

Effects of Src Homolog Phosphatase-1 (SHP-1) on Janus Kinase 2 (JAK/STAT) and
Phosphatidylinositol 3-Kinase (PI3-kinase) Pathways in 7 and 28 day Renal-Wrap Hypertension

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Poe Lwin

Abstract

Effects of Src Homolog Phosphatase-1 (SHP-1) on Janus Kinase 2 (JAK/STAT) and Phosphatidylinositol 3-Kinase (PI3-kinase) Pathways in 7 and 28 day Renal-Wrap Hypertension

By

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Advisor: Amy Banes-Berceli Ph.D.

Hypertension, or high blood pressure, affects millions of patients every year and is associated with many types of cardiovascular diseases. While there are many drugs available to treat hypertension only 40% of the patients on medication currently have their blood pressure controlled. One problem that contributes to this low effectiveness medication is that there is a paucity of knowledge about the molecular mechanisms involved in the regulation of blood pressure and the process of pathogenesis that leads to end organ damage. For example, the molecular mechanisms involved in increasing blood pressure are not the same ones that maintain the increase over time. In this project we studied the Janus Kinase (JAK)/Signal Transducers of Activated Transcription (STAT) and the Phosphoinositide kinase (PI3-kinase) pathways. These two pathways have been linked to altered function associated with other forms of hypertension but their roles and their regulation in these disease processes are not well understood. We chose to use two time points in our study (7 and 28 days). The reasons for this are to look at development vs established mechanisms. The main purpose of this thesis is to identify the levels of JAK2/STAT and PI3-kinase pathway activity. In this research, male Sprague-Dawley rats will be induced with hypertension by renal wrap and sacrificed at days 7 and 28. Aorta, other blood

vessels and cells from these rats will be collected and studied by myography and Western Blot to explore the relationship between the JAK2/STAT and PI3-kinase pathways. These data clearly demonstrate that these pathways are altered during hypertension and result in function changes in the vasculature. Although many drugs being used to treat hypertension are currently available in the market, 60% of the hypertensive patients do not have their disease progression under control which leads to end organ damage. By studying these pathways and their roles, we aim to identify potential new drug targets to control hypertension.

Introduction

High blood pressure or hypertension is known as the “silent killer” for it is an asymptomatic disease which leads to many health complications and even death. It has been found that 25% of adults in America are affected by hypertension. Furthermore, some ethnic groups such as the African Americans have the highest chance (about 33.3%) to be affected by hypertension. Risk factors for hypertension include lack of exercise, obesity, smoking, diabetes mellitus, high sodium diet, family history of hypertension, and gender (Matthews & Bellenir, 2004).

There are two types of hypertension: primary and secondary. Primary hypertension occurs when the underlying cause for high blood pressure is unknown or not able to be assigned to a single specific cause. Most of the cases of hypertension in the United States are primary hypertension. On the other hand, secondary hypertension is a symptom of other diseases such as chronic kidney disease, thyroid or parathyroid disease, and renovascular disease (Matthews & Bellenir, 2004).

Whether it is primary or secondary, hypertension can lead to many other complications, for instance, cardiovascular disease, stroke, or kidney failure. The human body is composed of many organ systems and hypertension can have effects on most of them because it affects the function of cardiac tissue and smooth muscles in the vasculature. This will result in decreased blood flow to the tissues and can cause ischemia and hypoxia which lead to tissue death.

Therefore, as hypertension can lead to development of many other diseases it needs to be treated more effectively. Currently there are many drugs to treat hypertension such as: diuretics and calcium channel blockers (CCBs) which target blood sodium level and blood volume, angiotensin converting enzyme inhibitor (ACEI), angiotensin receptor blocker (ARB), direct

renin inhibitor (DRI), and beta-blocker which target renin-angiotensin system (RAS), and alpha blocker, beta blocker, and central alpha agonists which target the sympathetic nervous system (Matthews & Bellenir, 2004). However, these drugs have not been effective in lower blood pressure in about 60% of the patients. Therefore, new therapeutic approaches need to be developed. One pathway that is targeted with some success is the Renin-Angiotensin System (RAS).

