Mechanisms of induced cardiovascular dysfunctions within diabetics.

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**Introduction: Arginase’s relation to collagen and diabetes.**

Disease states are extremely complicated physiological processes that can arise from a variety of disrupted processes within the body. Small perturbations in normal cell function can lead to global physiological damages, especially as time progresses without addressing the problem. Diabetes is no different. While the direct effect of destroyed β cells within the pancreas is the destruction of the body’s ability to produce insulin and thus regulate blood sugar levels, the ensuing complications fill books and affect some researchers entire life’s work. It has been known that diabetics have high blood pressure and one of the root causes of this results from capillariés and blood vessel’s inability to respond to dilatory stimuli. Surgical removal of these vessels from diabetics reveals the increased rigidity of these vessels compared to non-diabetics. This is a classic disease state condition where disrupted functioning of one system or protein has devastating secondary effects that branch throughout various organ systems of the body. Research has proposed that the cause of the increased vessel rigidity seen in diabetics may be due to the activation of the protein angiotensin and subsequent activation of arginase. Activation of these proteins is thought to lead to increased levels of collagen deposition around blood vessels in both the vascular and renal systems. This increased deposition disrupts blood vessels ability to dilate and thus prevents blood vessels from effectively controlling blood pressure\(^1\). Subsequent, long-term damage in the kidney and vascular system is believed to occur.

*Collagen deposition and its effect upon blood vessels and human physiology:* Collagen is the most abundant fibrous protein within the human body; in fact, it is the most abundant
protein within the human body\(^3\). Its sheer concentration throughout the body is testament to its physiological significance. Skin, bone, tendons, cartilage, various connective tissues and even teeth are all partly made up of collagen. Therefore, the mechanisms that control the production of collagen need to be regulated thoroughly. Its function varies from that of globular enzymes that speed the creation of physiological molecules and products. Collagen acts as connective fibers between cells and essentially acts to hold cells and tissue in place. It is believed that 80-90% of collagen within the body is fibrillar collagen\(^4\). In the context of the vascular system, this fibrillar collagen acts as a connective tissue that surrounds blood vessels and influences the blood vessel’s ability to dilate or constrict. By altering the amount of collagen that surrounds a blood vessel, collagen fibers will determine how effectively the blood vessel will respond to varying vasodilators such as nitric oxide.

Various classes of collagen are found within the body, and each localizes within a particular physiological environment. Fibrillar collagen is classified into four types: Type I, Type II, Type III, and Type IV. Despite different functions, they are all very similar in structure. In addition to fibrillar collagen, fibril associated collagen, sheet forming/anchoring collagen, transmembrane collagen, and host defense collagen are other classes of collagens with distinct functions. How the collagen fibers interact with each other and the extracellular matrices around their cells of origin is the basis for their varied classifications.

Within the fibrillar collagen classes, collagen type can be varied via three general mechanisms: first, by altering the number or length of the triple helical sections of distinct fibers; second, by changing the components that interrupt or interact with the
fibers as the segments progress; lastly, by altering the covalent modifications that line the \(\alpha\)-chains. By altering these varying components, the distinct types of collagens (such as Type I and III) are produced. Type I collagen is found in tendons and is the most abundant of all collagens. It can be found in skin, tendon, bone, ligaments and interstitial tissues. Type III collagen is the most important collagen concerning the cardiovascular system due to it being the predominate collagen type located around blood vessels\(^5\). Type I collagen often associates with Type III collagen to connect blood vessels to varying tissues. This association helps to facilitate diffusion of blood into tissues by creating an established and firmly held network of cells adjacent to blood vessels.

All types of collagen are extracellular proteins that extend from cells and reach into the extracellular matrix (ECM). Cells secrete the components of collagen which are then assembled outside of the cell. The amount of collagen that extends from a cell and then connects with the surrounding matrix greatly influences whether or not a cell is capable of movement or cellular expansion. In accordance with this, the amount of collagen found around the epithelial cells of the cardiovascular system greatly influences a vessel’s ability to dilate or contract. A noted increase in collagen deposition around any vascular cell, and thus around a blood vessel, would decrease that entities ability to move or respond to dilatory/motility stimuli.

