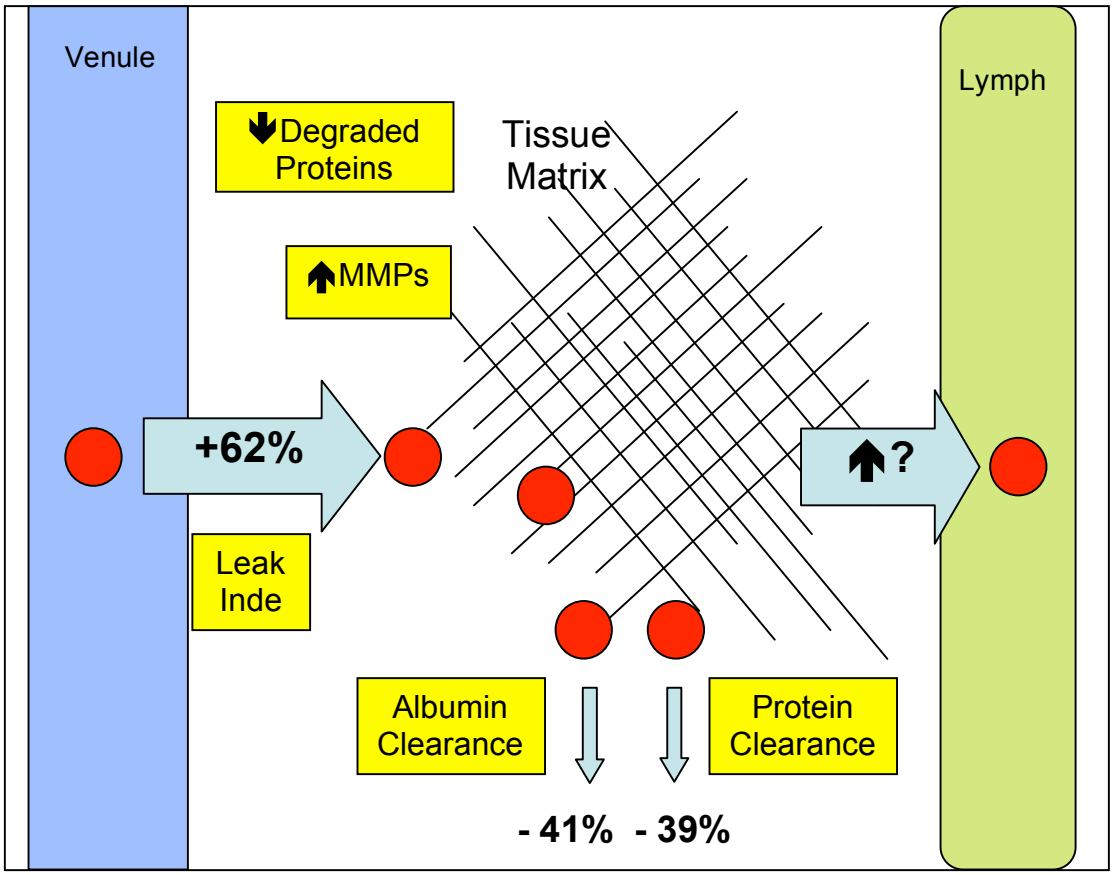


Figure 25: Schematic diagram of microvascular exchange in rat mesentery of adult males. The circles represent albumin and proteins. MMPs = matrix metalloproteinases.

Juvenile Males:

To determine whether the increase in the venular albumin leakage in the adult males was due to the testosterone, we repeated the experiments in sexually immature, juvenile male rats. Our finding was that the venular albumin leakage was significantly increased with insulin suffusion in the juvenile male rats (**Figure 10**). Treatment with insulin also prevented the decrease over time that was observed in the control group (**Figure 11**). This suggests that under control conditions, as we observed this same effect in the adult male (**Figure 3**), the vessel tends to become tighter over time. Thus the insulin-induced increase in venular albumin leakage was not due to testosterone, since the juvenile male rats have significantly decreased plasma testosterone than the adult males (**See Chapter 7**).

There was no significant change in the total protein clearance in juvenile males with insulin treatment (**Figure 12, 13**). Yet we detected an increase in albumin clearance with insulin suffusion (**Figure 14**). The albumin clearance over time looks similar to the kinetics observed in the venular albumin leakage, where the clearance of BBS suffused animals decreased and the insulin treated animals remained constant throughout the experiment (**Figure 15**). Again, this indicates that under control conditions, the permeability tends to decrease in the mesenteric microvasculature, and insulin abolishes that effect.

Our data also demonstrates that while protein clearance stayed constant, the albumin clearance increased with insulin suffusion. This implies there was an uncoupling of the relationship between albumin clearance and total protein

clearance with insulin. Thus albumin is regulated via a different mechanism in regards of its clearance from the tissues, compared to other plasma proteins. This uncoupling of the albumin and protein clearance response may be attributed to the different regulation of clearance for albumin from the rest of the interstitial proteins. In fact, protein transport between plasma and lymph has been observed in the dog, between albumin, fibrinogen, and dextran [103].

We also observed a slight increase in the amount of small peptides with insulin treatment (**Figure 16**), implying that there was increased degradation of the proteins with insulin treatment.

Overall, we found in our study that insulin suffusion significantly increased the venular albumin leakage in the juvenile males. We also found that only the albumin clearance was increased with insulin, as the total protein clearance remained the same (**Figure 26**). Thus albumin is regulated via a different mechanism than that of the rest of the proteins.

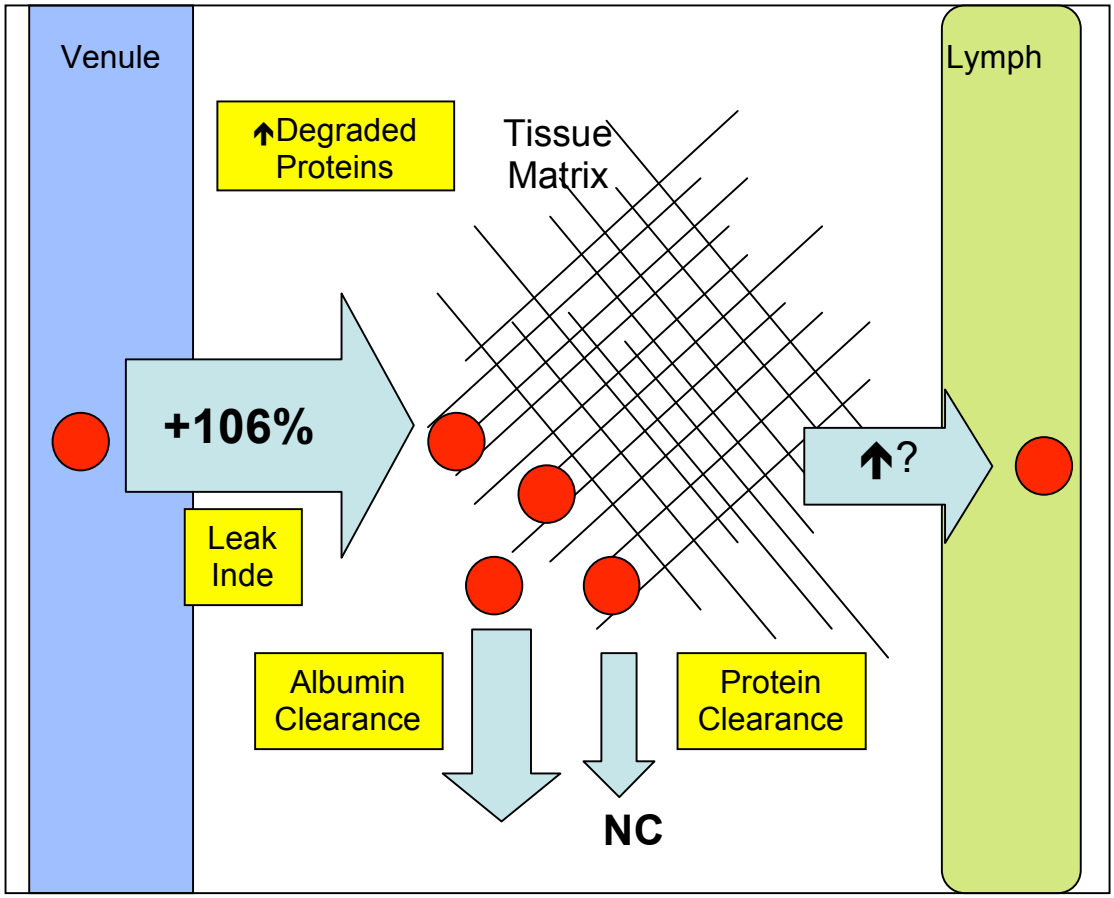


Figure 26: Schematic diagram of microvascular exchange in rat mesentery of juvenile males. The circles represent albumin and proteins. MMPs= matrix metalloproteinases.

Adult Females:

As we expected, we did not observe any significant changes in the venular albumin leakage with insulin suffusion in the adult female rats (**Figure 17, 18**). Insulin also time-dependently the decreased the total protein clearance by 23% (**Figure 19, 20**), but not the albumin clearance (**Figure 21, 22**). As we observed with the juvenile males, the data demonstrate differences in the handling of albumin from the other plasma proteins. The MMP-2 and -9 activities were not different with insulin treatment in the suffusate of the adult female mesenteric tissue (**Figure 23).**

Thus the lack of venular albumin leakage and albumin clearance responses to insulin by the adult females acts as a time and as a surgical control for the responses seen in the other two groups (**Figure 27**).

