

Characterization of 5-Hydroxytryptamine (5-HT, Serotonin) Receptors in the Kidneys of Type I
Diabetic Rats

Submitted by

Marissa Matthews

To

The Honors College

In partial fulfillment of the
requirement to graduate from

The Honors College

Mentor: Dr. Amy Banes-Berceli, Ph.D

Department of Biology

Oakland University

1 March 2014

Table of Contents

<u>Section</u>	<u>Page(s)</u>
Abstract	3
Introduction	4-11
Diabetes	4
Kidneys	6
Smooth Muscle	7
Serotonin and Diabetes	8-10
Hypothesis	10
Methods and Materials	11-15
Results and Figures	15-32
Discussion	33
References	34-35

Abstract:

Diabetes Mellitus commonly known as diabetes, is an epidemic in the United States accounting for numerous deaths due to complications each year. These complications include hypertension, cardiovascular disease, stroke, kidney failure, and many others. Diabetes also leads to a decrease in the quality of life for millions of patients every year because of amputations, daily injections of medication, daily monitoring of blood sugar levels, impaired wound healing, and fatigue. There is still no effective cure for diabetes and treatment options are extremely limited. We investigated the potential role of elevated levels of 5-hydroxytryptamine (5-HT, Serotonin) and its receptors observed in the plasma and kidney tissue of Type I diabetic rats. Hopefully by understanding these elevations and their physiological implications, we will be able to create better clinical treatments. These treatments will have a variety of impacts on both a clinical and economical level. It is important to use an intact physiological system in order to see the timing of when damage actually appears and to mimic the events in the intact organisms. The interconnectedness of the hormones, circulatory system, kidneys, etc. cannot be mimicked by any other means of study. By better understanding the role 5-hydroxytryptamine in the kidneys and the potential resulting damage, we will hopefully be able to find a way to treat these conditions.

Introduction:*Diabetes*

Diabetes mellitus, commonly referred to as diabetes, is a group of metabolic diseases that is characterized by high levels of glucose in an individual's blood. It can arise in two different forms commonly known as types. Type I is an insulin dependence form which is an autoimmune disease in which an individual's insulin producing pancreatic beta cells are destroyed. These beta cells are located in the islets of Langerhans. This leaves the pancreas unable to produce insulin, therefore leaving the cells of the body in a starvation state that calls for the intake of more glucose in order to maintain a homeostatic balance. The patient has an elevated level of blood glucose but no insulin with which to stimulate the cells to take it into themselves. Therefore, the glucose remains in the blood and is unusable to the cells. This state of hyperglycemia results in increased levels of reactive oxygen species, hormone alterations, formation of advanced glycation end products (AGEs) among other changes. All of these changes result in damage to the organs. Individuals with this disease usually present with hallmark symptoms of polyuria, polydipsia, polyphagia, and hyperglycemia (Rubin 2013). The symptoms can have a very abrupt onset and usually occur during childhood. This form of Diabetes Mellitus was called Juvenile Diabetes previously.

Type 2 diabetes is characterized by initial insulin resistance. Insulin resistance occurs when an individual's body cells fail to utilize the insulin properly. This results in high levels of plasma insulin (hyperinsulinemia). The defect in this disease is thought to be altered intracellular signaling mechanisms which detach the insulin receptor from the rest of the signaling cascade. This type is often caused by risk factors like obesity or high lipid levels, with 80% of cases being

attributed to obesity (Rubin 2013). After a prolonged period of time, this can cause the pancreatic cells to lose function and leads to insulin dependence. Although type 2 diabetes has the same symptoms as type 1, the onset of the symptoms are more gradual and subtle. This type also has a larger concordance with familial history, with more than 60% of individuals affected.

According to the 2011 National Diabetes Fact Sheet, 25.8 million children and adults currently have diabetes in the United States. Approximately another 79 million individuals have pre-diabetes; this is described as having blood glucose levels that are higher than normal, but not yet high enough to be diagnosed with diabetes. Currently there is no cure for Type I diabetes because we cannot replace the lost pancreatic Beta cells. These cells are responsible for the production of insulin and serve to maintain blood glucose levels in the body. Without them, Type I diabetics are subjected to taking insulin injections or being put on an insulin pump. Insulin isn't available in pill form because it has a peptide structure that is easily degraded by peptidases in the mouth; therefore, it must be injected directly into the bloodstream in order to interact with the receptors on the surface of the cells (Type 1 Diabetes 2011). This can be very disruptive to everyday activity and negatively impacts the quality of life for the individual.

Along with the everyday use of insulin, diabetics are at a very high risk for a wide variety of complications. These complications are life-threatening and in many cases cause death. As of 2007, diabetes contributed to more than 230,000 deaths. With diabetes the risk of cardiovascular disease is 4 times higher than the average population. Along with cardiovascular disease, it also accounts for more than 60% of non-traumatic lower-limb amputations and 60% to 70% of diabetics have mild to severe forms of nervous system damage. Along with these frightening complications, diabetics account for 44% of all new cases of kidney failure in 2008 (Diabetes Statistics 2011).

Hyperglycemia causes major microvascular changes of renal glomerular structure as well as the tubular structure. These two parts of the kidneys are responsible for all of the filtration and reabsorption which occurs in the kidneys. Therefore, it is important to determine the mechanism(s) by which this damage occurs. These numbers have been steadily increasing over the years and will continue to increase as many of the pre-diabetics become diabetics. As medical professionals see an increase in kidney failure and end-stage renal disease, as well as an increase in the cost of care, there is a push to study how type 1 diabetes affects the kidneys both structurally and physiologically.

Kidneys

Within the human body, the two kidneys sit retroperitoneal on the back abdominal wall, meaning they sit behind the abdominal cavity lining. Within the kidneys there are about a million structural elements called nephrons. Each contains an initial filtration element called the renal corpuscle and then a tubule extending from the renal corpuscle. These elements are made of epithelial tissues and smooth muscle that facilitates their functions. The corpuscle contains a webbing of interconnected capillary loops called the glomerulus that sits inside of the fluid filled Bowman's capsule. As blood flows from the afferent arteriole into the glomerulus, it is filtered through a series of membrane filters which allow most solute and solution to pass but not larger organic molecules such as proteins. From there the filtrate is transferred to the proximal tubule, where amino acids, glucose, ions, and other important elements are reabsorbed. In diabetics, this process is affected and not all of the glucose filtered out of the blood can be reabsorbed by the kidneys. This causes the glucose to be present within the urine and is a clinical manifestation of diabetes. The filtrate then goes through the loop of Henle, distal convoluted tube, and the

collecting duct where the filtrate is concentrated and diluted continuously until it is transferred to the ureter as urine (Wieldmaier 2011).

The kidneys are a very important and essential organ within the human body. They function to regulate the balance of water, inorganic ions, and acid-base concentration within the blood. These actions are critical to the proper control of blood pressure. While also working to remove harmful metabolic waste products from the blood and secrete them in the urine; along with metabolic waste they also remove foreign chemicals. The kidneys also produce a variety of essential hormones and enzymes like erythropoietin, renin, and 1,25-dihydroxyvitamin D. In their function to maintain homeostasis, the kidneys also are essential to gluconeogenesis (Wieldmaier 2011). This is an important process that works to maintain proper glucose levels, and it can be severely altered in diabetics.

Diabetic microvascular disease is thought to be responsible for many complications in diabetics including renal failure. Arteriolosclerosis and capillary basement membrane thickening are characteristic vascular changes observed in diabetes (Rubin 2013). This can be very detrimental in the kidneys because they are very vascular in nature. The glomerular capillaries can become thickened and lead to impaired filtration and absorptive capabilities. Also with damage to the kidneys, there is an alteration in the release of renin. This causes an increase in blood pressure and impaired ion exchange which can once again have very detrimental effects on the body (Banes-Berceli 2011).

Smooth Muscle

Smooth muscle lines almost all of the organs of the body including major blood vessels like the aorta, renal arteries, and veins. Smooth muscle cells are under autonomic nervous system

control which means that they are controlled without any conscious input. They are mostly used for housekeeping functions in the body like peristalsis of the esophagus or movement of blood through the arteries. Smooth muscle is non-striated, mononucleated tissues that usually presents with a layer of endothelial cells that help it to respond to the environment. They contain cross bridges of myosin anchored by dense bodies.

Their contractions are normally regulated by an influx of calcium that phosphorylates myosin of cross bridge cycling of the muscle tissues (Vander 2011). However, serotonin (5-hydroxytryptamine, 5-HT) has a peripheral effect as a smooth muscle mitogen that causes contractions. Another important mechanism of smooth muscle is the stimulation of endothelial cells by acetylcholine that leads to relaxations. The endothelium has a wide variety of functions in the body as it aids in the clotting of blood, prevents the inappropriate clotting of blood, regulates the growth of smooth muscle cells, vasodilation, vasoconstriction, filtering (like those in the kidney), and it provides structure to cellular membranes. For this study, its ability to induce relaxation was most important for our ability to assess its health and function.