The RAS system is thought to be present in humans during the early evolution stage as a safety measure so that when times of starvation fell upon them, they were able to tolerate low salt and water conditions and keep sufficient blood pressure in order for them to survive. In modern times, human are struggling with this system because majority of their diet contains high sodium levels, and when RAS is being activated in their bodies, it could trigger the onset of hypertension (Matthews & Bellenir, 2004). Angiotensin II (ANG II) is part of the RAS which is responsible for regulating the amount of salt (NaCl) and water reabsorption and potassium secretion in the kidneys. Overproduction of ANG II causes the kidney to secrete potassium and reabsorb NaCl and water more than the necessary amount, which expands the blood volume and increases the venous return, end diastolic volume, stroke volume and cardiac output. ANG II is also a vasoconstrictor which increases total peripheral resistance. Both of these sets of changes lead to increased blood pressure. Since ANG II causes high blood pressure, many of the hypertension drugs are designed to target this hormone with angiotensin-converting enzyme inhibitors (ACE inhibitors). ACE inhibitors decrease the amount of ANG II being produced, therefore lowering the blood pressure in the patients.

However, these RAS system drugs are not always effective at reducing the blood pressure and protecting the end organs like the kidneys, heart and blood vessels from pathological

changes. These data suggest that inhibition of ANGII's action at the receptor level is not enough as a therapeutic approach. The intracellular signaling pathways activated by ANGII must also be targeted to increase the efficacy of the medicinal approach. ANGII can utilize many intracellular pathways. However, the intracellular signaling pathways Janus Kinase 2/Signal Transducer and Activator of Transcription (JAK2/STAT) and Phosphatidylinositol 3-Kinase/Protein Kinase B (PI3-kinase/AKT) have been discovered to be involved in the development of hypertension and are linked to ANGII.

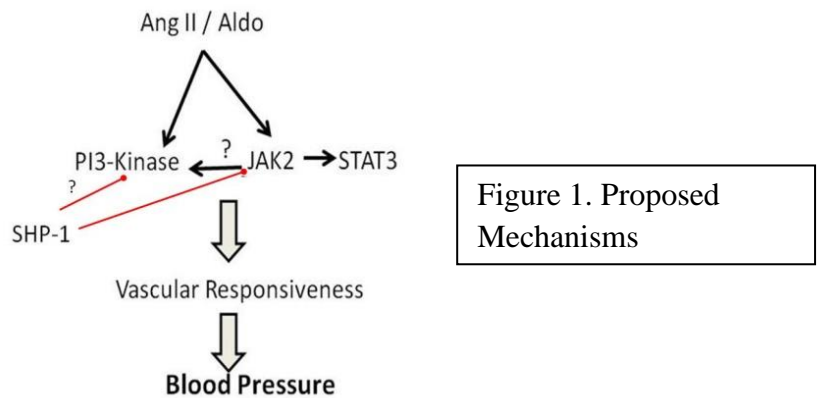
The JAK2/STAT signaling pathway is a second-messenger system. The JAK2/STAT cascade begins when a ligand binds to an inactive cytokine dimer receptor or G-protein coupled receptor on the plasma membrane of the target cell. Binding of the ligand causes the cytokine receptor to dimerize and allows the attachment of JAK2 in the cytosol. When JAK2 attaches to the receptor, it becomes activated and phosphorylates the tyrosine residues of the receptor. The phosphorylated receptor then allows the STATs to bind to its phosphorylated sites, and therefore activating them. Phosphorylated STATs are then released from the receptor to become STAT homo and heterodimers which enters the nucleus (Nelson & Cox, 2013). STAT dimers scan the DNA to look for the consensus sequence known as gamma activated sites (GAS). Binding of STAT dimers to GAS can affect gene transcription which could lead to expression of certain traits, cell growth or apoptosis. RAS activation of the JAK2/STAT3 pathway *in vivo* has been shown to cause hypertension by retaining NaCl in the blood, and led to remodeling of blood vessels in male Sprague-Dawley rats (Banes-Berceli et al., 2007 & Banes-Berceli et al., 2011). These data were later supported by work in a mouse model of hypertension based on ANGII-infusion (Brands et al., 2010) and in a mouse model where JAK2 was genetically knocked out or removed in vascular smooth muscle cells (Kirabo et al., 2011).

In healthy tissue, PI3-kinase/AKT affects the blood pressure by phosphorylating the enzyme endothelial nitric oxide synthase (eNOS), which produces the vasodilator nitric oxide (NO). eNOS convert Arginine into citrulline and NO. NO triggers the production of cyclic GMP which can activate protein kinase that causes the muscles of the heart and the blood vessels to relax (Nelson & Cox, 2013). PI3-K has also been shown to be uncoupled from AKT and interacts with L-type calcium channels leading to altered vascular function and hypertension. Further studies of these two signaling pathways could help us understand more about how they work to cause hypertension and the resultant end organ damage.