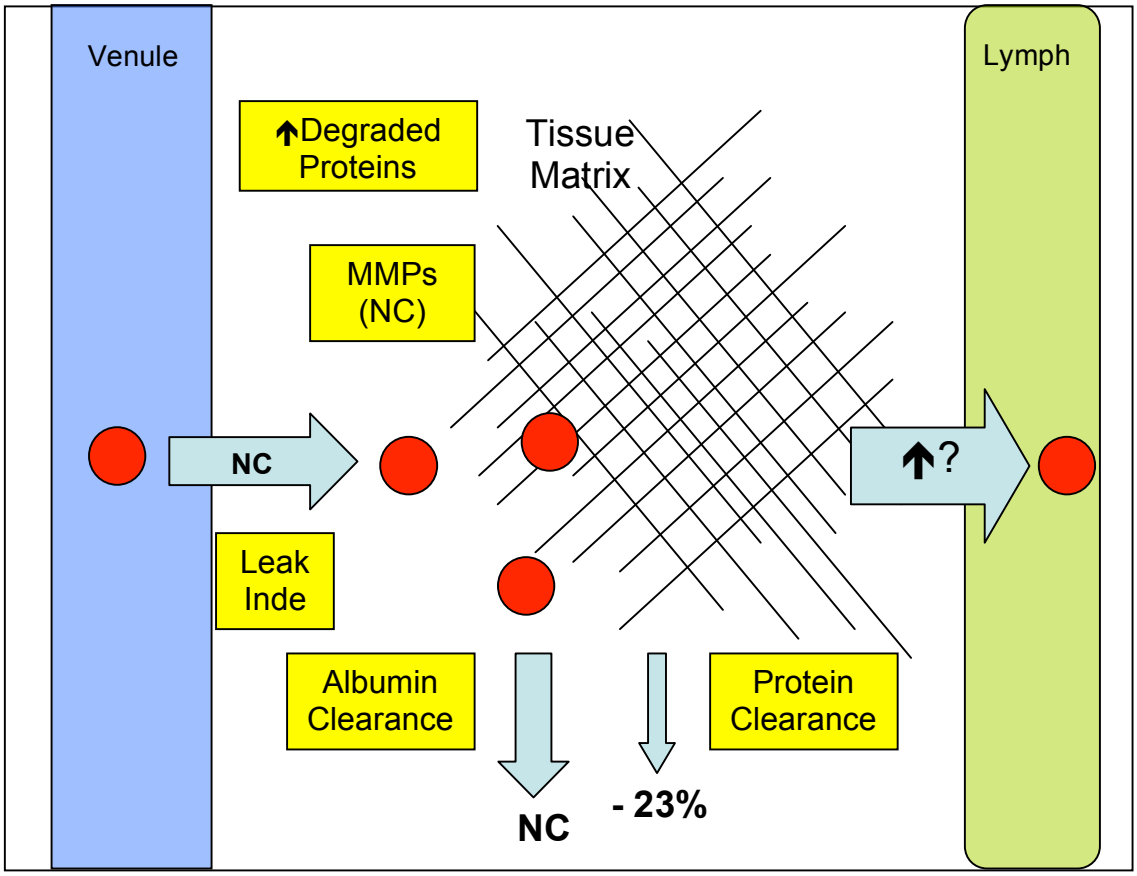


Figure 27: Schematic diagram of microvascular exchange in rat mesentery of Adult females. The circles represent albumin and proteins. MMPs= matrix metalloproteinases.

CHAPTER 7: DISCUSSION OF SEXUAL DIMORPHISM AND BASAL CHARACTERISTICS

Venular Albumin Leakage

Previous study in our lab has shown that skeletal muscle venules from adult males had the highest basal permeability values out of the three other animal groups that were studied (>adult females = juvenile males = juvenile females) [84]. Thus we predicted that we would observe a similar result with respect to albumin leakage in mesenteric venules. But contrary to expectation, it was the venules from the juvenile males that differed from the other two groups. In fact the basal leakage in the juvenile males was lower (N=6; 0.18 ± 0.02) than either the adult males (N=7; 0.34 ± 0.06; $p<0.05$) or the adult females (N=7; 0.29 ± 0.02; $p<0.05$) (**Figure 28**). This outcome implies that venular permeability differs with organ (i.e. skeletal vs. mesentery). At this point one could speculate that the difference in basal permeability for a given age and sex group reflects differences in the metabolic status of the two organs, i.e. skeletal muscle represents an organ with a greater metabolic demand relative to the mesentery.

In addition the solute permeability values (P_s) measured in Wang's thesis are not exactly equivalent to the albumin leakage values reported in this thesis. One major difference is that Wang used rat serum albumin (RSA) instead of bovine serum albumin (BSA) in this study. In previous work from the laboratory it was found that P_s to RSA > P_s to BSA [104].

Further, gel electrophoresis studies to determine whether the physicochemical properties of albumin varied by species demonstrated while the molecular mass of BSA was slightly greater than RSA, there were distinct differences with respect to the relative charge carried by the albumins. BSA is more anionic than RSA [66, 105]. Thus RSA may not be cleared from the venules in the same manner as BSA. The molecular basis for the tighter mesenteric venules of the juvenile males remains to be determined.

Total Protein Clearance

Basal total protein clearance in juvenile males (N=6; 3573 ± 322 ng/ml) was significantly lower than that from adult females (N=6; 4894 ± 396 ng/ml; $p<0.05$). Of interest, though, the value was not significantly lower than adult males (N=6; 4273 ± 345 ng/ml) (**Figure 29**). The rate of decline of basal total protein clearance in juvenile males was greater than the other two groups (**Figure 30**). Given that juvenile males had lower basal total protein clearance from the intact mesentery implies that juvenile animals retain more of their large proteins in the vascular compartment relative to the adult animals**.**

One outcome would be less of a driving force for filtration (and a lower potential for edema formation). The lower rate of albumin and protein clearance from the tissue is also consistent with the observed venular albumin leakage in that the juvenile males have lower microvascular exchange**.** Further, in the case of the juvenile males, the basal exchange characteristics of the venules match the behavior protein clearance.

Albumin Clearance

No significant differences in basal albumin clearance existed among the three animal groups (AM: N=6, 157 ± 19 ng/ml; AF: N=6, 157 ± 11 ng/ml; JM: N=6, 156 ± 12 ng/ml) (**Figure 31**). Interestingly, there were no differences with respect to albumin clearance, given that we observed the lower venular leak and the lower protein clearance in the juvenile males relative to the other groups. This outcome suggests that albumin movement within the tissue differs from other proteins in plasma and that simply measuring one of the three parameters would fail to provide an accurate picture of what is happening in the intact tissue.

Plasma Testosterone

As expected, basal plasma testosterone concentration was significantly higher in the adult males (median ± SEM; 96.2 ± 22.7 ng/dl) relative to both the juvenile males (45.8 ± 12.3 ng/dl; $p<0.05$) and adult females (3.0 ± 2.6 ng/dl; $p<0.05$) (**Figure 32**). The juvenile males had a higher testosterone than the adult females, but was not considered statistically significant due to the low "N" for the adult females (N=2). Thus, a future testable hypothesis is that the responses to insulin specific to the adult males reflect mechanisms mediated by the higher plasma testosterone concentration.

Plasma Estradiol and Progesterone

The average basal plasma estradiol level of the adult females (median ± SEM; 34.0 ± 5.8 pg/ml) was higher than that of the adult males (24.6 ± 2.2 pg/ml), although it was not statistically significant (p=0.06) (**Figure 33**). Of interest, the basal estradiol level of the juvenile males (28.6 ± 0.8 pg/ml) was comparable to that of the adult females. The values in the juvenile males varied significantly less than that of the cycling adult females (± 3% versus ± 17%, respectively). Thus, the basal juvenile properties and responses to insulin may reflect a combination of both androgen- and estrogen-dependent mechanisms.

As expected, adult females (median ± SEM; 29.3 ± 5.2 ng/ml; p<0.01) had a significantly higher basal plasma progesterone level than both the juvenile males (9.3 ± 1.5 ng/ml; p<0.05) or adult males (8.3 ± 2.6 ng/ml; p<0.01) (**Figure 34**).

Plasma Insulin

There was no significant difference in the control plasma insulin levels among the three animal groups (median ± SEM; AM: 1.3 ± 0.1 ng/ml, JM: 1.2 ± 0.1 ng/ml, AF: 1.3 ± 0.1 ng/ml) (**Figure 35**). This is consistent to the clinical studies where male and female patients had similar insulin levels [106].

Plasma Glucose

The plasma glucose levels in the Juvenile males (median ± SEM; 122.0 ± 11.2 mg/dl) were significantly higher than that of the adult males (95.0 ± 2.5

mg/dl; $p<0.05$), although not statistically different from adult females (117.0 ± 7.7 mg/dl) (**Figure 36**). The difference between the adult males and females did not reach significance. The reason for the higher basal plasma glucose in juvenile rats is uncertain as the feeding, housing, and handing regimes did not differ between the three groups. The lack of sex differences in basal plasma glucose concentration amongst the adult animal groups is consistent with published studies involving both humans [106] and rats [107].

Mean Arterial Blood Pressure (MAP)

Of interest, the mean arterial pressure (MAP) measured in the internal carotid artery, was significantly higher in the adult males (N=7, median ± SEM, 146.2 ± 1.0 mmHg) compared to both the juvenile males (N=6, 125.8 ± 1.9 mmHg; $p<0.01$) and adult females (N=7, 129.3 ± 2.1 mmHg; $p<0.01$) (**Figure 37**). Although the mean MAP was higher in the adult males, MAP decreased at a slower rate (0.09 ± 0.02 mmHg/min; $p<0.05$) than the juvenile males (0.18 ± 0.04 mmHg/min) or the adult females (0.20 ± 0.04 mmHg/min) (**Figure 38**).