5-HT and Diabetes

Scientists have begun investigating the physiological and anatomical influence of diabetes on the kidneys with many interesting discoveries being made. One of these discoveries is that the levels of 5-HT are elevated in the plasma of diabetic patients (Watts SW 2005). Along with increased levels of the neurotransmitter itself, there is also an increased expression of the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors (Banes AKL 2004). This is very interesting because the expressions of these receptors are not readily seen in the kidneys under normal physiological conditions. This leads to many questions regarding, whether 5-HT causes damage to the tissue of

the diabetic kidneys, if the increased 5-HT levels and 5-HT receptor levels are a compensatory response to diabetes-induced damage, or if these results are not physiologically linked.

5-HT is a hormone produced by the pineal gland, enterochromaffin cells in the digestive system, serotonergic neurons in the central and peripheral nervous systems, and is stored in blood platelets and nerve terminals (What is Serotonin? 2011). Its major metabolite is 5-hydroxyindoleacetic acid. After the body uses 5-HT it is transported to the liver where it is broken down, the metabolites are then excreted in the urine after passing through the kidneys. In the central nervous system, it works to help regulate learning, mood, sleep, anxiety, appetite, and the constriction of blood vessels. While in cells, it acts as a growth factor that plays a pivotal role in vasoconstriction and wound healing.

Approximately 80% of the human body's 5-HT is found in the enterochromaffin cells of the gut. Here it is utilized to help regulate the movement of the smooth muscle. 5-HT works in the smooth muscle cells to cause vasoconstriction. If the smooth muscle cells of the arteries and veins are stimulated by increased levels of 5-HT in diabetes then this could lead to organ and vessel damage and an increase in blood pressure. Increased blood pressure is an independent risk factor for kidney failure. More research is necessary to help bridge the gap between the elevated levels of 5-HT and the damaged inflicted on the kidneys.

5-HT works through at least sixteen types of receptors within the body, in the kidneys. We are interested in whether there is altered expression of the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Under normal physiological conditions all three of the type 2 receptors use the phospholipase C G-coupled protein mechanism within cells (Torics 2014). The 5-HT_{2A} receptor is usually found in the forebrain, caudate nucleus, hippocampus, vascular smooth muscle, and blood platelets. Its main function is with the neuronal depolarization, smooth muscle contraction

and platelet activation. They also play a role in appetite control, thermoregulation, and sleep. 5-HT_{2B} receptors are usually found in the brain, gut, heart, kidneys, and lungs and like 5-HT_{2A} it works with contractions. Finally, 5-HT_{2C} receptors are apparent in the choroid plexus, cortex, and limbic system. Their main function is with hypolocomotion, hypophagia, and anxiety (Torics 2014).

Studies have shown that the clinical actions of 5-HT_{2A} receptors may actually involve 5-HT_{2C} receptors or a combination of 5-HT_{2A} and 5-HT_{2C} receptors. For the most part, the specific role of 5-HT_{2B} receptors is unknown. The 5-HT_{2B} receptors exhibit about 70% homology to 5-HT_{2A} and 5-HT_{2C} receptors, and, like 5-HT_{2A} receptors, appear to couple functionally to phosphoinositol hydrolysis. There is some evidence that 5-HT_{2C} receptors may also be linked to stimulation of cGMP production (Glennon 2000). However, 5-HT has a 300 fold higher affinity for the 5-HT_{2B} receptor than it does for the 5-HT_{2A} or 5-HT_{2C} receptors. This means that small increases in the levels of 5-HT would preferentially activate the 5-HT_{2B} receptor first.

While individuals have researched 5-HT, they have not looked much into their effect on diabetics. 5-HT in diabetes has been studied in only a few different ways. The first study showed 5-HT_{2A} receptors during vascular smooth muscle contractions *in vitro* or cell cultures. These function to activate the JAK/STAT pathway and stimulate smooth muscle cell proliferation (Banes 2004). The use of a 5-HT_{2A} receptor antagonist was used to decrease blood glucose levels in type 2 diabetic rat models *in vivo*. It has also been shown to reduce the markers of kidney damage in type 2 diabetic patients in Japan. Since plasma 5-HT levels are elevated in diabetics, activation of these receptors may participate in the development of diabetic complications.

Based on the previous studies, I tested for the amounts of 5-HT and the expression of receptors 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} in the tissues of both Type I diabetic rats and normal, control rats. I hypothesized that there would be increased levels of 5-HT receptors in the renal tissues of the Type I diabetic rats as compared to the normal control animals. Also, I tested the blood vessels to see the extent of damage done by the disease.

Methods:

Streptozotocin Induced Diabetes

Male Sprague-Dawley rats were rendered diabetic by a single intravenous injection of streptozotocin, or STZ, with a dosage of 60 mg/kg made up in fresh 0.1M citrate buffer, pH 4.5. Age-matched control rats received buffer only. The diabetic state was confirmed 48 hours later by measuring the blood glucose level, from arterial blood obtained via a catheter, using an accucheck glucometer. All rats given STZ with a blood glucose concentration exceeding 140 mL/dL were considered diabetic. Diabetic rats were treated with two units of insulin per day to prevent ketoacidosis. Rats were monitored daily for fluid intake, urine output, and food intake. The urine samples were taken daily to check for proteinuria.

Isolation and homogenization of vessels for biochemical studies

The arteries and kidneys were isolated and cleaned of connective tissues and debris, pulverized and homogenized in ice cold buffer (50 mmol/L Tris · HCl, pH 7.4, 0.1 mmol/L EDTA, and 0.1mmol/L EGTA, 250 mmol/L sucrose, 10% Glycerol) in the presence of protease inhibitor (1 mmol/L PMSF, 1 µmol/L pepstatin A, 2 µmol/L leupeptin and 0.1% aprotinin,

sodium fluoride, vanadate). Protein concentrations were determined by standard Bradford assay using bovine serum albumin, BSA, as the standard.

Animal Experimentation

All animal studies were conducted with the approval of the Oakland University Institutional Animal Use Care Committee in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All animal procedures were approved and followed in accordance with the institutional guidelines of the committee. Male Sprague-Dawley rats were purchased from Harlan. Survival surgery was done with sodium pentobarbital anesthesia and using aseptic techniques the rats were allowed to recover from surgery in clean, warmed polypropylene cage. Following recovery, rats were transferred to individual metabolic cages in the laboratory animal facilities. Buprenorphine (0.01-0.05 mg/kg) was administered pre- and post- operatively to minimize and control pain and discomfort. All surgical equipment, catheters, and pumps were sterilized prior to use. The incision site was shaved and sterilized with betadine and a drape was placed at the site of the incision.

Vascular Catheterization

Briefly, under sodium pentobarbital anesthesia and using aseptic techniques, a non-occluding catheter will be inserted into the abdominal aorta via a midline abdominal incision. The insertion point will be sealed with cyanoacrylate adhesive and the catheters were placed in the vena cava through a femoral vein *via* separate incision. Both catheters were tunneled subcutaneously to the scapular region and exteriorized. There, they were routed through a stainless steel button that was implanted subcutaneously. All incisions were infiltrated with

penicillin G procaine and Sensorcaine and the rats were allowed to recover from surgery in a clean, warmed polypropylene cage. Following recovery, the rats were transferred to individual metabolic cages in the laboratory animal facility. Catheters were routed through a stainless steel spring connected to the scapular button and the opposite end of the spring were connected to an AirFlyte electrical swivel mounted about the cage, with an Instech dual-channel hydraulic swivel connected in series. The catheters were passed through the swivel center up to the hydraulic swivel. The venous swivel channel was connected to a Harvard infusion pump and a continuous intravenous infusion was started. The arterial swivel cannal was connected to a pressure transducer mounted on the cage exterior. Blood pressure was monitored in freely moving, conscious rats by this mechanism. The arterial catheter was flushed daily with 0.5 mL of 0.9% saline and filled with heparin solution.

Isolated Vessel Studies

Rat thoracic aortas, renal arteries, superior mesenteric arteries and femoral arteries were cleaned of debris and connective tissue but with endothelium intact and cut into 3-4mm rings and suspended in Danish Myo Technology myographs. The endothelium was left intact, knowing it has receptors for 5-HT which would mimic actual physiological conditions. Myograph chambers contained 5 mL of physiological salt solution with 5% CO₂-95% O₂ bubbled at a constant rate.