Previous research in Dr. Banes-Berceli's lab has shown that when JAK2 inhibitor is administered along with ANG II in rats, there is no development of hypertension (Banes-Berceli et al., 2011). It has also been discovered that ANG II inactivates a phosphatase named Src Homolog Phosphatase-1 (SHP-1), and decreased functioning of SHP-1 triggers the hyperphosphorylation of JAK kinases (Jiao et al., 1996), which means SHP-1 plays an important role in down regulating JAK2/STAT pathway. Data also supports a role *in vitro* for an interaction of SHP-1 with PI3-kinase (Cuevas et al., 1999). Whether this interaction is observed *in vivo* is not yet known. If both JAK2 and PI3-kinase signaling pathways are altered *in vivo* by loss of the function of one protein, SHP-1, then these are critical data which will further our understanding of the disease processes.

All of these studies mention previously have centered on artificial methods of ANGII increases. This project will utilize the renal wrap model of hypertension. This is a model in which ANGII is increased physiologically over time by the body to compensate for a loss of kidney function. These pathways have never been studied in this model previously.

Therefore we tested the hypothesis that in the renal wrap model of hypertension there would be elevated levels of JAK2 and PI3-K pathway activation which is necessary for the development of hypertension. We propose an interaction between these pathways at the level of SHP-1 as noted in Figure 1.



Methods

Animals:

All 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 (IACUC protocol 11101). All animal procedures were approved and followed in accordance with the institutional guidelines of the Animal Use and Care Committee. Male Sprague-Dawley rats (325-350 g) were purchased from Harlan (Madison, WI). Until surgery, animals were kept in clear plastic boxes with free access to standard rat chow (Teklad) and tap water. These studies utilized male Sprague Dawley rats (325-350 g body wt.) and methods Dr. Banes-Berceli has employed previously for chronic iv. infusion and 24 hr hemodynamic monitoring. Survival surgery was done with sodium pentobarbital anesthesia and using aseptic technique 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 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, pumps etc. was to be sterilized prior to use. The incision site was shaved and sterilized with betadine, followed by a rinsing with alcohol and a sterile drape was placed at the site of the incision. The surgeon wore sterile gloves and a mask (Brands et al 2010).

Renal Wrap Hypertension: Figure 8 Renal Wrap/Contralateral Nephrectomy:

All animals involved in the study underwent either control (sham surgery) or renal wrap procedures to produce hypertension. This surgery is performed by exposing a kidney through a retroperitoneal incision. After the fat is gently removed from the kidney, a figure-8 wrap is placed around the kidney with 0-suture. The contralateral kidney is also removed through a separate incision on the opposing side. The blood vessels and ureter are ligated with 4-0 suture before

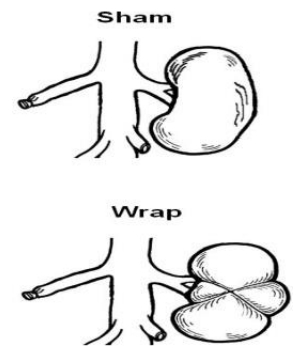


Figure 2. Diagram of Renal Wrap Protocol

excising of the kidney. The sham operation consists of only the unilateral nephrectomy. The muscle layer will then be closed with 3-0 absorbable suture. The skin is closed will be closed with autoclips or 4-0 nylon suture. Two weeks post-surgery the autoclips or suture are removed. The anesthetic used is isoflurane anesthetic, *via* an anesthetic machine. The animal is continually monitored for depth of anesthetic, *via* toe pinch and watching breathing. Buprenorphine (0.02-0.05 mg/kg) will be used both pre- and post- operatively to alleviate painful procedures. Once recovered from surgery the animals in this study remained on this regimen for 7 days and 28 days prior to use (Brands et al 2010).

Vascular Catheterization: Briefly, under sodium pentobarbital anesthesia and using aseptic technique, a non-occluding catheter was inserted into the abdominal aorta *via* a midline

abdominal incision. The insertion point was sealed with cyanoacrylate adhesive and the catheter will be exteriorized through the lateral abdominal wall. A second catheter was placed in the vena cava through a femoral vein *via* a 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 (Brands et al 2010).