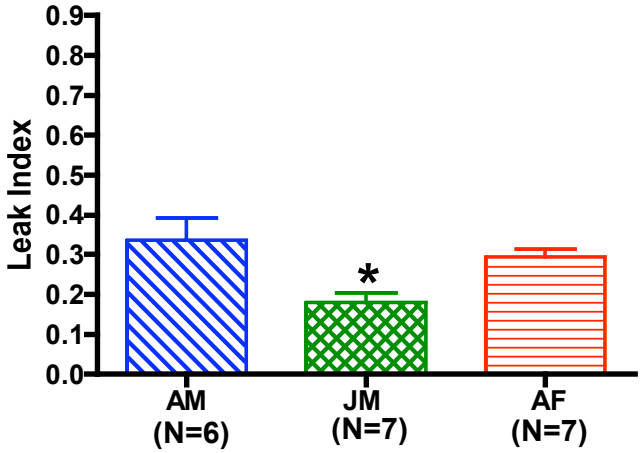


Figure 28: Comparison of basal venular albumin leakage across the three animal groups. The basal venular leak of the Juvenile males (JM=Crosshatches) was significantly lower than both adult males (AM: Diagonal lines) and adult females (AF: Horizontal lines) (*p<0.05). The bars are means ± SEM.

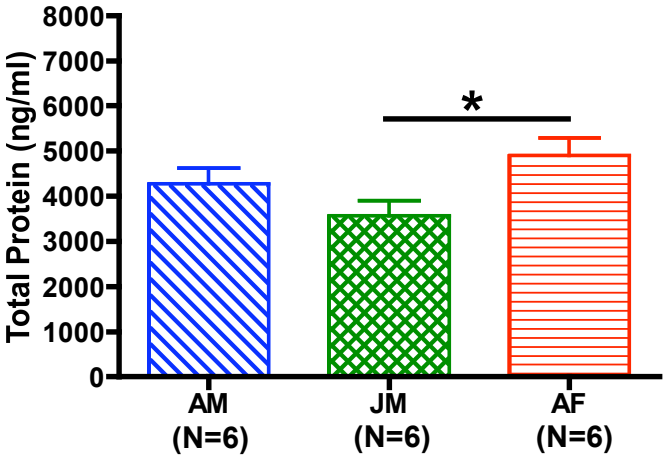


Figure 29: Comparison of basal total protein clearance from the mesenteries of the three animal groups. The basal total protein clearance of the Juvenile males (JM: Crosshatches) was significantly lower than that of the adult females (AF: Horizontal lines) (*p<0.05), but not that of the adult males (AM: Diagonal lines). The bars are means ± SEM.

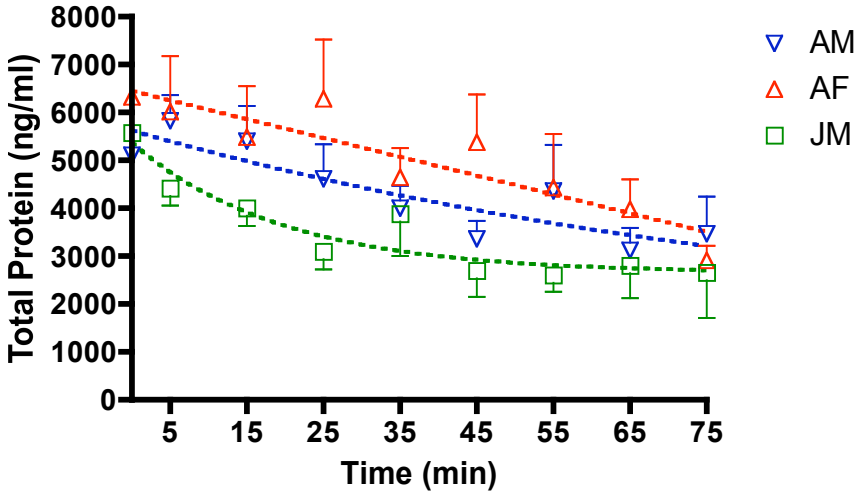


Figure 30: Comparison of the kinetics of total protein clearance from the mesenteries of the three animal groups. Data for adult males (AM, inverted triangles), adult females (AF, triangles) and juvenile males (JM, squares) are Mean ± SEM

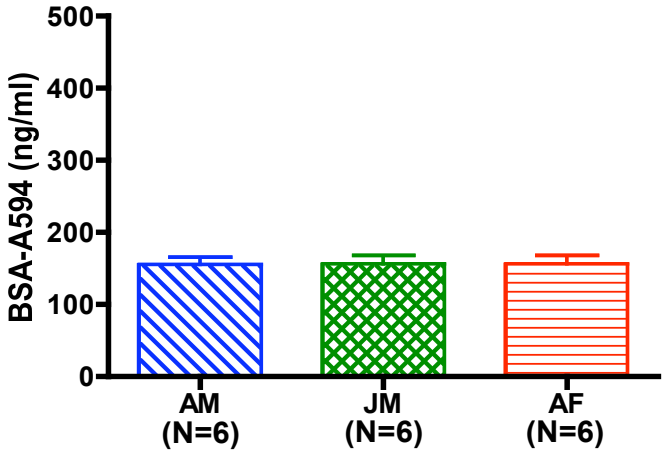


Figure 31: Comparison of basal albumin clearance from the mesenteries of the three animal groups: Juvenile males (JM: Crosshatches), adult females (AF: Horizontal lines), and adult males (AM: Diagonal lines). There was no significant difference between the three groups. The bars are means ± SEM.

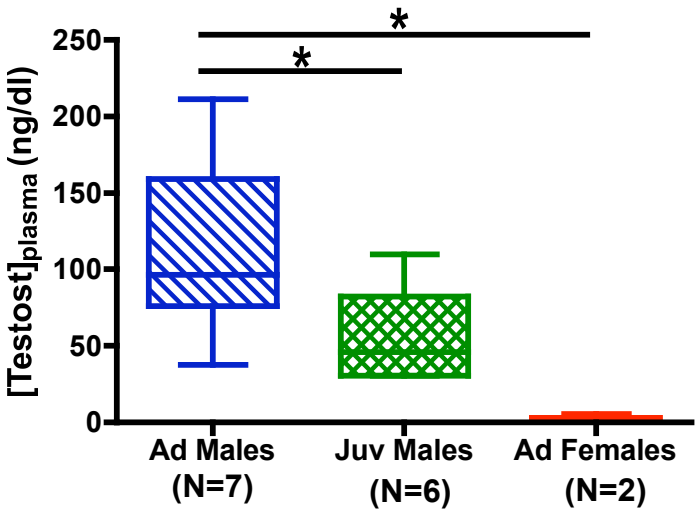


Figure 32: Comparison of plasma testosterone levels across three animal groups: Juvenile males (JM: Crosshatches), adult females (AF: Horizontal lines), and adult males (AM: Diagonal lines). Plasma testosterone of the AM was significantly higher than JM or AF (*p<0.05). Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

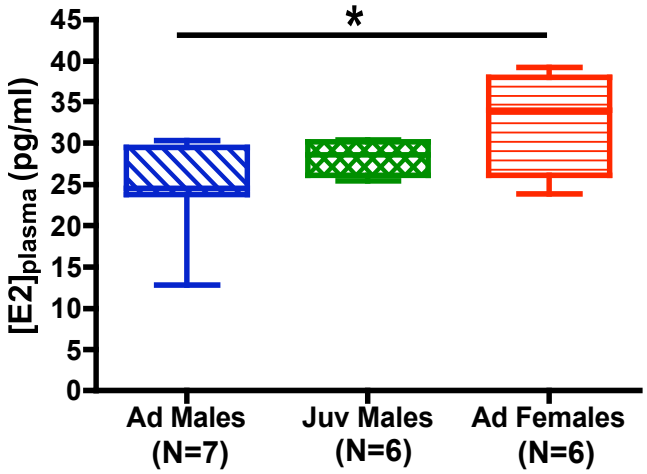


Figure 33: Comparison of plasma estradiol levels across three animal groups: Juvenile males (JM: Crosshatches), adult females (AF: Horizontal lines), and adult males (AM: Diagonal lines). Plasma estradiol was higher in the AF than AM (*p=0.06) and did not differ from JM. Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

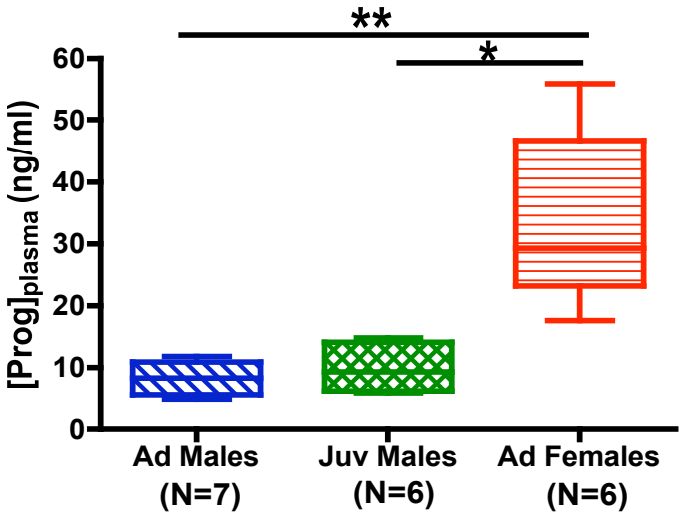


Figure 34: Comparison of plasma progesterone levels across three animal groups: Juvenile males (JM: Crosshatches), adult females (AF: Horizontal lines), and adult males (AM: Diagonal lines). Plasma progesterone of the AF was significantly higher than JM (*p<0.05) and AM (**p<0.01). Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

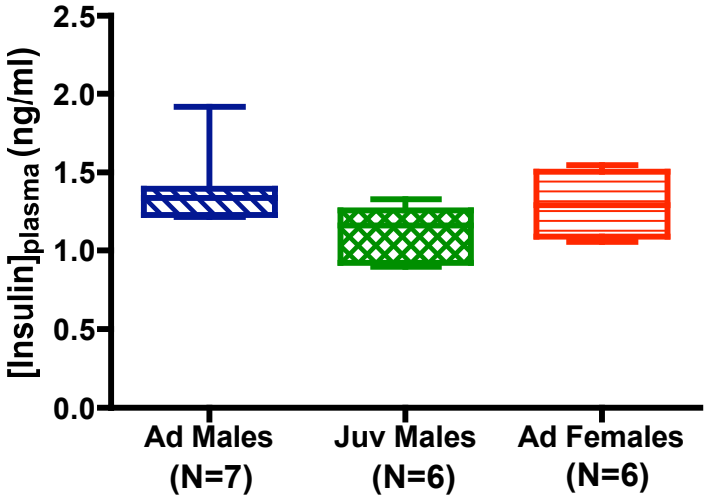


Figure 35: Comparison of plasma insulin levels across three animal groups: Juvenile males (JM: Crosshatches), adult females (AF: Horizontal lines), and adult males (AM: Diagonal lines). There was no significant difference between the three groups. Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