*The extracellular environment and its relation to collagen:* Collagen is assembled in the extracellular environment. It extends from cells and connects cells to the network of fibers and proteins that localize outside of cells. One of the major targets of collagen within the extracellular matrix is the basal lamina. The basal lamina is a mesh like
weaving of extracellular matrix components that consists mainly of Type IV collagen, laminins, perlecan, and entactin (Figure 1).

![Molecular structure of a basal lamina](image)

Figure 1: Diagram of the basal lamina. Note the different molecules that constitute its structure.

Type IV collagen is an interesting collagen type in that it is both a rodlike protein (the typical form of collagen) and a globular protein. By having rod-like and globular sections, Type IV collagen is capable of branching and forming the backbone of the basal lamina. It essentially makes a web-like structure that allows various proteins extending from cells to anchor. In doing so, cells adhere to the extracellular environment and are prevented from moving. Once this web is formed, numerous other proteins associate with the collagen network and provide more complex and specific attachment sites for anchoring proteins that extend from cells. Laminins are one of these and are the main adhesion molecule type within the basal lamina. They resemble an anchor with a small
hook at the base of their structure; a large bar crosses the main chain of the protein and increases the protein’s surface area for binding. The anchor-like structure of the protein allows it to adhere to the backbone of the basal lamina, Type IV collagen, and also allows it to bind to integrins and collagen from surrounding cells. Perlecan cross-links layers of the extracellular matrix and may also bind to cell surface molecules. It is important in maintaining the three dimensional meshwork that that defines the basal lamina. Entactin plays a similar role in that it connects all of the components of the basal lamina and helps to interconnect all of the ECM components.

Integrins and collagen proteins associate with lipid rafts in the cell membrane in order to increase a cell’s ability to adhere to the ECM. Lipid rafts are hardened portions of the cell membrane and can act as superior anchor points (versus the normal lipid membrane); the rigidity of lipid rafts occurs due to increased concentrations of cholesterol and sphingolipids within the rafts. The increased concentration of sphingolipids within the raft domains allows for a higher concentration of mostly to fully saturated lipids. Saturated lipids lack the kinks found in unsaturated lipids, which allows for greater lipid membrane packing and increased hydrophobic interactions. This creates more rigid lipid domain that has been compared to a raft (hence the name) floating in the fluid membrane.

Conceptually, lipid rafts can be understood in terms of an oil versus butter analogy. Imagine trying to pull a stick (integrin/collagen fiber) through oil (unsaturated lipids) versus butter (saturated lipid). The highly saturated butter has a more solid, rigid structure than the unsaturated oil. Thus, it makes sense that anchor proteins would localize to the more rigid, saturated areas of the cell membrane.
Within the vascular system, vascular endothelial cells constitute the main cell type that line and surround blood vessels. The amount of collagen deposition around these endothelial cells helps to determine how effectively the blood vessels can respond to various stimuli (such as nitric oxide). These stimuli range from contractionary to dilatory, and vessels must be able to respond all types in order to maintain healthy blood pressure. In response to dilatory signals, blood vessels increase their internal diameter and allow greater volume of blood to flow through a given cross-section in a unit time. In doing so, blood pressure is effectively decreased as a greater throughput (due to the increased blood vessel diameter) is inversely related to pressure.