Aortic rings and rings from superior mesenteric arteries were placed under 30 mN of passive tension and equilibrated for 30 minutes. Rings from the renal and femoral arteries were placed under 15 mN of passive tension and equilibrated for 30 minutes. Viability was determined by measuring a contraction obtained by adding a maximal concentration of KCl. After peak

contraction was achieved, the baths were washed by removing the PSS and exchanging it for fresh until baseline tension was reached and then the vessels were rested for 30 minutes.

Immunohistochemistry

Aortic rings and kidneys were placed into freezing molds with Tissue-Tek OCT cryogenic freezing medium and snap frozen with liquid nitrogen. Samples were then sectioned on a cryostat and the thickness of the sections was 16 μ m. The sections were then placed on the slides and incubated with the appropriate primary and secondary antibodies. We used green fluorescent protein conjugated secondary antibody and used 4', 6-diamidino-2-phenylindole for nuclear staining. Fluorescence images were obtained with a scanning confocal microscope.

Western Blotting

The lysates of tissue samples were sonicated and centrifuged at 13000 RPM, 4 C for 15 minutes. The supernatant was boiled for 5 minutes and separated on SDS-polyacrylamide gels with 2-mercaptoethanol as the reducing agent and bromophenol blue as the tracking dye and then transferred onto nitrocellulose membranes.

Incubation was done overnight at 4°C using either antibodies specific for 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors were used as primary antibodies. Blots were washed once with TBS-t for 20 minutes and twice with TBS for 10 minutes. Membranes were incubated for 1 hour at room temperature using either mouse or rabbit anti-IgG horseradish peroxidase as the secondary antibody in BSA/TBS-T solution. Membranes were developed with enhanced chemiluminescence in a darkroom using autoradiography film to visualize labeled bands. After

developing, membranes were also incubated in stripping reagent. Blots with tissue samples were also probed for β -actin to ensure equal protein loading in all lanes.

Data Analysis and Statistics

Data are presented as means with standard error of the mean for the number of trials in parentheses. Statistical analysis for the Western protein blot data was carried out with the Graph Pad Prism program. When comparing two groups, the appropriate Student's t test was used. One-way ANOVA followed by a Student-Newman-Keuls post hoc test were performed when comparing three or more groups to determine significance. Band density was quantified using the program NIH image.

Results:

Contractile and Relaxation due to Receptors

In relation to the contractile effects of 5-HT, we examined a variety of different vessels that included the thoracic aorta, renal arteries, superior mesenteric, and femoral arteries. Along with the contractile effects, it was necessary to also consider the relaxation stimulated by acetylcholine. Figure 1 illustrates the contractile effects of 5-HT on the thoracic aorta obtained from both the control animals and the diabetic animals after both 14 and 28 days of treatment. The x-axis shows the concentration of agonist, while the Y axis is the contraction measured in mg of tension produced relative to the initial KCl-induced contraction. These graphs provide a way to normalize the contractile responses for the various tissues and ensure viability of the samples. In this particular figure an increase number of contractions would show and increase in function of the tissue, which therefore could be caused by the increase in receptors or a change in

Contractile Effects of 5-HT in Thoracic Aorta Obtained from Control, 14 day Diabetic and 28 day Diabetic Rats

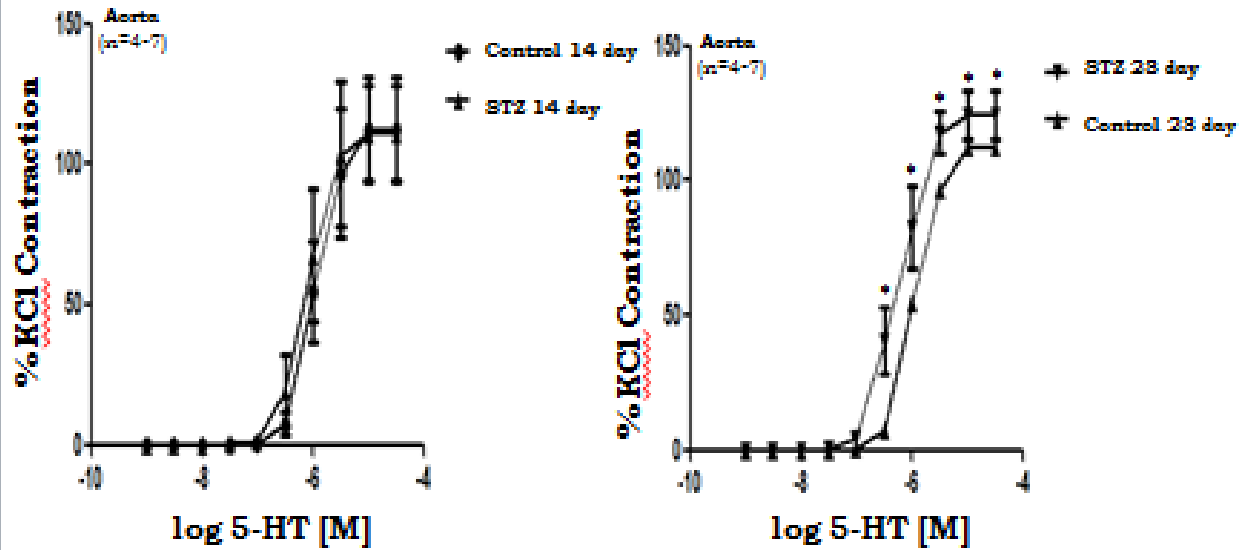


Figure 1: Contraction in rat thoracic aorta with 5-HT. Effects of 5-HT-induced contraction in rat thoracic aorta obtained from 14 day and 28 day control and 14 day and 28 day diabetic rats.

Values are shown as Mean \pm SEM. N=4-7

the sensitivity of the receptors to the agonist. There is not a profound difference in the 14 day responses, but there is in the 28 day data. Figure 2 shows the relaxation response of the thoracic aorta. The vessels are first contracted and then subjected to acetylcholine, if the endothelium is intact we should see a phase of relaxation. In this case, there is a slight decrease in the relaxation abilities of the 14 day STZ specimens. In the case of the 28 day diabetic rats, there is a significant decrease in the relaxation abilities in relation to the control group (Figure 3).

For the renal arteries, there isn't much of a difference between the 14 day groups, but the 28 day groups show a slight difference in contractile response (Figure 4). However, there is a profound difference in relaxation ability between the controls and the tissues from the two diabetic groups (Figures 5 & 6). The superior mesenteric and femoral arteries were just taken for the 28 day group. For the contractile effects, there was not much of a difference between the diabetic group and the controls (Figure 7). For the superior mesenteric arteries, the relaxation abilities were profoundly altered (Figure 8). This also held true for the femoral arteries (Figure 9).

These data suggest that an increase in the activation of 5-HT-induced signaling pathways can potentially stimulate a contractile response in rat thoracic aortic rings. This induction was enhanced in renal, superior mesenteric, and femoral arteries were compared to the aorta, but not in between the control and diabetic groups. However, these vessels show a significant shift in the relaxation of smooth muscle which is indicative of endothelial damage. Our data also illustrated that an increased sensitivity of renal, superior mesenteric, and femoral arteries to 5-HT to reach the maximum contraction compared to the aorta could be due to the increase in expression of 5-HT₂ receptors in peripheral blood vessels.

Effect of Acetylcholine (ACh) in Thoracic Aorta Obtained from Control and 14 day Diabetic

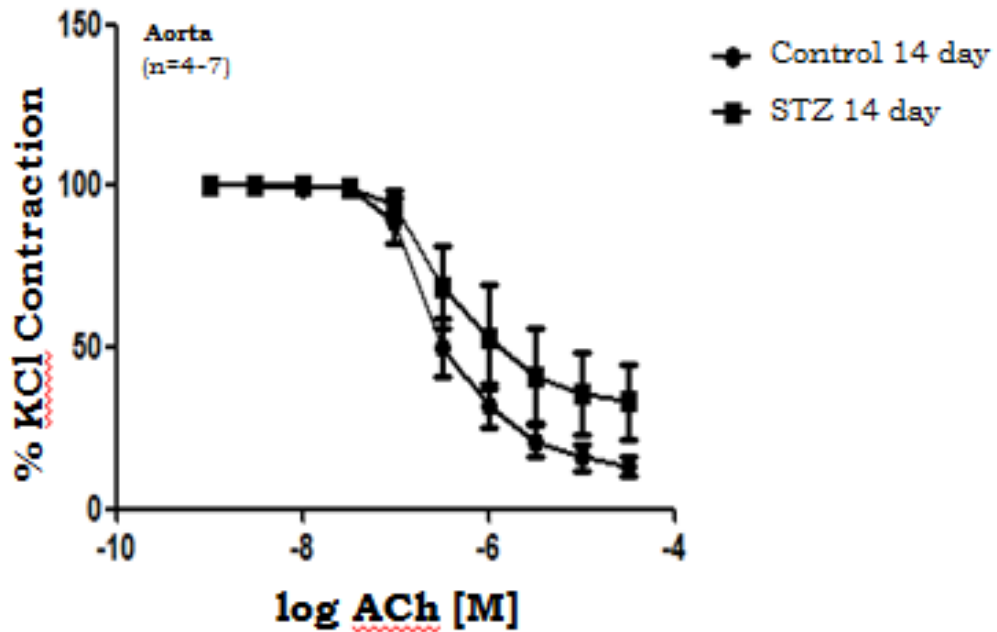


Figure 2: Relaxation in rat thoracic aorta with Acetylcholine. Effects of ACh-induced relaxation in rat thoracic aorta obtained from 14 day control and 14 day diabetic rats. Values are shown as Mean \pm SEM. N=4-7