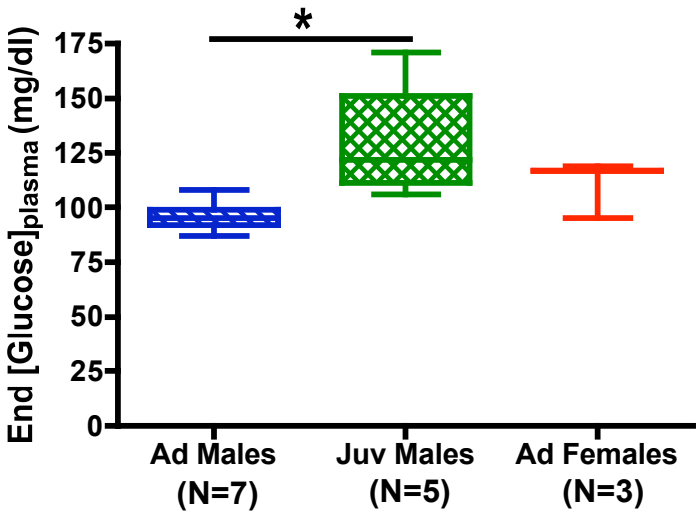


Figure 36: Comparison of plasma glucose levels at the end of the experimental protocols with insulin treatment for the three animal groups: Juvenile males (JM: Crosshatches), adult females (AF: Horizontal lines), and adult males (AM: Diagonal lines). JM had significantly higher plasma glucose than AM (*$p<0.05$). Values depicted as 25^{th}, 50^{th} and 75^{th} percentile bounded by the 10^{th} and 90^{th} percentiles.

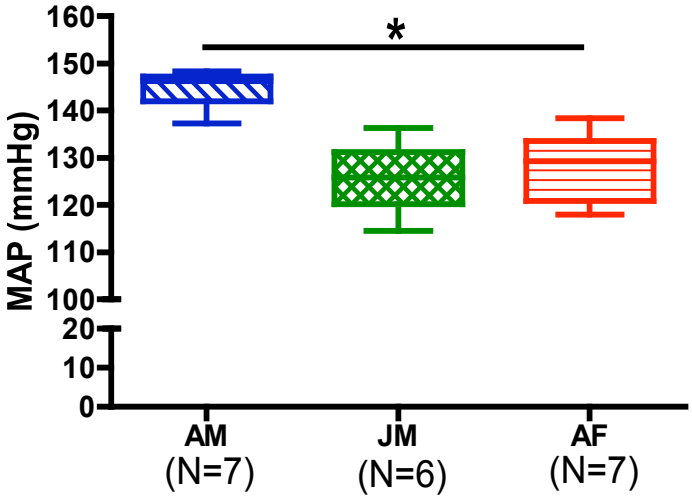


Figure 37: Comparison of mean arterial pressure (MAP) across three animal groups: Juvenile males (JM: Crosshatches), adult females (AF: Horizontal lines), and adult males (AM: Diagonal lines). AM had a higher MAP than AF (*p<0.01). Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

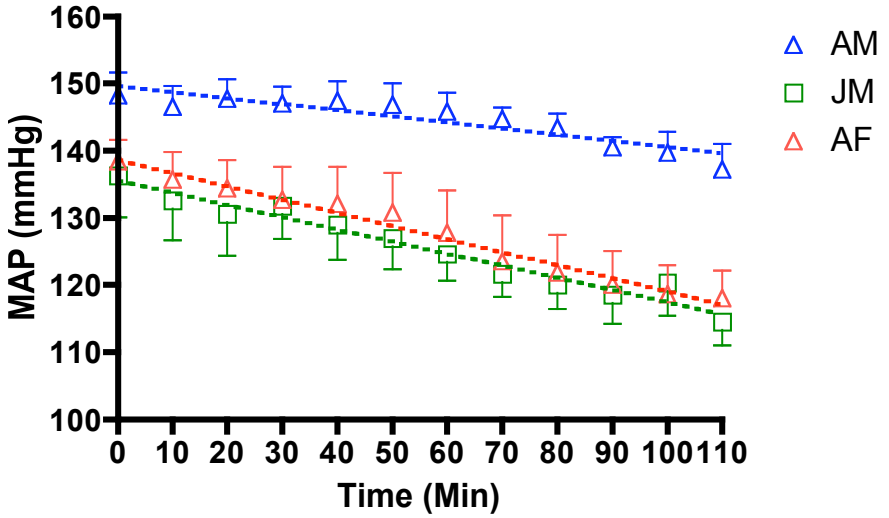
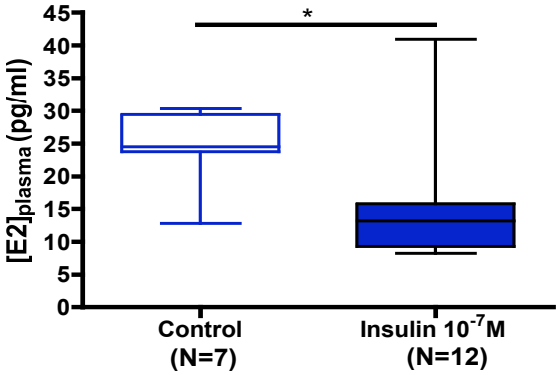
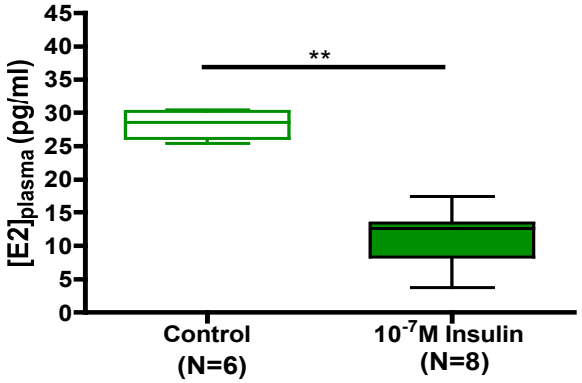


Figure 38: Comparison of mean arterial pressure (MAP) over time across three animal groups: Juvenile males (JM), adult females (AF), and adult males (AM). MAP in AM was higher than either AF or JM and decreased at a slower rate than JM and AF. Data are Mean ± SEM.

A)



B)



C)

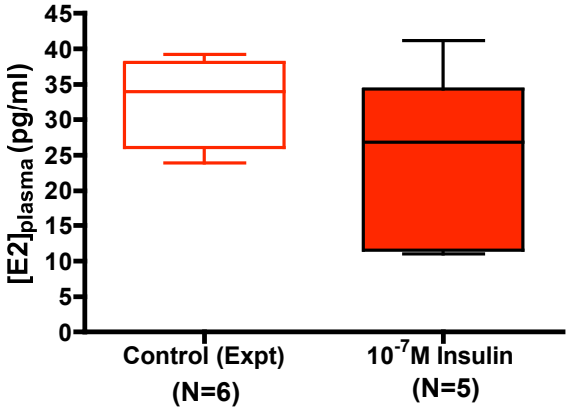


Figure 39: Plasma estradiol in adult males (AM: Blue) (**A**), juvenile males (JM: Green) (**B**), and adult females (AF: Red) (**C**). Plasma estradiol levels in adult males and juvenile males were decreased with insulin suffusion (*p<0.05; **p<0.01). Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