*The importance of collagen’s structure:* Collagen is a rod-shaped molecule that is approximately 3,000 Å long and 15 Å wide. From the difference in its length versus width, its ability to cover long distances becomes apparent. The protein comprises nearly 1,000 amino acids, but despite its size, the amino acid sequence is not very diverse. Nearly every third amino acid is a glycine residue; proline and hydroxyproline residues appear with nearly the same frequency as glycine. The most common sequence repeat within the amino acid chain is glycine–proline–hydroxyproline. Lysine and hydroxylysine may also be found in place of the proline/hydroxyproline pairs, but this is less common. This may seem like an odd and simplistic sequence for a protein, but this short repeat creates a unique structure that gives collagen its interesting physiological characteristics.
In proline, the amino acid’s nitrogen and α-carbon are linked in a ring like structure by three methylene (-CH$_2$-) groups. This structure constricts the movement of the amino acid chain and essentially causes a kink to occur within the peptide sequence. Hydroxyproline is similar except that it has a hydroxyl group on the central methylene carbon atom of the proline side chain. The sequence repeat of glycine-proline-hydroxyproline causes a tight spiral to form due to the repeated kinks formed with each proline/hydroxyproline pair. These three residues create one turn of the helix. This spiral helix is unique and has been aptly named the collagen helix. No hydrogen bonding occurs within the tightly packed collagen helix due to the limited spatial constraints created by the repeated, very tight proline turn. However, stabilization of the helix does occur due to steric interactions between adjacent prolines and hydroxyprolines. The large pyrrolidine rings on these residues create significant contacts between adjacent residues, and these repeated contacts occur throughout the strand.

To form collagen strands, three collagen helices wrap around one another (Fig 2). Each helix can essentially be considered a subunit, and the association of three helices forms the quaternary structure of the protein. However, collagen can be either homotrimeric or heterotrimeric,
so the different sequences of the collagen strands determine which type of collagen is formed.

The space between the helices is very small because of their tight association. Thus, the necessity for a glycine residue every third residue now becomes logical: it is the only residue capable of fitting into such a small space. The hydroxyprolines, with their hydroxyl group are capable of hydrogen bonding to one another on the exterior surface of the superhelical cord structure, and this further increases the tensile strength of the protein.

In order to construct collagen fibers and strands, cells need the precursor amino acids to assemble these proteins. Clearly, the most essential molecule for collagen assembly is proline. Proline is not an essential amino acid because the body readily makes it from molecules already present within our bodies. One means of making this amino acid involves arginase and the breakdown of the amino acid L-arginine.

*Arginase structure and the protein’s relation to collagen:* Arginase is an essential enzyme in mammalian species because of its role in nitrogen removal via the urea cycle. Arginase is a key enzyme in this cycle because its converts L-arginine into L-ornithine and urea. Via L-ornithine, arginase is also indirectly involved in the formation of proline and polyamines, two molecules essential for collagen deposition and cell proliferation, respectively.

Arginase comes in two isoforms, and a distinction between the two is necessary in understanding arginase’s role within diabetes. Named arginase I and arginase II, the two isoforms are encoded by different genes, and while performing the same reaction, are
localized in different organs and tissues throughout the body where they regulate varied processes.

Due to its role as the body’s filter, the liver naturally accumulates high levels of nitrogen and nitrogen containing amino acids that need to broken down into water soluble molecules that can be effectively flushed from the body. Not surprisingly, given its role in nitrogen removal, large amounts of arginase are found in the liver to help complete the conversion of nitrogenous compounds into urea, which is effectively transported out of the body. Arginase I predominates in the liver and is a cytosolic protein. Small amounts of arginase I may also be found in some epithelial cells and could have a minor effect on collagen deposition in these cells.

Arginase II is a mitochondrial isoform of the protein. It localizes mainly in extrahepatic cells, but low levels of the protein have been found within the liver. The expression and activity of either isoform in extrahepatic cells provide the most insight into how arginase functions and causes dysfunction within diabetic patients. However, before addressing how arginase causes damage in diabetes, it is important to understand how arginase operates.

*The mechanism of arginase:* Arginase is a metalloenzyme that employs a binuclear manganese cluster within its active site. It is a homo trimeric protein and each subunit has its own manganese bi-nuclear cluster. Figure 3 reveals the symmetry of the enzyme. It can also be seen that the Mn\(^{2+}\) - Mn\(^{2+}\) cluster is positioned on a \(\beta\)-sheet that orients one of the manganese atoms farther into the protein’s three-dimensional structure\(^2\). As Figure 4 shows, the manganese atoms are labeled A and B. Manganese A is coordinated by Asp-
124, Asp-128, Asp-232 and His-101. His-126, Asp-124, Asp-232, Asp-234 and the bridging hydroxide coordinated manganese B. A hydroxide ion lies in the space between the two manganese ion and bridges the two metals. Manganese-A assumes a square pyramidal geometry, while manganese-B assumes an octahedral geometry with Asp-234 acting as a “monodentate bridging ligand”\(^2\). Asp-124 is a “syn-syn bidentate bridging ligand”\(^2\) that acts as another bridge between the metal ions. This structure allows the bridging hydroxide to be positioned favorably for nucleophilic attack on the L-arginine substrate.