Vessel isolation: At the conclusion of the experimental protocol, each rat was euthanized by pentobarbital overdose; then, the animal was infused with ice cold saline to remove blood and immediately after this, heart, kidneys, thoracic aorta, renal arteries, and mesenteric artery will be extracted and flash frozen in liquid nitrogen to preserve protein integrity. These frozen tissues were then transferred to a freezer until they could be analyzed. Alternatively, tissues are placed into PSS for *ex vivo* contractility studies. After being cleaned of fat and connective tissue four rings are cut for the myograph studies (see methods below), and the remaining tissues are separated into endothelium denuded (endothelium removed by scrubbing with a moist cotton swab) tissue to study the vascular smooth muscle cells. The remaining tissue is fixed and the endothelium removed by gently separating it from the basement membrane. A portion of the removed cells are then stained and the rest processed as described below for western blot analysis (Banes-Berceli et al., 2007).

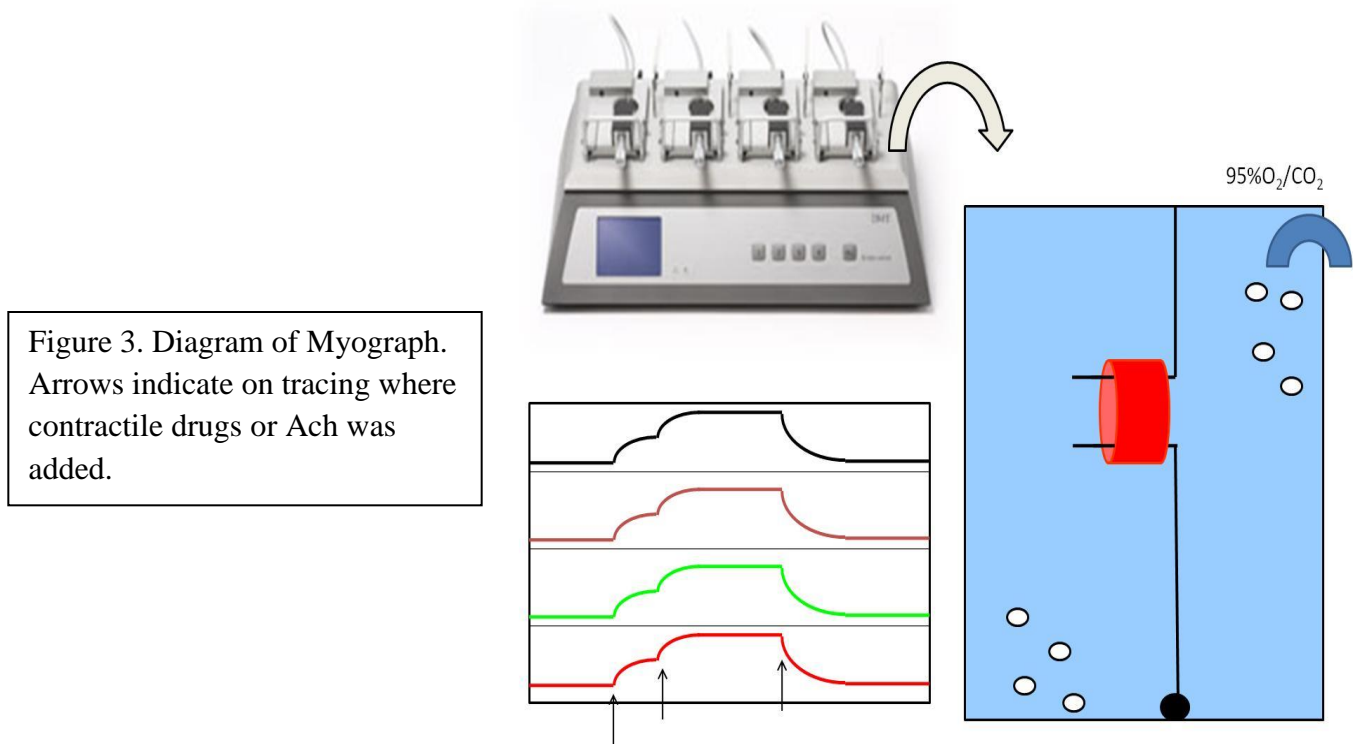
Tissue homogenization for protein work: Tissues obtained from rats treated were quick frozen with liquid nitrogen, pulverized in a liquid nitrogen-cooled mortar and pestle and solubilized in a 255 mM sucrose/10mM Tris buffer (pH 7.4) with protease (0.5 mM PMSF, 2mM EGTA, 10 µg/ul aprotinin and 10 µg/ml leupeptin) and tyrosine phosphatase (1 mM sodium orthovanadate)

inhibitors. Homogenates were centrifuged (14,000g for 10 minutes, 4°C) and supernatant total protein (Bio-Rad) were measured (Banes-Berceli et al., 2007).