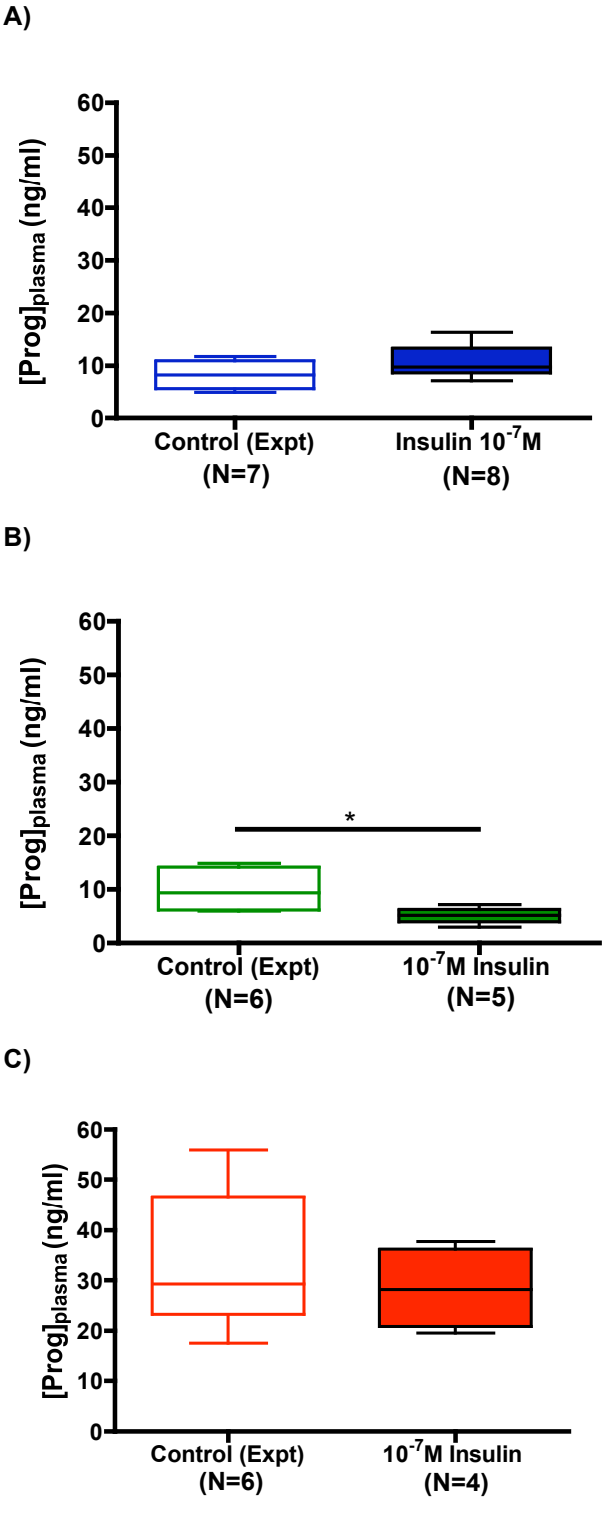
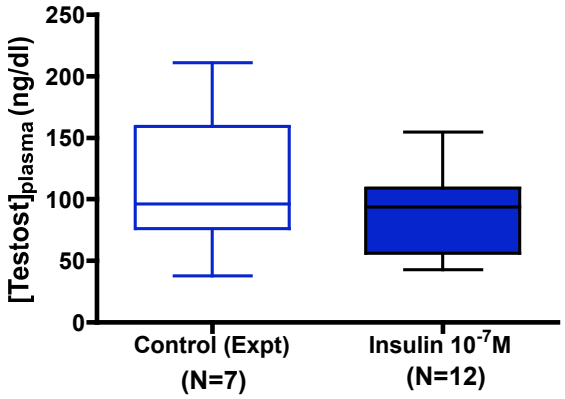
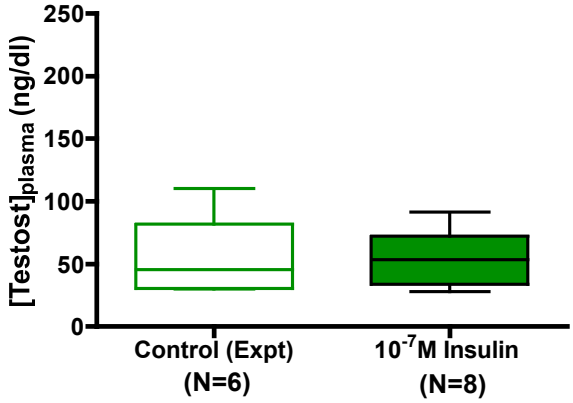


Figure 40: Plasma progesterone in adult males (AM: Blue) (**A**), juvenile males (JM: Green) (**B**), and adult females (AF: Red) (**C**). Plasma progesterone levels in juvenile males were decreased with insulin suffusion (*$p<0.01$). Values depicted as 25^{th}, 50^{th} and 75^{th} percentile bounded by the 10^{th} and 90^{th} percentiles.

A)



B)



C)

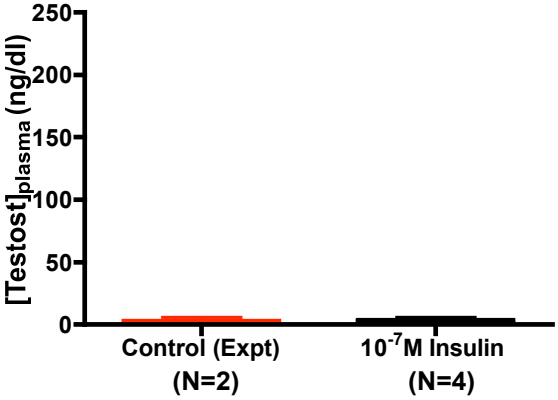
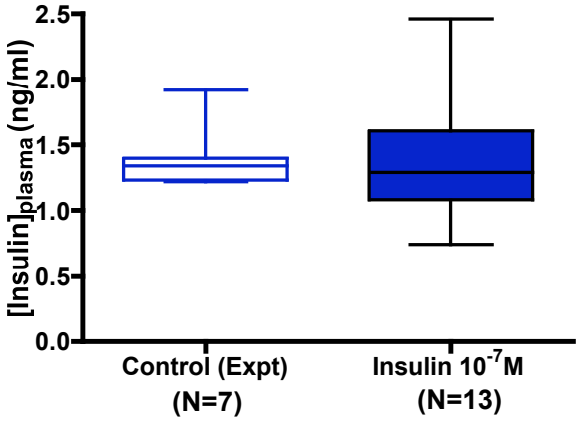
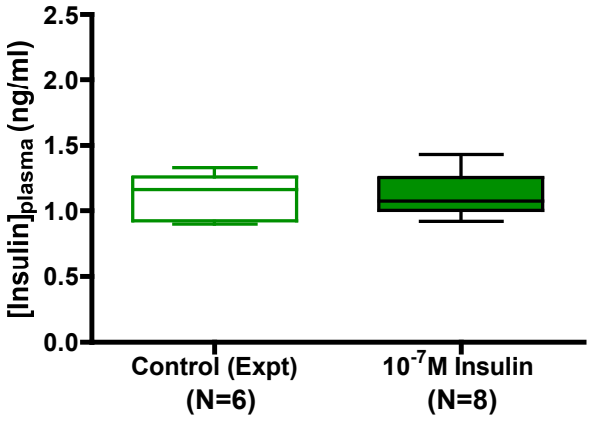


Figure 41: Plasma testosterone in adult males (AM: Blue) (**A**), juvenile males (JM: Green) (**B**), and adult females (AF: Red) (**C**). Plasma testosterone levels were unchanged with insulin suffusion. Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

A)



B)



C)

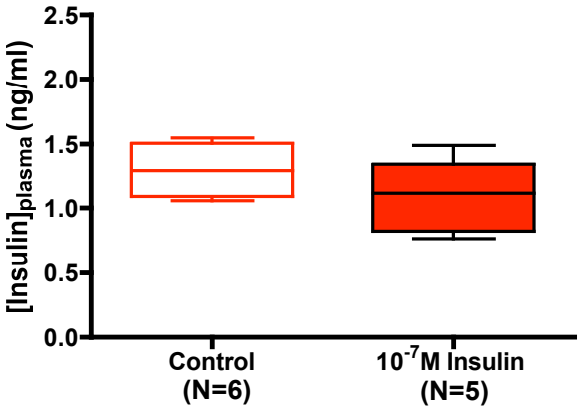
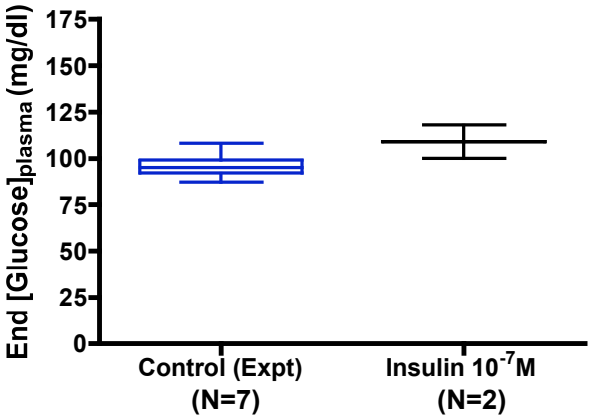


Figure 42: Plasma insulin in adult males (AM: Blue) (**A**), juvenile males (JM: Green) (**B**), and adult females (AF: Red) (**C**). Plasma insulin levels were unchanged with insulin suffusion. Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

A)



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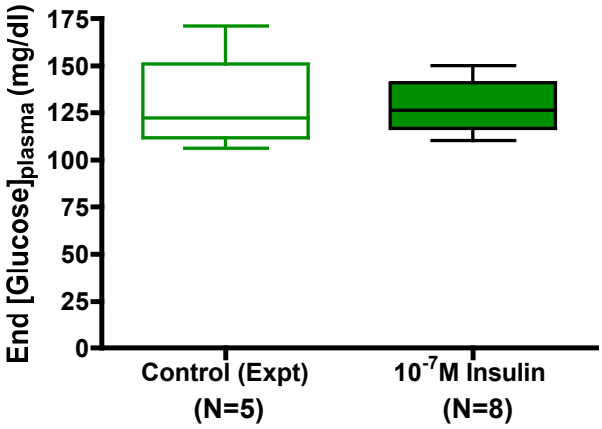


C)

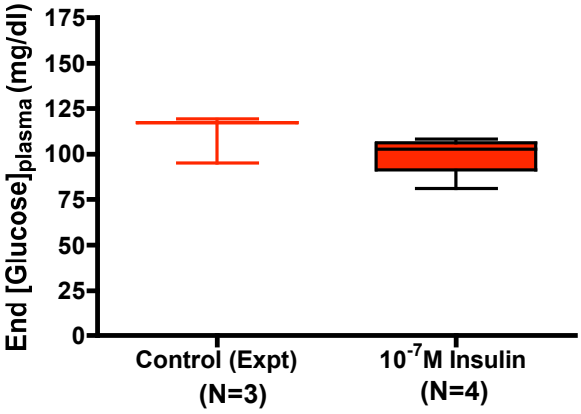
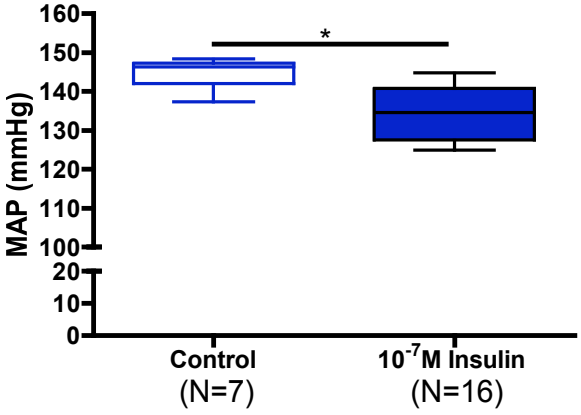
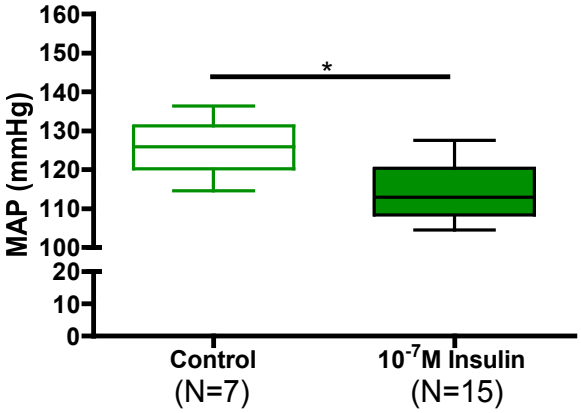