![Figure 3: Three dimensional ribbon model of the arginase protein](image)

![Figure 4: Binuclear manganese active site found within arginase\(^2\).](image)
As Figure 5 shows, the hydroxide ion attacks the carbon atom central to arginine’s guanidino group. Collapse of the tetrahedral intermediate breaks the carbon nitrogen bond to the rest of the arginine molecule; L-ornithine is thus formed and leaves the active site. The previously bridging hydroxide ion (now a keto group on the urea molecule) is released by the manganese ions freeing the urea from the active site. Water then enters the active site where it replaces the previously bridging hydroxide and is subsequently deprotonated to restore catalytic function. In this manner, arginase converts L-arginine, with its high concentration of side chain nitrogen, into urea and ornithine. As mentioned earlier, urea is a small, soluble molecule that is excreted from the body. The remaining carbon chain of L-ornithine is retained within the body to be recycled in various physiological processes. Thus, the arginase reaction not only prevents ammonia toxicity, but also creates the
precursor molecules to several biologically essential compounds. The protein is thus multifunctional, but when its function is altered as it is in many disease states, its activity can have very damaging effects.

As shown in Figure 7, L-ornithine is the precursor molecule to both L-proline and polyamines. On the biosynthetic route to proline, ornithine aminotransferase (OAT) catalyzes the reaction that creates L-pyrroline-5-carboxylate which can then be converted into L-proline. Via L-ornithine, it is apparent how increased arginase activity can lead to increased collagen deposition. If arginase activity is increased, then L-ornithine concentrations will also be increased, leading to elevated amounts of proline, the key precursor to collagen. Thus, arginase activity stokes the furnaces of the collagen building machine, but why in diabetics is arginase upregulated?
Angiotension regulation and its effects in diabetics: Within diabetics, a marked increase in angiotensin II has been noted, and angiotensin II has been implicated in many complications that are common to diabetic patients, especially complications related to hypertension and insulin intolerance. Angiotensin I is initially synthesized as the zymogen, angiotensinogen. Angiotensinogen has a primary structure of 452 amino acids, and it is synthesized in the liver, heart, blood vessels, kidneys, and adipose tissue. After its biosynthesis, the protein is released into the circulatory system, where the highly specific protease renin converts it into angiotensin I (Ang I). Ang I is then converted into angiotensin II by angiotensin-converting enzyme. Individuals with Type II diabetes are often obese and have large deposits of adipose tissue (fat cells) that produce highly elevated levels of the angiotensinogen. The initiation of diabetes within these individuals is largely believed to occur due to the increased activity of angiotensin II that results from increased angiotensinogen production.

Figure 8: Angiotensin production pathway and its varying cell receptor targets.
Elevated angiotensin II production occurs when excessive amounts of adipose tissue experience constant hyperglycemic conditions. The induced stress (caused by constant hyperglycemic conditions and the ensuing saturation of adipose tissue with glucose) causes inflammation of adipose tissue; in response, these stressed cells produce abnormally high levels of angiotensin. The elevated production of this hormone by adipose tissue initiates two distinct conditions that are equally harmful. Insulin signaling fails due to a decrease in total insulin production (via pancreatic \( \beta \)-cell cell death) which is compounded by the development of insulin intolerance.