Effect of Acetylcholine (ACh) in Thoracic Aorta Obtained from Control and 28 day Diabetic Rats

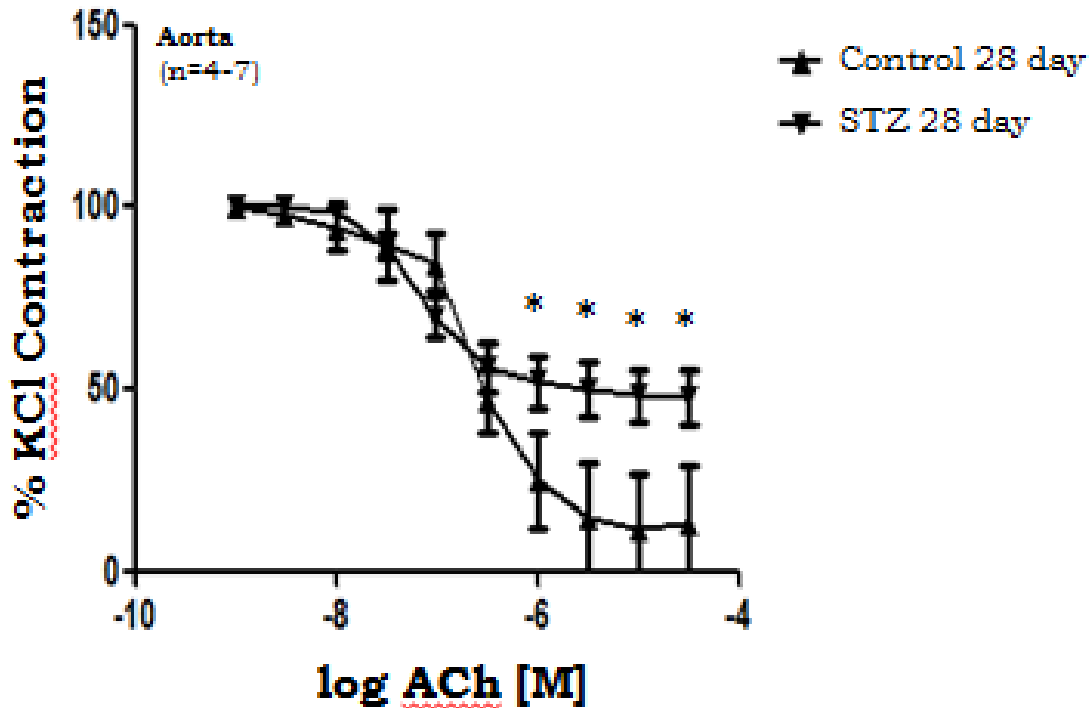


Figure 3: Relaxation in rat thoracic aorta with Acetylcholine. Effects of ACh-induced relaxation in rat thoracic aorta obtained from 28 day control and 28 day diabetic rats. Values are shown as Mean \pm SEM. N=4-7

Contractile Effects of 5-HT in Renal Arteries Obtained from Control, 14 day Diabetic and 28 day Diabetic Rats

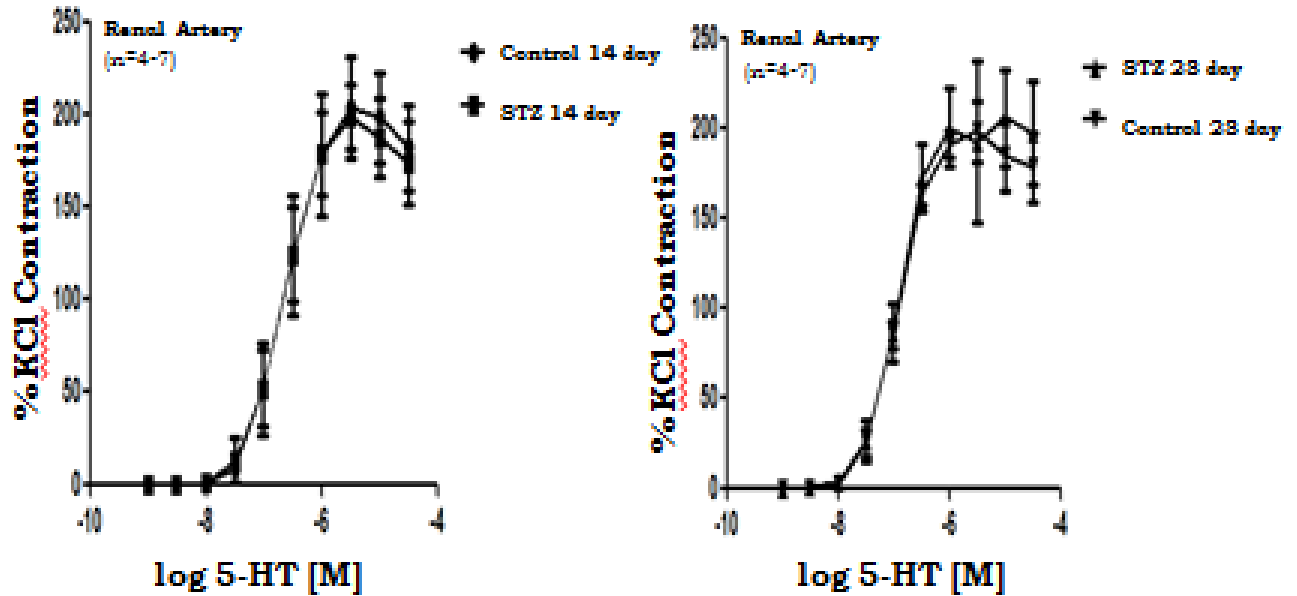


Figure 4: Contraction in rat renal arteries with 5-HT. Effects of 5-HT-induced contraction in rat renal arteries obtained from 14 day and 28 day control and 14 day and 28 day diabetic rats.

Values are shown as Mean \pm SEM. N=4-7

Effect of Acetylcholine (ACh) in Renal Arteries Obtained from Control, 14 day Diabetic Rats

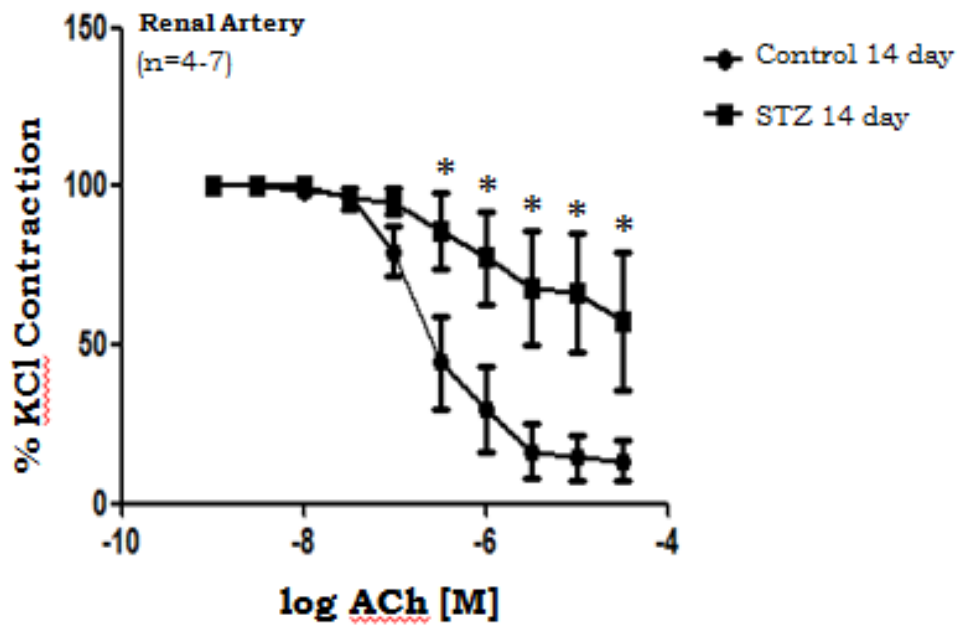


Figure 5: Relaxation in rat renal arteries with Acetylcholine. Effects of ACh-induced relaxation in rat renal arteries obtained from 14 day control and 14 day diabetic rats. Values are shown as Mean \pm SEM. N=4-7. * Denotes statistical significance when compared to control