Figure 43: Plasma glucose in adult males (AM: Blue) (**A**), juvenile males (JM: Green) (**B**), and adult females (AF: Red) (**C**). Plasma glucose levels were unchanged with insulin suffusion. Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

A)



B)



C)

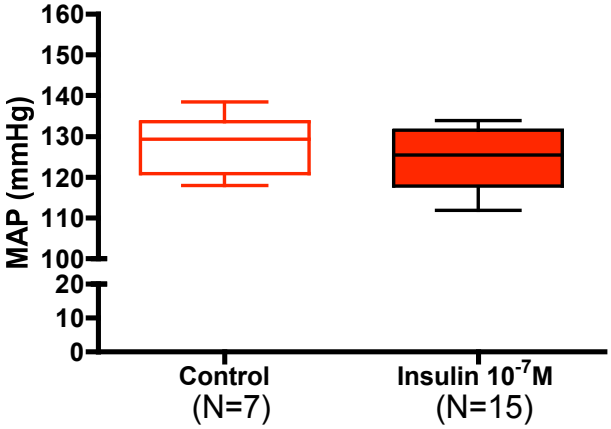
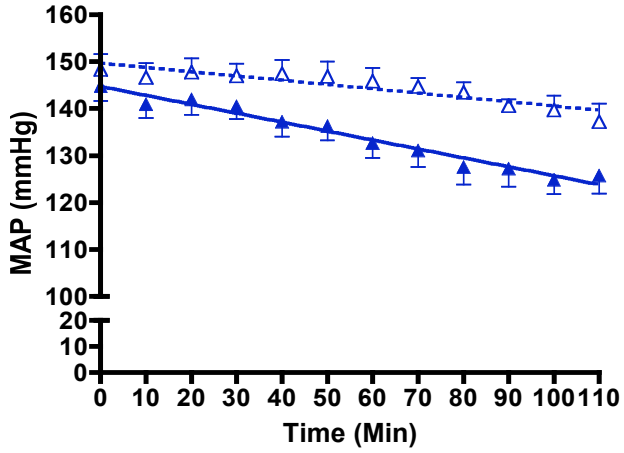
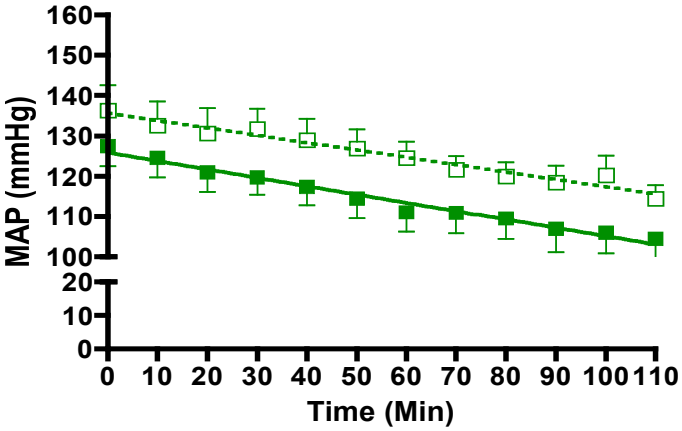


Figure 44: Mean arterial pressure (MAP) in adult males (AM: Blue) (**A**), juvenile males (JM: Green) (**B**), and adult females (AF: Red) (**C**). MAP was decreased in AM and JM with insulin suffusion (*p<0.01). Values depicted as 25th, 50th and 75th percentile bounded by the 10th and 90th percentiles.

A)



B)



C)

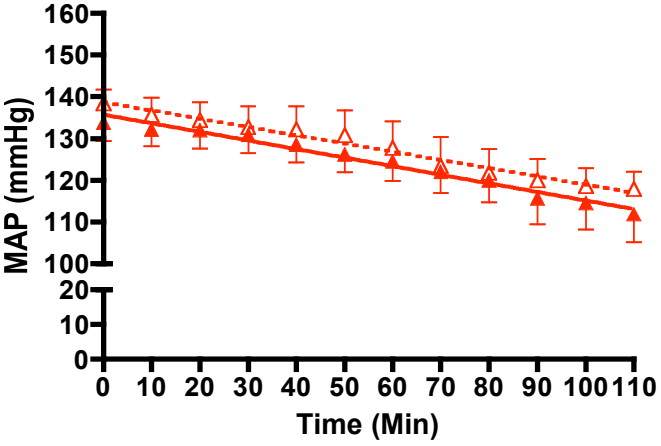


Figure 45: Mean arterial pressure (MAP) in adult males (AM: Triangles) (**A**), juvenile males (JM: Squares) (**B**), and adult females (AF: Upside-down triangles) (**C**). The controls for each group is depicted as empty symbols and its linear regression with dashed lines. Those that received insulin-treatment for each group is shown as filled symbols and solid lines for its linear regression. MAP declined at a faster rate than control in with insulin suffusion (*$p<0.01$). Data are Mean ± SEM.

CHAPTER 8: DISCUSSION OF SEXUAL DIMORPHISM IN RESPONSE TO HIGH-DOSE INSULIN

Venular Albumin Leakage

Our hypothesis had been that exposure to high-dose insulin would result in an increase exchange of macromolecules from the microvasculature of the autoperfused mesentery. In the case of the male groups, indeed the venular permeability response to insulin was an increase. Our further expectation had been that the responses would not differ with sex or age of the animals. With respect to age we found that high-dose insulin had a greater effect on venular permeability in the juvenile than on the adult males [106 vs. 62% increase for juvenile male (**Figure 10**) and adult male (**Figure 2**), respectively]. This outcome could imply a greater susceptibility of young, sexually immature animals to the negative effects of insulin, especially with respect to venular exchange function. This conclusion is in fact consistent with the clinical literature in that adolescents (and prepubescents) have increased risk of developing insulin resistance [42-44, 74, 108]. In addition, it would be worth determining the dose response relationship in the two groups (juvenile and adult males) to determine whether the curve is shifted to the left and/or the maximum responses differ between the juvenile and adult males in future studies.

Contrary to the null hypothesis, that there would be no sex differences with respect to the permeability responses, our data clearly demonstrated a complete absence of a leakage response to insulin in venules from adult

females. This outcome suggests that the venules, and perhaps other vascular elements, in adult females are protected from the effects of high dose insulin. This conclusion is consistent with recent literature showing females to not be affected by the observed in the males [107]. One suggestion, therefore, is that the protective effect may be due to estradiol in females [91-93] and/or the negative effects of testosterone [77, 106]. In fact, there have been correlations made with respect to insulin resistance in post-menopausal women and hyperandrogenism [109, 110]**.**

Also, given the higher levels of progesterone in the adult females relative to either group of males (**Figure 34**), without further study, it cannot be ruled out that either progesterone contributes to the responses and/or moderates the action of estrogen.

Total Protein and Albumin Clearance

There was no change in the total protein clearance with high-dose insulin treatment in juvenile males (**Figure 12**), although it was decreased in both groups of adult animal groups (**Figures 4 and 19**). Albumin clearance was increased following insulin exposure in the juvenile males (**Figure 14**), which was contrary to what we observed in adult males, where the albumin clearance was decreased (**Figure 6**). As with venular albumin leakage, albumin clearance was unchanged by high-dose insulin treatment in the adult females (**Figure 21**). The lack of response in the adult females can also be attributed to estrogen and/or progesterone “protection”.

Relationship Between Venular Leakage and Clearance

The responses, with respect to venular leakage and the clearance, to insulin did not go in opposite directions in the juvenile males (**Figures 10 and 12**) as observed in the adult males (**Figures 2 and 4**). Instead, there was an uncoupling of the albumin and protein clearance response with insulin suffusion in juvenile males, where the total protein clearance remained unchanged with insulin (**Figure 12**), but albumin clearance was increased (**Figure 14**). As mentioned above, we also observed this uncoupling in the adult females, as there was a decrease in the total protein clearance (**Figure 19**), but the albumin clearance was unchanged with insulin (**Figure 21**). Again, we reiterate the notion that protein clearance reflects albumin clearance and that both are a result of leakage from the venular elements of the mesenteric microvasculature is not correct. Further, the results from the three groups of data demonstrate that albumin homeostasis in this tissue must be regulated by a different mechanism(s) from the other proteins, and that the mechanism(s) remain to be elucidated. How albumin distribution in the vascular and tissue compartments is attained will not only influence the oncotic forces across the vessel wall in health and disease but further influence the transport of a multitude of materials, from ions to lipids to drugs, carried by albumin [111].

To further evaluate the differences among the three animal groups, we also measured their plasma for components that we had reason to believe may

influence the responses to high-dose insulin. These were the sex hormones, estradiol, progesterone, and testosterone, in addition to insulin and glucose. The goal was to know whether their levels differed between the control and experimental animals and whether they were altered in response short-term mesenteric treatment with regular insulin.