Angiotensin II does not physically attack the \( \beta \)-cells of the pancreas, but instead initiates damage via an adaptive method of energy storage that occurs in obese patients. Since obese individuals already have huge stores of adipose tissue, their body attempts to prevent further storage of energy within this tissue. Angiotensin II works to accomplish this by preventing adipocyte differentiation\(^9\): upon an influx of excess calories after a large meal, the inhibitory effects of angiotensin II prevent pluripotent fat cells from differentiating into adipose cells. Thus, influxes of glucose or other calories cannot be properly stored in adipose cells. Instead, the glucose and glycogen molecules are held within the body and circulate until another location that can store the excess energy is found. Consequently, excess calories begin to accumulate in abnormal cell types within the liver, pancreas, muscles and other tissues. When large amounts of fat are deposited in the pancreas, the \( \beta \)-cells that produce insulin stop functioning correctly, and damage ensues; this phenomenon is known as lipotoxicity and is believed to be one of the cause
of Type II diabetes. The body thus begins to produce less insulin due to the pancreatic damage.

In addition to a decrease in total insulin production, the body’s cells become less sensitive to insulin signaling. This insensitivity may occur via decreased insulin cell surface receptor concentrations or via increased cross-talk between the angiotensin AT₁ receptor and insulin receptors. Thus, due to decreased insulin production and increased insulin insensitivity, insulin signaling is virtually abolished in obese individuals leading to Type II diabetes.

It probably seems slightly paradoxical that the creation of fat cells would prevent the formation of diabetes, especially when Type II diabetes is initiated by obesity in the first place. However, the differentiation and development of fat cells must be taken in context. Adipose tissue normally acts as a storage site for excess calories. With improper functioning of these cells, excess calories will accumulate at sites not normally intended.

Figure 9: ACE inhibitor function within the body. Notice the increase in preadipocyte differentiation when ACE inhibitors are used."
for energy storage, like the pancreas where lipotoxicity occurs. To help combat this condition which starts with the increased influx of angiotensinogen, recent drugs have targeted the angiotensin converting enzyme (ACE). These drugs are known as ACE inhibitors and prevent the transformation of Ang I into Ang II. This should prevent the lipotoxic effects of Ang II and restore normal insulin signaling. Preadipocytes (adipocyte pluripotent cells) will again differentiate and become fat cells that can store elevated levels of food calories normally⁹.

If these drugs are not used, pancreatic β-cell damage and an increase in angiotensin II activity will affect a variety of intracellular processes. If both insulin and angiotensin II (Ang II) are bound to receptors on the same cell, intracellular crosstalk between receptor-activated signaling molecules occurs¹⁰. Ang II binding causes intracellular production of TGF-β and plasminogen activator inhibitor (PAI-1)¹⁰. In normal cells, one of insulin’s major secondary messengers is inositol 3-phosphatidyl kinase (PI3K)¹⁰. This kinase activates endothelial nitric oxide synthase (eNOS), which produces the visodilator nitric oxide in vascular endothelial cells. PAI-1 decreases PI3K’s

![Figure 10: Cross talk mechanism between angiotensin II and insulin via downstream messengers from their respective receptors](image-url)
ability to phosphorylate eNOS and thus prevents eNOS activation, leading to a drop in nitric oxide levels.

Nitric oxide is of extreme importance to vascular endothelial cells due to its ability to act as a major inducer of vasodilation. Via vasodilation, blood vessels are able to expand and allow increased blood flow. Blood pressure can accordingly be reduced as blood vessel diameter and blood throughput increase accordingly.

eNOS produces nitric oxide from L-arginine (Figure 11). When Ang II induced PAI-1 inhibits PI3K, the ability of the eNOS to produce NO from L-arginine is greatly reduced and L-arginine pools increase (Fig 7). In response to elevated arginine levels, arginase activity is upregulated which sets the stage for increased proline and collagen production.

In conclusion, much of the damage in hypertensive diabetics results from improper vasomotor control of blood vessels that results when vascular remodeling occurs and when the production of vasodilators is altered. In diabetics, this vascular remodeling and the decrease in vasodilators is caused via a variety of mechanisms, but angiotensin II is believed to be the main culprit. As we have seen in obese individuals,
angiotensin II levels are increased, which reduces eNOS activity. Nitric oxide production in vascular endothelial cells is greatly reduced, and the effects of one of the main vasodilators is greatly hampered. Simultaneously, L-arginine levels and arginase activity increase which leads to the increase in collagen deposition as vascular remodeling occurs. This two fold attack greatly affects the circulatory system’s ability to regulate blood pressure, and the hypertensive state observed in diabetics ensues.