Effect of Acetylcholine (ACh) in Renal Arteries Obtained from Control and 28 day Diabetic Rats

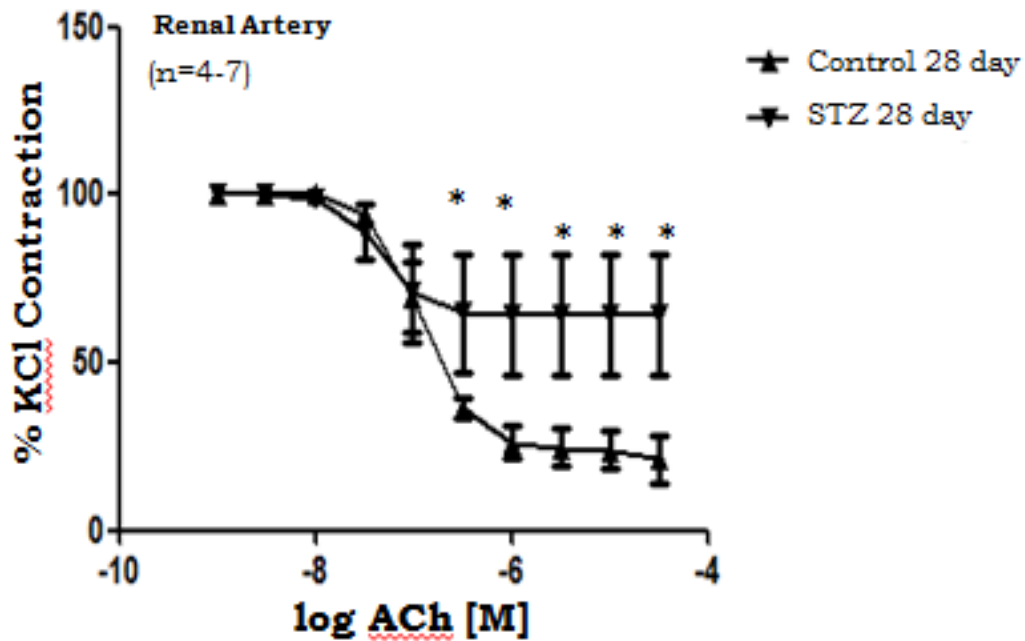


Figure 6: Relaxation in rat renal arteries with Acetylcholine. Effects of ACh-induced relaxation in rat renal arteries obtained from 28 day control and 28 day diabetic rats. Values are shown as Mean \pm SEM. N=4-7. * Denotes statistical significance when compared to control

Contractile Effects of 5-HT in Superior Mesenteric and Femoral Arteries Obtained from Control and 28 day Diabetic Rats

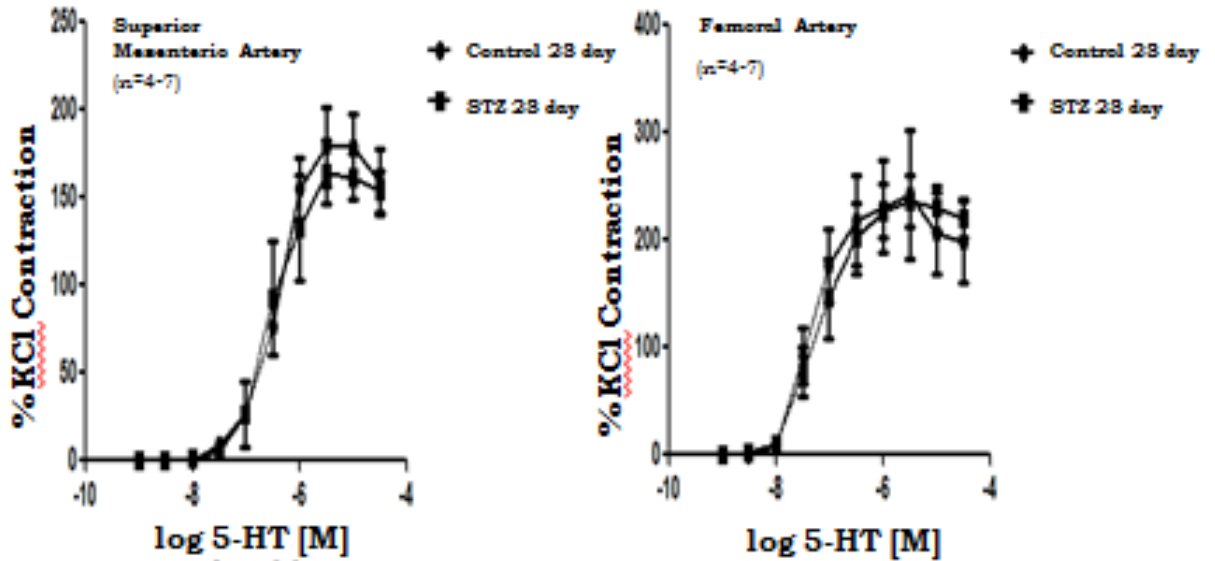


Figure 7: Contraction in rat superior mesenteric and femoral arteries with 5-HT. Effects of 5-HT-induced contraction in rat superior mesenteric and femoral arteries obtained from 28 day control and 28 day diabetic rats. Values are shown as Mean \pm SEM. N=4-7

Effect of Acetylcholine (ACh) in Superior Mesenteric Arteries Obtained from Control and 28 day Diabetic Rats

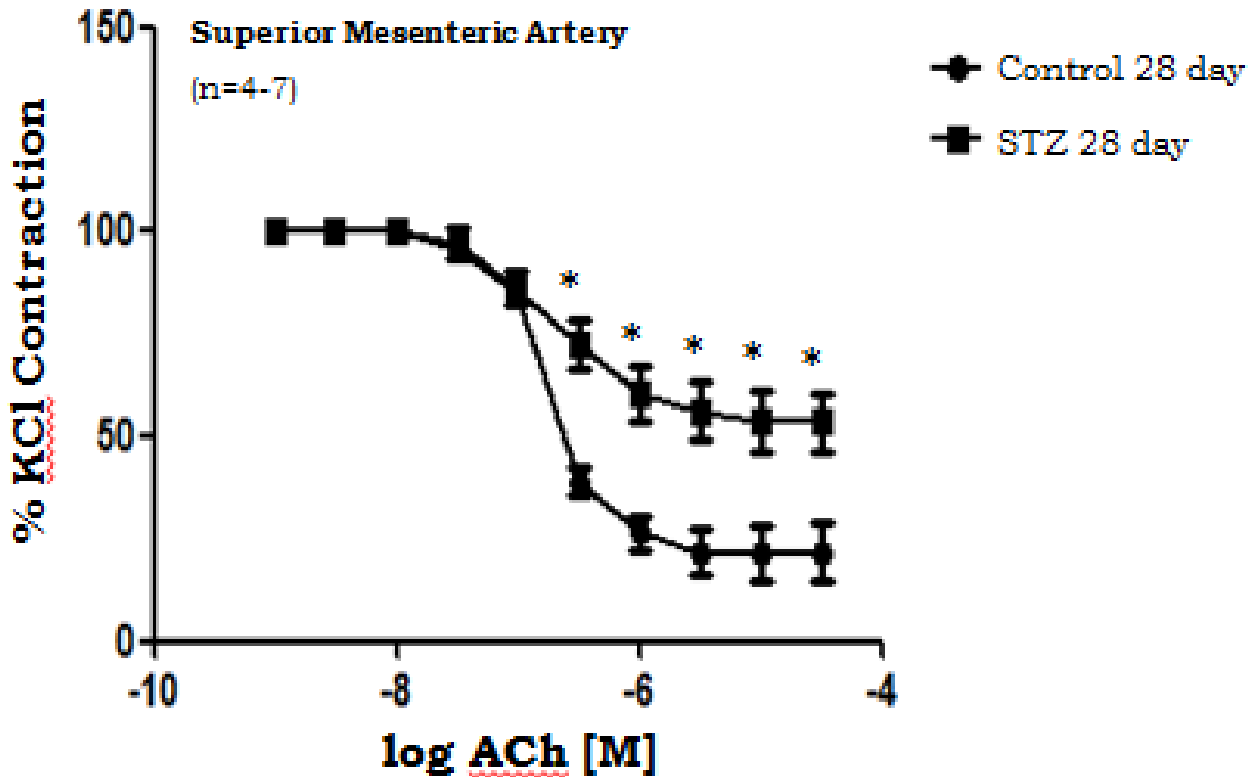


Figure 8: Relaxation in rat superior mesenteric artery with Acetylcholine. Effects of ACh-induced relaxation in rat superior mesenteric artery obtained from 28 day control and 28 day diabetic rats. Values are shown as Mean \pm SEM. N=4-7. * Denotes statistical significance when compared to control