Plasma Estradiol and Progesterone

75 minutes of insulin exposure resulted in a significant reduction (~50%) in the levels of plasma estradiol levels in both adult (median ± SEM; Control: 24.5 ± 2.2 pg/ml vs. Insulin: 13.2 ± 2.7 pg/ml; $p<0.05$) and juvenile (Control: 28.6 ± 0.77 pg/ml vs. Insulin: 12.5 ± 1.6 pg/ml; $p<0.01$) male animal groups (**Figure 39A** and **39B,** respectively). This apparent robust change in the metabolism or clearance of estradiol from the plasma of the male animals with insulin treatment was a surprise. There was no significant difference in the plasma estradiol of adult females (**Figure 39C**).

Of note, while plasma progesterone was unchanged by insulin treatment, *per se*, in either the adult male or female groups (**Figure 40A** and **40C**, respectively), we observed that in plasma progesterone levels decreased significantly in the juvenile male rats after insulin treatment (median ± SEM; Control: 9.3 ± 1.5 ng/ml vs. Insulin: 5.1 ± 0.7 ng/ml; $p<0.05$) (**Figure 40B**). In aggregate these data could imply that some of the differences in responses of the juvenile males reflect withdrawal of an acute response to progesterone.

Plasma Testosterone

Our evaluation of the plasma testosterone levels after exogenously applied insulin, showed that it did not lead to increases in the testosterone levels in the three different animal groups (**Figure 41A-C**).

Plasma Insulin

Plasma insulin levels after 75 minutes of exogenous application of high dose insulin were shown to not change in any of the three groups of animals (**Figure 42A-C**). First and foremost this demonstrates that the responses to application of insulin to the suffusate solution initiated local, rather than global, responses to the hormone. Had the plasma levels risen it could be argued that the myriad of systemic responses (especially hypoglycemia and hypotension) lead to changes in Starling Forces and delivery of exogenous tissue factors which could alter macromolecule flux. Further, the outcome of plasma insulin remaining constant in the face of high-dose insulin is consistent with observations from peritoneal dialysis (PD) studies in animals and human patients where high concentration of insulin does not lead to hyperinsulinemia [112].

Another possibility for the “failure” in altering plasma insulin levels may be explained by the asymmetric transport properties of the microvascular wall. In a previous section (**Chapter 7**), we mentioned that previous studies have shown that large molecules flow out of the mesenteric microvessels unidirectionally, and must be taken up by the lymphatics to go back into the blood [101]. Yet insulin is a small molecule of ~6 kDa, but was insulin not taken back into the blood. The

data are more consistent with insulin, acting like the large molecules, also being taken back up into the blood via the lymph, and is transported across the blood to the interstitium unidirectionally or via transport-mediated processes [17, 25-28]. As conflicting reports exist with respect to the transport of insulin across the vascular wall (See **Chapter 1**), further research is required to delineate the mechanisms. Possible means of investigating this question are provided in the Future Studies section of the General Discussion (**Chapter 9**) of this dissertation.

Plasma Glucose

Had insulin been moving into the systemic vasculature and been acting at its target tissues in these healthy animals, we would have expected plasma glucose levels to have changed in the treated animals relative to the controls and to have decreased with time of exposure. Instead, we did not observe any differences in the plasma glucose levels in any of the three groups with insulin suffusion (**Figure 43A-C**). This result has two outcomes: First and foremost, the implication is that none of the responses to insulin are a consequence of the profound effects of hypoglycemia. Second, as with the measures in the previous section the fact that glucose levels remained unchanged during exogenous insulin administration is consistent with the fact that plasma insulin levels were also unchanged by this treatment route. Moreover, given that i.v., subcutaneous, and oral administration of insulin results in changes in both parameters, it is apparent that the mesenteric microvascular

barrier handles insulin in a manner that restricts its movement from the tissue space into the vascular space.

In the case of hyperglycemia, an acute increase in plasma oncotic pressure will occur, thereby drawing fluid into the circulation and limiting net outward flux - obviously not expected in the face of high insulin. One clinical study showed that severely obese patients with insulin resistance with normal glucose tolerance have the same degree of endothelial dysfunction as type 2 diabetic patients, suggesting insulin resistance has a more detrimental influence than hyperglycemia [113]. Thus the hyperinsulinemia that often occurs in the early phase of insulin resistance appears to be more influential in the stimulation or maintenance of the complications leading to type 2 diabetes than hyperglycemia.

Sex differences with respect to MAP

Insulin suffusion resulted in small, but significant, reduction in the overall mean systemic arterial pressure (MAP) for the adult (N=7, mean difference ± SEM; 10.3 ± 2.3 mmHg; $p<0.01$) (**Figure 44A**) and juvenile (N=6, 11.1 ± 2.9 mmHg; $p<0.01$) (**Figure 44B**) males. No change in MAP was observed in the adult females (N=7, 3.4 ± 3.0 mmHg) (**Figure 44C**). For the adult males, the rate of MAP reduction was significantly faster in the presence of insulin (mean slope ± SEM; 0.19 ± 0.03 mmHg/min; $p<0.05$) relative to the controls (0.09 ± 0.02 mmHg/min; $p<0.05$) (**Figure 45A**). The rate of change was unaffected by insulin in the juvenile males (Control: 0.18 ± 0.04 mmHg/min; Insulin: 0.21 ± 0.04

mmHg/min) (**Figure 45B**) or the adult females (Control: 0.20 ± 0.04 mmHg/min; Insulin: 0.20 ± 0.04 mmHg/min) (**Figure 45C**).

These results run contrary to a previous study where treatment with high-dose insulin resulted in hyperinsulinemia and an *increase* in the blood pressure in the males, but not the female rats [107]. Possible discrepancies may be that in the latter study, the insulin was administered subcutaneously and exposure time was much longer (5 weeks), whereas in our study, insulin was applied directly to exposed mesentery for 75 minutes. The lack of change in MAP in the female rats with insulin treatment is consistent with findings in the literature that there are no changes in blood pressure due to estradiol [90, 107].

Our results are in line with previous studies that indicate the increased microvascular permeability in metabolic syndrome patients are not due exclusively to changes hemodynamics. One study demonstrated that the increased permeability observed in metabolic syndrome patients are not always hypertensive [114]. Another study showed that pressure elevations in the mild-to-moderate range are only loosely related to transcapillary escape rate [115]. In fact, microvessel rarefaction (and reduction in flux secondary to a reduction in surface area available for exchange) has been shown in the forearm skin of hypertensive patients [116]. One study showed that transcapillary escape rate of albumin in metabolic syndrome patients with hyperinsulinemia (and hypertension) was higher than hypertensive patients [94]. Thus the increased permeability in metabolic syndrome patients is not due exclusively to changes

hemodynamics and likely illustrates changes in the microvascular barrier and/or changes in the interstitial clearance of albumin.

CHAPTER 9: GENERAL DISCUSSION AND CONCLUSION

Our primary question was whether insulin, itself, could regulate macromolecular exchange in the microvasculature. By inference, if insulin alters the exchange barrier for macromolecules it could also regulate the rate and amount of insulin that traverses from the vascular space, into which it is secreted from the pancreas, to the target tissues where it is involved in the regulation of glucose transport. The work in this dissertation demonstrates multiple means whereby insulin influenced movement of albumin. Therefore it was imperative that we determine whether the responses we observed were the direct action of insulin at the level of the microvascular exchange vasculature of the mesentery or secondary to insulin's actions at a global level.

Mechanisms for increased microvascular permeability by insulin:

There are several possible sites involved with regulation of barrier properties where insulin could interact to change microvascular permeability. The mechanisms thought to regulate permeability are: transcelluar transport via caveoli (a.k.a. vesicle) shuttling or fusion and paracelluar transport via changes in junctional protein number/position and/or via changes in endothelial cell structure via cytoskeletal contraction/relaxation. Insulin may be incurring permeability changes physically or structurally on the microvascular wall (i.e. junctional proteins, cytoskeleton) or at the level of the signaling (i.e. via caveoli, contraction, fusion).

Evidence for Insulin altering caveoli transport:

When insulin binds to its surface receptors on the target organs (skeletal muscle and adipose tissue), it initiates a cascade of molecular events involving the activation of signaling factors via phosphorylation. The bound insulin-receptor complex becomes autophosphorylated, leading to phosphorylation of the intracellular insulin receptor substrates (IRS-1 and 2). The phosphorylated IRS substrates then bind and to the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). The subsequent activation of the PI 3-kinase pathway leads to increased amino acid transport, and the metabolic functions such as glucose uptake and glycogen synthesis. Insulin receptors have been identified in vascular endothelial cells [16]. Additionally, vascular smooth muscle cells have been shown to undergo similar signaling processes upon the binding of insulin as observed in the target organs [117].

Further, Jiang et al [118] have shown that insulin signaling is defective in the microvessels of male obese hyperinsulinemic Zucker rats. Stimulation with high insulin (10^{-7}M *ex vivo*) resulted in decreased phosphorylation of insulin receptors in the microvessels despite unchanged protein levels. Association of p85 subunit to IRS protein was also decreased in obese rats compared to lean rats. These outcomes indicate that insulin signaling occurs in vascular tissues, and that in the presence of high insulin (e.g. the hyperinsulinemic animal model) there is decreased activation of the downstream signaling factors. Thus high insulin appears to suppress the signaling activities of the insulin receptor on endothelial cells.

Association of insulin signaling factors and caveoli has been observed in adipocytes [119]. Thus the defective insulin signaling response to high insulin may in turn influence changes in the caveoli transport of proteins. Caveolae are cell-surface invaginations believed to play a vital role in the regulation of transcytosis and compartmentalization of signaling proteins in endothelial cells (ECs) [119, 120]. Caveolin-1 (Cav-1), an isoform shown to be present in endothelial cells, has been identified as the main protein making up the caveolae [121]. Insulin receptors have been associated with cav-1 in the vascular endothelium [20], implying that insulin action can regulate caveoli transport. Wang et al also showed that insulin interacts with caveoli in the vascular endothelium within 10 minutes of infusion. Also, disruption of the caveoli with the cholesterol-binding agent, filipin, reduced the uptake of labeled insulin by the vascular endothelium [20]. Cav-1 knock-out (KO) mice were also shown to have increased circulatory clearance to radio-iodinated albumin [122] and developed insulin resistance [123]. Thus caveolin appears to play an important role in the regulation of insulin action, and alters macromolecule distribution between the tissue and vascular spaces (thus changing macromolecule exchange by altering permeability).