**Experimentally determined arginase induced vascular damage within diabetic model.**

To determine the effects of diabetes-induced arginase damage upon the vascular system, a mouse model was employed to mimic the conditions observed within diabetic patients. This lab attacked the problem of arginase upregulation via two approaches. The first approach to control arginase upregulation is through drug administration. The second approach involves genetic regulation where the arginase II gene is completely knocked out in all mice (except the wild type control) while other mice have varying degrees of arginase I knockouts. Using both approaches in concert with varying methods of diabetes induction, the laboratory hoped to elucidate arginase’s role in vascular damage in the context of diabetic cardiovascular disease.

**Experimental Methodology**

*Rat lines:* Three male Sprague-Dawley rat lines (240-265g) were made diabetic with streptozotocin (STZ) treatment. One Sprague-Dawley control line was also used. Controls were received vehicle only treatment. Two of the three diabetic lines received L-
McCutcheon (50mg/kg/per day, orally) or simvastatin (5mg/kg/per day subcutaneously, in phospho-saline buffer) simultaneously with STZ. Eight weeks after diabetes diagnosis (controls in an according time span), rats were sacrificed. Liver, aorta and heart were harvested. The aorta tended to be used immediately after sacrifice to ensure accurate dilatory response.

**Mouse lines:** Four week mouse models (described below) were used within the genetic control study. Four week mice from each line were sacrificed and their liver, heart and aorta were isolated and preserved by liquid nitrogen flash freezing until use.

**Measuring dilatory response:** Vessel’s dilatory abilities were measured using a wire myograph. The aorta was opened and rung over two force probes that measure the decrease in pressure as increased dilation occurs. Contraction was initially induced with thromboxane A2 analog U46619 at a final concentration of $6 \times 10^{-5}$ M; subsequent administration of ACh in final concentrations of $1 \times 10^{-11}$ M, $1 \times 10^{-10}$ M, $1 \times 10^{-9}$ M, $1 \times 10^{-8}$ M, $1 \times 10^{-7}$ M, $1 \times 10^{-6}$ M, and $1 \times 10^{-5}$ M determined a vessel’s ability to dilate in response to vasodilators. The largest percent change in dilation is expected to occur around addition of $1 \times 10^{-7}$ M ACh (final concentration). Readouts were recorded on a linked computer.

**Arginase activity determination:** To determine arginase activity, urea content within the tissues was determined. Tissues were frozen in liquid nitrogen, pulverized, and combined (1:4 wt:vol) with ice cold lysis buffer (50mmol/L Tris-HCl, 0.1 mmol/L EDTA and EGTA, pH 7.5) containing proteases. Samples were then homogenized on ice. Samples were centrifuged at 14,000 g for 20 minutes and the supernatant was removed for enzyme
assay. Urea concentrations within the various organs were measured using a UV-Vis spectrophotometer set to measure wavelength at 285 nm.

*Collagen staining:* Heart samples were sliced and placed upon paraffin slides. Collagen within the tissue was stained using Massons Trichome agent. The samples were deparaffinized and rehydrated via subsequent washings with 100%, 95% and 70% alcohol. Samples were then washed in distilled H2O and stained in Weigert’s iron hematoxyling solution (1g hematoxyling:100mL 95% alcohol). After the initial stain, samples were washed in warm tap water for 10 minutes followed by a quick rinse in distilled water. The second stain was performed using Beibrich scarlet acid fuchsin solution (Biebrich solution [1% aqueous 90 mL], acid fuschin [1% aqueous 10 mL], and glacial acetic acid [1 mL]) for 15 minutes. The samples were washed post staining with distilled water. Differentiation was then performed using phosphomolybdic-phosphotungstic acid solution (5% phosphomolybdic acid [25 mL], 5% phosphotungstic [25 mL]) for 15 minutes. Samples were then immediately added to aniline blue solution and stained for 10 minutes, followed by a rinse in distilled water. Dehydration was quickly performed using 95% ethyl alcohol; samples were cleared with subsequent xylene washes. Slides were then mounted with resinous mounting medium. Upon staining, collagen fibers appeared purplish blue. Stained samples were then observed under a digital microscope. Sample pictures were taken as physical displays of collagen deposition.