Effect of Acetylcholine (ACh) in Femoral Arteries Obtained from Control and 28 day Diabetic Rats

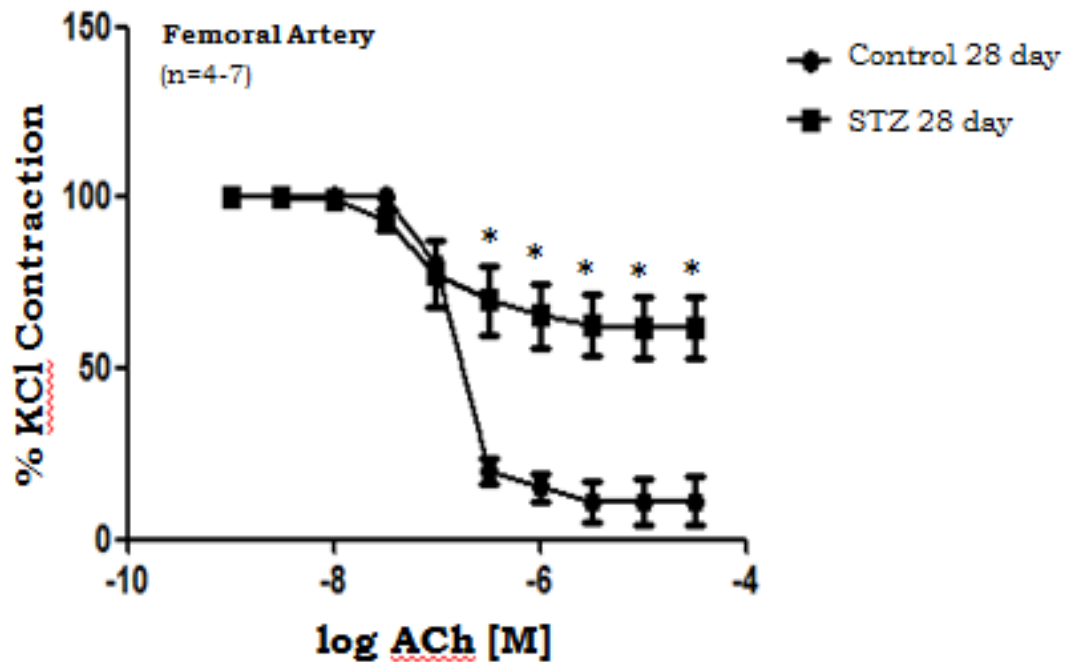


Figure 9: Relaxation in rat femoral arteries with Acetylcholine. Effects of ACh-induced relaxation in rat femoral arteries obtained from 28 day control and 28 day diabetic rats. Values are shown as Mean \pm SEM. N=4-7. * Denotes statistical significance when compared to control

Western Blot and Immunohistochemical Analyses

Our western blots showed that there was no significant increase in the expression of 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors in the aortas from the type 1 diabetic rats when compared to the aortas from the control rats. This was apparent in both the 14 Day and the 28 Day diabetic groups (Figures 10 & 12). However, our western blot data showed an increase in the overall expression of 5-HT_{2A} and 5-HT_{2B} receptors in the type 1 diabetic kidneys, but not in the expression of the 5-HT_{2C} receptors proteins (Figures 11 & 13). Also the degree of up regulation was more pronounced in the 28 day group kidneys when compared to the 14 day group kidneys.

Immunohistochemical analyses also support the data shown in the western blots. Figures 14 and 15 illustrate the expression of 5-HT_{2A} receptors in a control kidney, a 14 day diabetic kidney, and a negative control with only secondary antibody. These figures show the up regulation of the receptor in the diabetics in relation to the control, as shown through an increase in the amount of green on the image. Figure 14 shows the expression of 5-HT_{2B} receptors of the 14 day diabetics, once again the tissues show an increase in the amount of green and therefore the amount of 5-HT_{2B} receptors. Finally figure 15 shows the expression of the 5-HT_{2C} receptors proteins. Here there is no apparent increase in green staining so therefore there is not a significant amount of up regulation seen in the tissues.

The immunohistochemical analyses of the 28 day studies show the same trend among the stained slides. In comparison to the control in their groupings and with the 14 day the receptors are up-regulated. However, with these studies there are more apparent receptors than in the 14 day study, once again supporting the idea that the up-regulation is more severe. These findings suggest that the duration of diabetes is extremely important and that the changes in the receptor protein occur prior to the occurrence of damage to the renal vasculature. We can conclude this

because there was no proteinuria observed in any of the samples from any of the groups at the time points examined. Thus, there was no overt kidney damage in any of these animals.

Levels of 5-HT receptors in Control Vs 14 Day Diabetic Rat Aortas

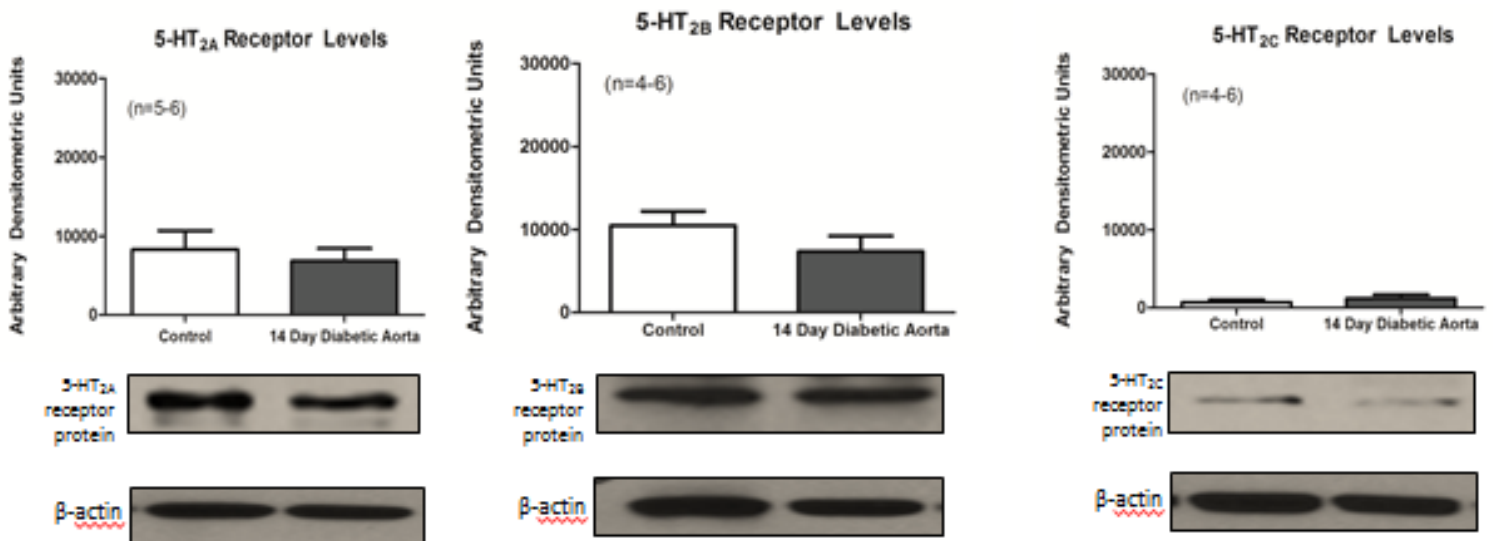


Figure 10: Levels of 5-HT₂ receptors in rat thoracic aorta obtained from 14 day control and diabetic rats. The top is densitometric analysis of Western blots. The bottom is representative Western blots probed for the three different receptors and for beta-actin as the loading control.