Insulin alters junctional protein organization and expression

Junctional proteins can alter microvascular permeability by having their expression either up- or down-regulated [124]. Cav-1 one has been shown to be regulate the expression of junctional proteins as it relates to microvascular

permeability [122, 125]. Cav-1 KO mice showed defects in the tight junction morphology [122]. Cav-1 Knock-down with siRNA increased lung microvascular permeability to albumin and resulted in increased width of interendothelial junctions (IEJs) at 4 days post siRNA injection, in particular in the venular endothelia [125]. The restoration of Cav-1 expression over time (8 days after post injection) resulted in normal caveolae number and closure of interendothelial junctions, as well as reduced microvascular permeability to albumin. Cav-1 down-regulation with siRNA in brain microvascular endothelium decreased protein expression of junctional proteins [occludin, zona-occludin-1 (ZO-1), VE-cadherin] and increased paracellular permeability to labeled dextran [126].

Other studies have shown that diabetic subjects have decreased microvascular junctional protein expression. In one study it was found that vascular endothelial (VE) cadherin-based adherens junctions were reduced in the retinal microvasculature in diabetic patients [127]. Impairment of tight junctions structures have also observed in diabetic retinal microvessels [30, 128]. Of interest, the metalloprotienase, MMP, isoforms -2, -9, and -14, have been associated with the breakdown of the tight junction protein, occludin, in increasing vascular permeability in diabetic retinopathy [129]. However, the aforementioned studies were performed on streptozosin-induced diabetics. Streptozosin creates a model for type 1 diabetes, and thus may not reflect the same mechanism for altered microvascular permeability found in insulin resistant or hyperinsulinemic subjects.

Therefore it appears that high-dose insulin inhibits insulin signaling in the endothelium and attenuates transcytotic transport via caveoli. The increases microvascular permeability may be due to an over-compensated response to the decreased transcellular transport, evidenced by the down-regulation of junctional protein expression and leading to increased paracellular transport.

Mechanisms for development of endothelial dysfunction (ED) by insulin

High insulin leads to increased deposition of protein in matrix over time The increased permeability of the venules to protein and the retention of the proteins in the tissue over long periods of time may lead to increased protein and lipid (via albumin) deposition in the vascular wall, altering matrix metabolic balance and leading to the formation of microangiopathic lesions [130]. The increased deposition of proteins leading to microvacular complications may predispose the endothelium and the surrounding tissue matrix for development of atherosclerosis and associated cardiovascular diseases (CVD). The association between insulin resistance and atherosclerosis has been well documented [131], and further research is needed to understand the link between these diseases.

We do not know whether it is appropriate to assume that increases in the microvascular permeability to albumin with high insulin also applied to the smaller peptide hormone, insulin itself. Thus we need to measure directly the microvascular permeability to dye-labeled insulin in perfused *ex vivo* microvessels.

Future Studies:

Measure lymphatic vessel permeability to albumin and insulin

Currently, a method for measuring accurately the permeability of the lymphatic vessels to substances from in the peritoneal fluid has not been developed. Our lab is in the process of devising and validating a technique for measuring the lymphatic wall permeability to labeled solutes, including macomolecules. Determining the effect of high insulin and/or flux of albumin on the permeability of lymphatic vessels will provide us a better picture about what is occurring and aid in obtaining understanding of microvascular exchange in the mesentery.

Measure the solute permeability (P_s) to insulin by perfusion of isolated microvessels with dye-labeled insulin.

To affirm that the increase in venular leakage for albumin also applies to insulin, we can investigate the permeability properties to albumin and to insulin in isolated microvessels *ex vivo* using the techniques available in our laboratory. Although attempts have been made using both *in vivo* and *in vitro* methods to measure the microvascular permeability to insulin and determine its mode of transport, the results have been conflicting. Some studies have concluded that insulin diffuses freely across the microvascular wall [21-24]. Others have shown insulin to be transported via a receptor-mediated pathway [25-28, 132]. Thus far, no studies have been performed in *in situ* perfused single microvessels, to directly measure the rate of insulin passage or provide any clue as to the route of

transport. The method utilized in our lab [62, 64, 65, 67, 68, 83, 84] will allow us to directly measure the flux of dye-labeled molecules across the microvessel wall.

Measure albumin leakage response to insulin after 75 minutes

Although the technique we used in this dissertation does not allow us to measure the albumin leakage past 75 minutes due to the deterioration of the preparation, we can measure this in isolated perfused venules where conditions are controlled. This will show us the response observed with insulin was after the preparation was fully equilibrated and stabilized. Also, **Figure 3** shows that leak index observed in insulin treated group follows the leak index responses for the control group for approximately 30 minutes before diverging in the responses.

Determine dose-dependent action of insulin

To determine the action of insulin, we can use different concentrations of insulin to suffuse the mesenteric venules and measure the albumin leakage response in the adult male, juvenile male, and adult female rats. We would expect to see increases in the venular albumin leakage with increasing insulin concentrations in the males.

Experimental Problems/Limitations of Methods:

Venules are not the only exchange vessels

The common assumption has been that the primary, if not the sole site for macromolecular exchange in the microvascular network is across the venular vessels. The remaining components, capillaries and arterioles, are assigned functions of volume flux for capillaries and regulation of blood flow (hence regulation of oxygen transport) for the arterioles. In fact there are now several studies demonstrating that macromolecules as large as albumin can flux across not only venules but capillaries [133] and arterioles in the porcine heart [65] and skeletal muscle of rats [64, 84] and mice [63]. Recent studies in this laboratory and in collaboration with the Sarelius laboratory at University of Rochester have demonstrated appreciable flux from both arterioles and venules in situ and in isolated microvessels [63]. The consequence of flux occurring from additional elements of the microvascular network is that there could easily be "uncoupling" of the venular leak data from the clearance data if the distributions of sites are altered by insulin directly or indirectly. Thus the contribution of arterioles and capillaries to the permeability must also be taken into account when evaluating overall microvascular permeability.

Mesenteric venules are different from the other venules in body

The mesenteric microvasculature is an excellent model for investigating the characteristics of permeability in the intact animal. The mesentery is easily accessible through an incision in the abdomen that can be draped over the

microscope pillar for visualization under constant suffusion with physiological buffer that remains viable for several hours. Much of the knowledge of mammalian microvascular functions, as they relates to water and solute permeability, has been derived from experiments performed on the *in situ*, mesenteric preparation [67, 68, 134, 135]. The mesenteric microvessels have also shown similar physiological responses to vasoactive agents as in other microvessel beds [4, 65]. Thus information acquired from this model will provide an approximate basis for the microvascular exchange function. Although differences in physiological function have been observed among different microvascular beds, the information gained from the mesentery in this dissertation will be relevant for clinical treatment the function of insulin at the level of the peritoneal interstitium and mesenteric vasculature, as it is the site of insulin infusion for peritoneal dialysis for many diabetic patients.

Effect of superfusing the mesentery with saline solution:

Barber et al has shown that superfusion with saline solution of the male rat mesenteric tissue matrix depletes the albumin concentration in the tissue [136]. Thus the decreased albumin concentration of the tissue with superfusion may have affected our study, in that the responses may not be physiologic. For future reference, we will be adding a certain concentration of albumin in our BBS to reproduce more accurately physiologic conditions.

Lack of Juvenile Female Rat Data

Juvenile female rats were not used in the studies due to difficulty in finding viable venules to use for assessment in the venular albumin leakage studies. The juvenile females at this age (<35 days) do not appear to have developed venules that are detectable in the mesentery windows, and thus could not be used for the experiments. On the other hand, it is expected that the clearance studies will be conducted on the juvenile females as it is also necessary to know whether insulin a) alters protein clearance in the young females and b) whether the amount or kinetics differ amongst the groups.

Exposure of venule to epi-illumination

To minimize light exposure to tissues, each measure of albumin leakage was performed during limited (<5 seconds) exposure to epi-illumination, controlled by a manual shutter. It was essential for the exposure time to be brief to prevent bleaching of the fluorescent dye, camera, and production of oxidants. Studies of photobleaching an influence of dye which photobleach (e.g. fluorescein isothiocyanide; FITC) on microvascular permeability in this preparation have been conducted in the laboratory [67]. To prevent the production of oxidants due to light exposure, we used Alexa-Fluor dyes, which has been shown to have minimal effects on oxidant production and have little effect on the physicochemical properties of the proteins to which it is bound [66].

The lack of detection of leakage in z-plane

Our calculation of the leak index only takes into account the x-y plane of what is recorded on our video screen. There is a possibility that increases in the fluorescence intensity (I) of the vessel (I_v) is not accompanied by increases in the tissue fluorescence intensity (I_t). This would lead to the I_v being quantitatively greater than I_t, resulting in a decrease the leak index (I_t/I_v), although in actuality, it has increased (qualitatively) in leakage.

Summary and Conclusions:

The data from this dissertation demonstrate that not only did insulin result in changes in macromolecule exchange in the intact, autoperfused mesenteric microvasculature, but that these changes were varied depending on the sex and the sexual maturity of the animal. Notwithstanding that these studies were acute (minutes to hour) in nature, they demonstrated the need for additional work and consideration of not only the sex of the individual, but their age. In addition the work in this dissertation demonstrated clear differences in the contribution of genomic (e.g. juvenile vs. adult) and sexual (e.g. adult male vs. female) components in the handling of macromolecule distribution between the blood and metabolizing tissue that are sensitive to the presence of insulin. Finally, this work illustrates that more than one mechanism is involved to bring living creatures into volume homeostasis in the presence and absence of insulin, and are themselves influenced by age and sex.

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VITA

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