*Methodology discussed:* Diabetes was induced within the mice using streptozotocin (STZ). STZ attacks and destroys the β-cells of the pancreas. This eliminates insulin
production in STZ-treated mice and Type-I like diabetic conditions develop over approximately four weeks. Four and eight week rat and mouse models were used in these experiments. Obviously, more pronounced symptoms are observed in the 8 week mice as untreated diabetes has a longer time to attack varying systems.

A blood vessel’s ability to respond to the vasodilatory stimulating chemical acetylcholine is indicative of blood vessel health. Acetylcholine induces nitric oxide production within the cell and accordingly should induce vasodilation. If increased collagen deposition has occurred around a blood vessel or if eNOS expression has been downregulated, decreased levels of dilation should be noted verses control, non diabetic animals. If decreased levels of dilation are correlated with increased arginase expression levels, this will suggest a role for arginase in vascular damage. Using the eight week rat aorta and the wire myograph, the ability of the rat aorta to respond to vasodilatory signals was determined.

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The control wildtype was a normal mouse with no genetic or health modifications. The AII -/- AI +/- control had its arginase II (AII) genes knocked out, but it had fully intact arginase I (AI) genes. The AII +/- AI +/- control had its arginase II genes fully knocked and one of its arginase I alleles knocked out. An AII/- AI -/- animal cannot be constructed as this is lethal. All diabetic animals were given diabetes via STZ administration. The diabetic wildtype had no genetic modifications. The diabetic AII -/-
AI+/+ had its arginase II genes fully knocked out but had fully intact arginase I genes. The diabetic AII -/- AI +/- animal had its arginase II genes fully knocked out along with one of its arginase I alleles. Using the knockouts in diabetic and control animals, it was hoped that the effects of arginase expression could be compared across the models for a more thorough understanding of the protein’s role in collagen deposition.

Results and Observations

As shown in Figure 12, control animals without diabetes responded most effectively to ACh induced NO vasodilation. The diabetic animal’s aorta could only relax approximately 20% in response to NO production. Administration of simvastatin or L-citrulline to diabetic animals restored most of the aorta’s ability to respond to NO production. Simvastatin was chosen because of its role in treatment of hypertensive diabetics. L-citrulline is believed to be an allosteric inhibitor of arginase, but the L-Citrulline treatment was chosen initially as more of a curiosity. It was known that L-citrulline is a precursor to L-arginine (Figure 7), as well as a product of the eNOS reaction (Figure 11), so its selection was not completely random, but its inhibitory effects were not suspected. The significant effects related to arginase activity prompted further testing within our lab.
To try and determine why the decreased response to vasodilatory stimuli occurs in the diabetic animals, heart slices were stained for collagen deposition. The collagen is stained blue/purple within the images. As Figure 13 and 14 show, the largest deposits of collagen occur within the diabetic animal (Figure 13 B and E). The blood vessel is surrounded by collagen and excess collagen extends deep into the cardiac tissue (Figure 13E). As shown in Figure 14, the diabetic animal has the largest surface area ratio of collagen. Inhibition of vessel dilation within diabetics seems logical due to increased collagen deposition. It also appears that treatment of diabetic animals with simvastatin reduces collagen around blood vessels. This decrease in collagen deposition in the drug treated diabetic animals is probably responsible for their increased dilatory response.
Figure 13: Heart cross sections stained for collagen deposition (blue). The circular units are blood vessels within the heart. Notice the deep blue/purple color of collagen deposition. A/D: Control. B/E: Diabetic. C/F: Diabetic with simvastatin treatment.

Figure 14: Surface area ratio of collagen deposition around blood vessels of control, diabetic, and diabetic simvastatin treated models.
In order to determine if there was a relationship between collagen amounts and arginase activity, levels of arginase activity were normalized to the levels observed in wild type control animals. In both the vascular and hepatic cells of diabetic animals, arginase expression is elevated. In the vasculature, it is increased to 150% of the control value while it is increased to 200% in the liver (Figure 15). The increase in arginase expression within diabetics correlates with the decrease in the blood vessel’s ability to respond to nitric oxide production. Also, note the decreased level of diabetic arginase expression in the diabetic animals treated with simvastatin or L-citrulline. Clearly, there is an inverse relationship between arginase activity and vessel dilatory ability, so that increased arginase activity may lead to a possible decrease in vessel dilation functionality.