Levels of 5-HT receptors in Control Vs 14 Day Diabetic Rat Kidneys

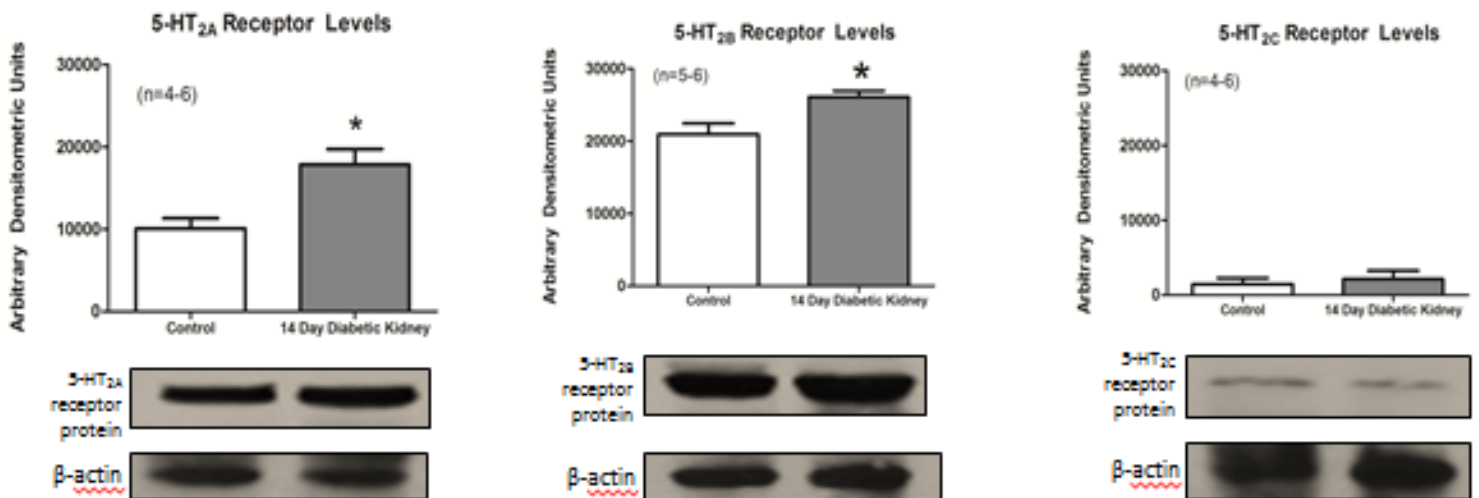


Figure 11: Levels of 5-HT₂ receptors in rat kidneys obtained from 14 day control and diabetic rats. The top is densitometric analysis of Western blots. The bottom is representative Western blots probed for the three different receptors and for beta-actin as the loading control.

Levels of 5-HT receptors in Control Vs 28 Day Diabetic Rat Aortas

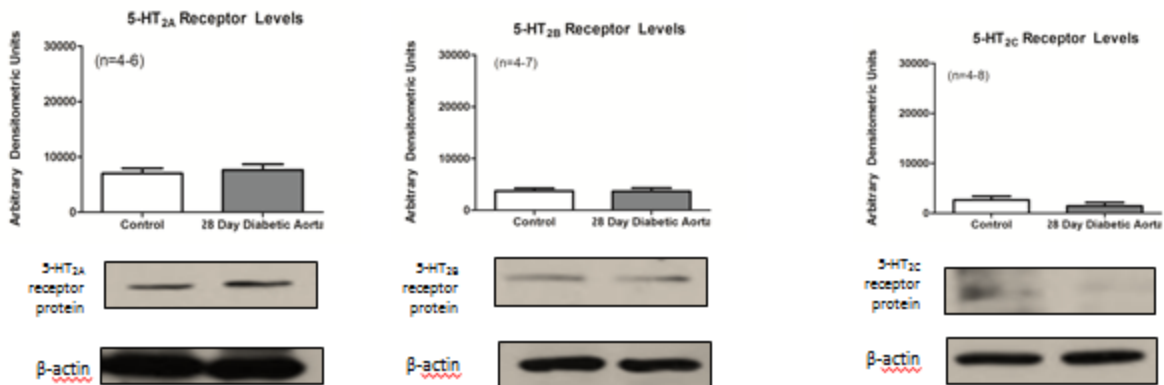


Figure 12: Levels of 5-HT₂ receptors in rat thoracic aorta obtained from 28 day control and diabetic rats. The top is densitometric analysis of Western blots. The bottom is representative Western blots probed for the three different receptors and for beta-actin as the loading control.

Levels of 5-HT receptors in Control Vs 28 Day Diabetic Rat Kidneys

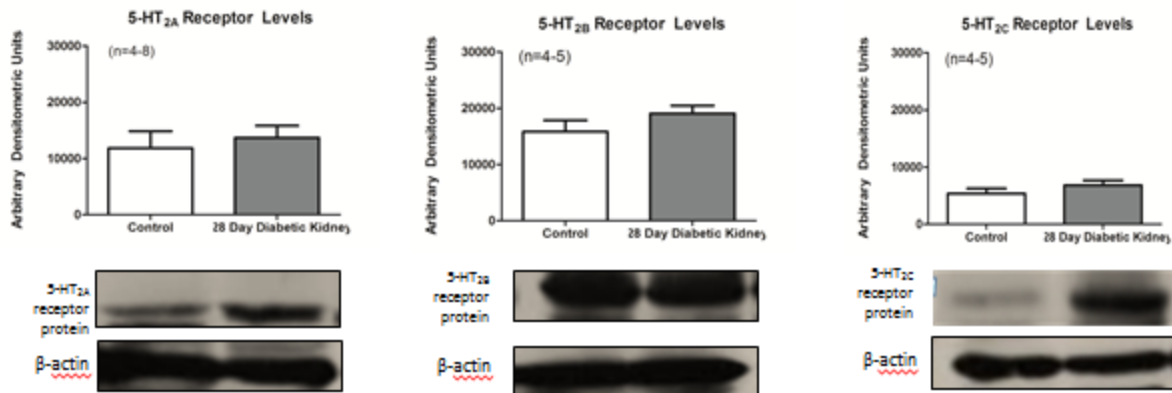


Figure 13: Levels of 5-HT₂ receptors in rat kidneys obtained from 28 day control and diabetic rats. The top is densitometric analysis of Western blots. The bottom is representative Western blots probed for the three different receptors and for beta-actin as the loading control.

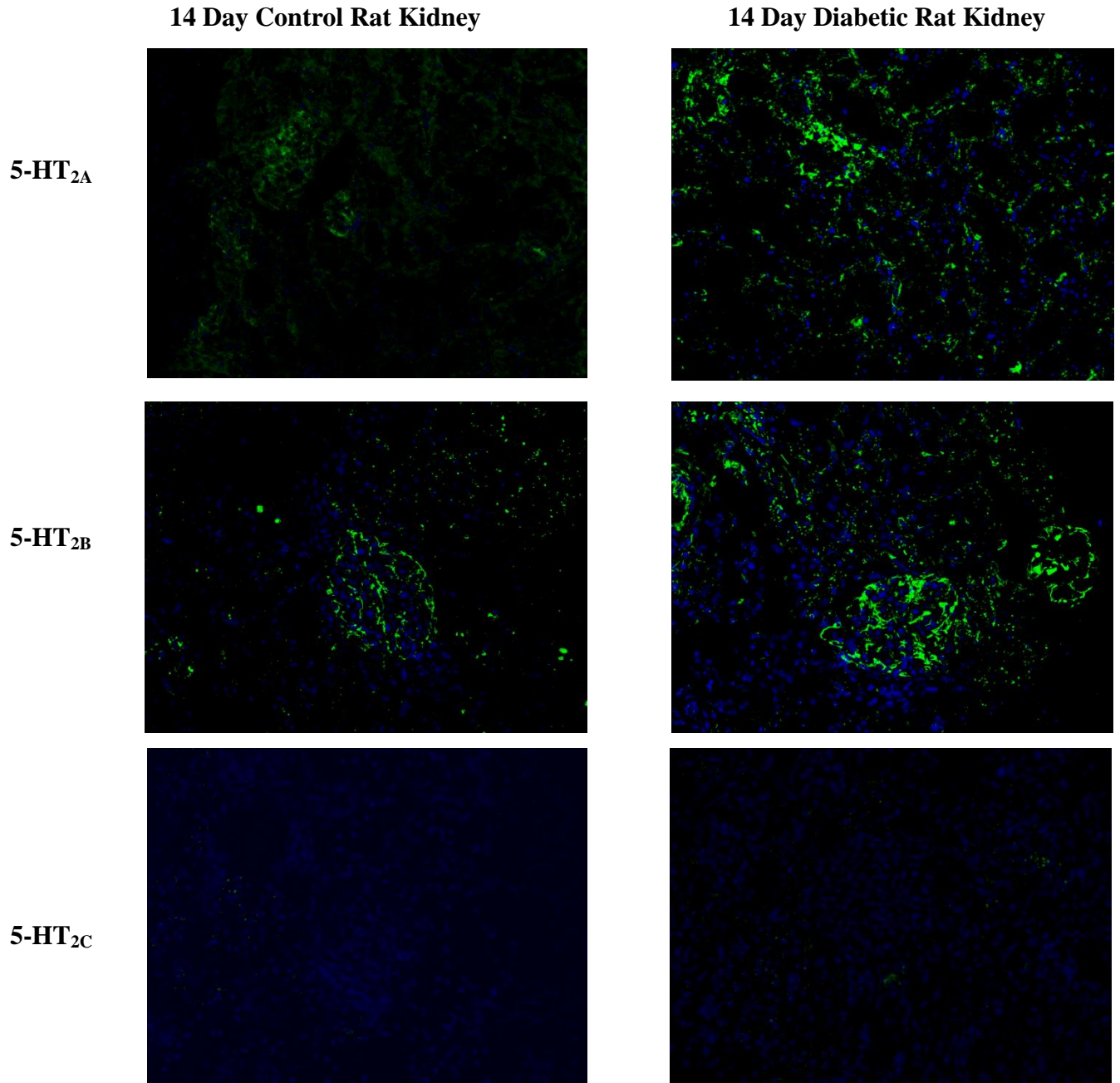


Figure 14: Expression of 5-HT₂ receptor proteins of each of the three subtypes (A, B, and C) in rat kidney obtained from 14 day control and diabetic rats. The IHC analyses were cut into 16 μ m sections for confocal staining. They were stained with green fluorescent protein which is the green and counter stained with 4', 6-diamidino-2-phenylindole which is the blue.

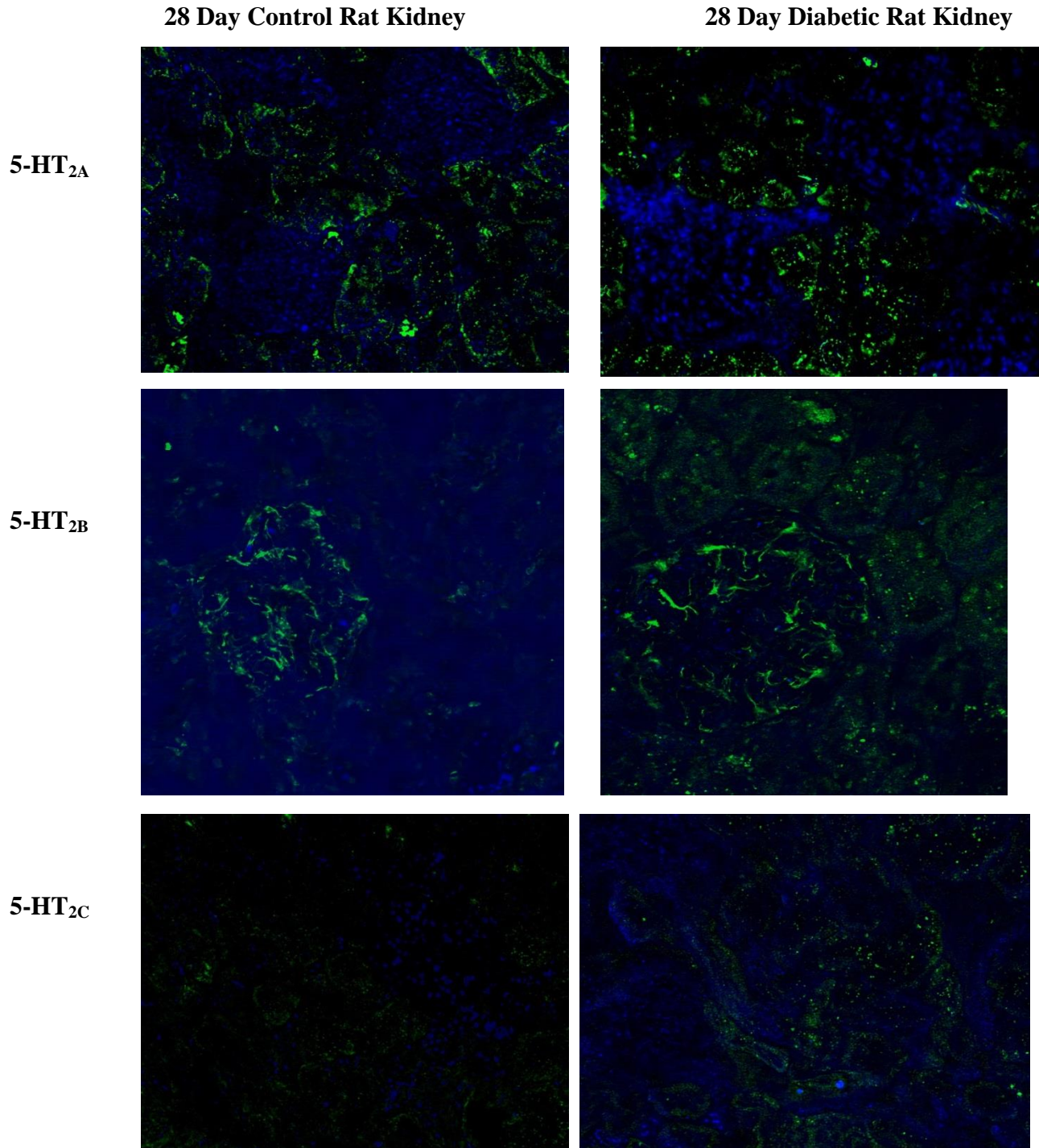


Figure 15: Expression of 5-HT₂ receptor proteins of each of the three subtypes (A, B, and C) in rat kidney obtained from 28 day control and diabetic rats. The IHC analyses were cut into 16 μ m sections for confocal staining. They were stained with green fluorescent protein which is the green and counter stained with 4', 6-diamidino-2-phenylindole which is the blue.

Discussion:

We were able to provide our first evidence using a myograph that 5-HT interactions at a physiological concentrations increases smooth muscle contractions that could increase the development of vascular complications. These increased plasma levels of 5-HT in diabetes could allow for the same interactions *in vivo*. In congruence with our data, the enhancement of 5-HT-induced intracellular signaling along with the alterations in the expression of the 5-HT₂ receptors most likely occur prior to the development of renal vascular injury as seen in diabetics. We can use this new understanding to provide a route for adjunctive therapies, as well as, potentially prevent the risk of renal failure and its associated vascular complications in cases of chronic diabetes.

The result of this research will help to better understand the physiological implication of 5-HT in the kidneys of diabetics. By learning more about the up-regulation of 5-HT₂ receptors at both a 14 day and 28 day time period, we can better understand how a prolonged case of diabetes can lead to more destructive outcomes. The experiment helped shed light on the understanding of renal damage in diabetes. Contractility studies worked to show possible reasons why there is an increase in 5-HT levels.

With more knowledge, researchers and pharmacologist will be able to synthesize a better, clinically relevant treatment for diabetes. With these better treatments there will come a decrease in costs, a decrease in the number of renal failure cases, and an increase in the quality of life for patients living with this disease. Also, with a better understanding of the disease researchers may be able to find more clinically relevant precautionary techniques for individuals who have already been diagnosed with diabetes. These techniques may in the end help to save thousands of lives.

Bibliography:

- Banes-Berceli AKL, Al-Azawi H, Proctor D, Qu H, Femminineo D, Hill-Pyror C, Webb RC, Brands MW. Angiotensin II utilizes JANUS KINASE 2 in hypertension, but not in the physiologic control of blood pressure during low salt intake. *S. Am J.* Aug 2011. 4-5.
- Banes AKL, Shaw SM, Tawfik A, Patel BP, Ogbi S, Fulton D, Marrero MB. Activation of the JAK/STAT pathway in vascular smooth muscle by serotonin. *Am J Physiol Cell Physiol.* 2004 (228). C805-C806.
- Diabetes Statistics. National Diabetes Fact Sheet. 2011.
- Fernandez M, Moran A, Marin ML, Roman LS. Mesenteric vasoconstrictor response to 5 hydroxytryptamine in the in situ blood autoperfused rat mesentery: involvement of 5-HT_{2B} and/or 5-HT_{2C} receptors. *European Journal of Pharmacology.* 2000; 401 (2). 221-227.
- Glennon, R., Dukat, M., & Westaemper, R. (2000). *Serotonin receptor subtypes and ligands* .
- Kanasaki K, Taduri G, Koya D. Diabetic Nephropathy: the role of inflammation in fibroblast activation and kidney fibrosis. *Fontiers.* 2013.
- Rubin, E. (2014). *Essentials of Rubin's pathology* (6th ed.). Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.
- Torics. (2014). *5-ht₂ receptors*.
- “Type 1 Diabetes: Insulin-dependent diabetes” *A.D.A.M Medical Encyclopedia. PubMed Health* (2011 June 28).

Watts SW, Thompson JM. Characterization of Contractile 5-Hydroxytryptamine Receptor in the Renal Artery of the Normotensive Rat. *The Journal of Pharmacology*. 2004; 309 (1).

165-172

"What Is Serotonin? What Does Serotonin Do?." *Medical News Today*. (2011 Aug 4).

Widmaier, E. P., Raff, H., Strang, K. T., & Vander, A. J. (2011). *Vander's human physiology: the mechanisms of body function* (12th ed.). New York: McGraw-Hill.