Figure 15: Relative arginase expression levels within blood vessels (vascular) and liver samples (hepatic).
As Figure 16 shows, vessel from the wildtype control mouse relaxed to approximately 60% of the maximum upon ACh stimulation. The control AII-/- AI+-/ actually dilated to a greater extent than did the wildtype control. The wildtype diabetic mouse was only able to respond approximately 25%. The KO of the AII-/- AI+-/- alleles appears to restore some of the vasodilatory capabilities of the vascular system in the diabetic animal (Figure 16, red filled-diamonds).

To determine if collagen deposition was responsible for the results above, heart cross sections were stained for collagen. As Figure 17 shows, the wildtype diabetic animal had the largest deposit of collagen surrounding its blood vessels.
It should be noted that some collagen is needed around all blood vessels to ensure proper positioning and anchoring within an organ. This is the reason for the small deposits of collagen around some of these blood vessels. However, the most significant aspect of these images is the increased level of collagen deposition noted in the diabetic models with the fewest arginase knock outs. The wildtype diabetic model has by far the most collagen deposition surrounding the observed blood vessel. If this can be correlated with increased arginase activity, the cases for arginase’s role in vascular damage will become more complete.

To determine arginase activity within the genetic mouse models, aortic arginase activity was assayed. All non-diabetic controls had roughly similar levels of arginase activity, but as Figure 18 shows, the diabetic wild type was shown to have increased arginase activity versus the controls. Knock out of the AII gene lead to a slight decrease in arginase activity within the aorta. However, the partial KO of AI significantly affected arginase activity within the aorta, suggesting that AI activity is particularly important within the vascular system.
Discussion: These experiments reveal several key points. In wild type diabetic rat and mouse models, arginase activity is elevated. Blood vessels with the most decreased vasodilatory capability also have the most significant increases in arginase activity. Moreover, increased levels of arginase activity correlate with an increase in collagen deposition surrounding blood vessels. This increase in arginase activity followed by increased collagen deposition is likely responsible for the decreased ability of blood vessel to dilate. In both the drug and genetic studies, when arginase expression was lowered, vasodilatory function was returned to near control levels and collagen synthesis around blood vessels mirrored control levels.
It is known that an increase in arginase activity causes an increase in L-proline concentration within cells. L-proline is a key precursor to collagen due to collagen’s considerable content of proline and hydroxyproline. If arginase is upregulated, an increase in L-proline will allow increased levels of collagen synthesis. This increase in collagen synthesis will lead to deposits of collagen around blood vessels and prevent effective dilation of the vessels. Upon simvastatin or L-citrulline drug administration to diabetic animals, reductions in arginase activity were noted. Simvastatin is known to combat hypertension within diabetic patients; results within this laboratory suggest that simvastatin’s method of hypertension reduction occurs due to decreased arginase activity and subsequent decrease collagen synthesis. L-citrulline is a known to be a precursor to L-arginine. L-citrulline was initially administered as a curiosity, but testing revealed that L-citrulline decreased arginase activity nearly as much as simvastatin. Observations of collagen deposition within these drug treated animals also revealed reduced deposition levels versus untreated diabetic animals. Similarly, vasodilatory function was retained when collagen synthesis was lowered by these drugs.

In summary, in diabetics, increased arginase activity is noted in the liver and vascular system. Without administration of cardiovascular preventative drugs (such as simvastatin), the increased activity of arginase is believed to induce hypertension and ensuing cardiovascular problems. Many of these complications arise from excessive collagen deposition that occurs around blood vessels.
Citations and Footnotes:


7: Caldwell, W. “Diabetes induced arginase activation causes cardiovascular and renal dysfunction and fibrosis.” (Figure generated in lab and used with permission). 2008.