Western Blotting: Tissue homogenate supernatant (4:1 in denaturing sample buffer, boiled for 5 minutes), were separated on SDS-polyacrylamide gels (SDS-PAGE; percentage varying on experiment) and transferred to Immobilon-P membrane. Membranes will be blocked [3-4 hours in Tris-buffer saline + Tween-20 (0.1%; TBS-T) containing 4% chick egg ovalbumin and 0.025% sodium azide] and probed overnight (4°C) with primary antibody. Blots were washed three times with TBS-T (30 minutes, 5 minutes, 5 minutes) and once with TBS (5 minutes). Dependent on the antibody either an anti-rabbit or anti-mouse horseradish peroxidase –linked secondary antibody (1:7500, Amersham Labs) was added for one hour and incubated with the blots at 4°C. Blots were washed using the described regimen. Enhanced chemiluminescence (Super Signals Ultra, Pierce) was used to visualize labeled bands (Banes-Berceli et al., 2007).

Myograph: The animals were euthanized and the thoracic aortae and superior mesenteric arteries removed. They were placed in a physiological salt solution (PSS) and cleaned of fat and connective tissue. The arteries were cut in half with half used for isolated tissue bath studies to determine the function of the endothelium and half being separated western analysis and immunohistochemistry. Isometric contractility experiments were carried out as previously described (Banes-Berceli et al., 2007). Aorta and superior mesenteric arteries will be cleaned, cut into helical strips, mounted on stainless steel holders and placed into tissue baths (5 mL) for isometric tension recordings using transducers connected to MacLab hardware and an iBook laptop computer (Figure 3). Strips will be placed under optimal resting tension and strips from control and hypertensive rats will be placed into the same bath to minimize experimental variation. Functional integrity of the endothelial cells will be evaluated by testing endothelium-

dependent relaxation to acetylcholine (1 μ M) in strips contracted with phenylephrine (10 nM). (Banes-Berceli et al., 2007).



Data analysis and statistics: Data are presented as means \pm standard error of the mean for the number of animals in parentheses. Statistical analysis for the western protein blot data were carried out with the Graph Pad Prism program (GraphPad Software, Inc. San Diego, CA). When comparing two groups, the appropriate Student's t test was used. One-way ANOVA followed by a Student-Newman-Keuls post hoc test was performed when comparing three or more groups to determine significance. Band density was quantified using the program NIH Image. A value of $p \leq 0.05$ for all studies was considered statistically significant.

Results

To verify our model of renal-wrap hypertension we first chronically measured the blood pressure in our rats. The renal-wrap hypertensive rats had significantly elevated blood pressures on both day 7 and 28 of the study (Figure 4). These data are consistent with data from previously data.

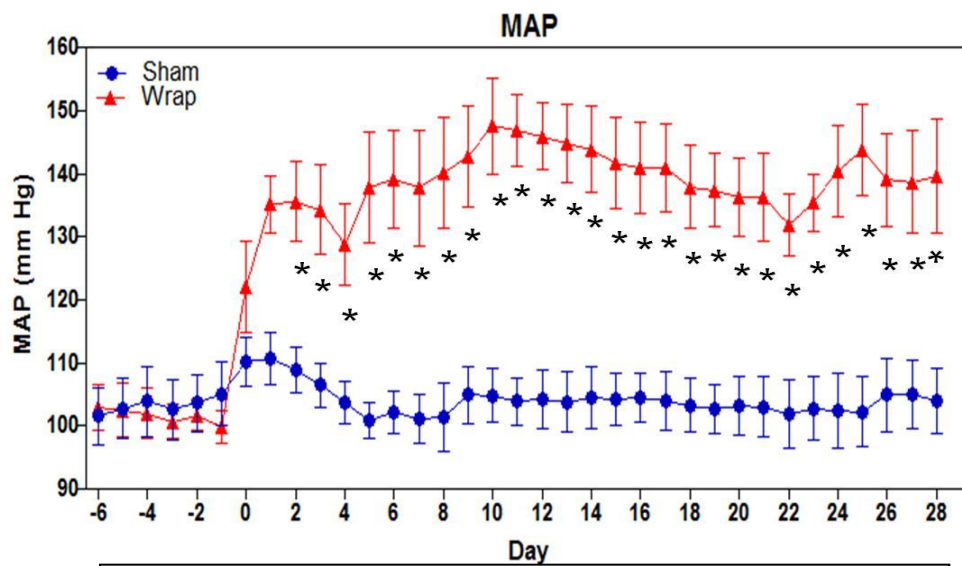


Figure 4. Mean Arterial Pressure (mm Hg) in control and renal-wrap hypertensive rats. N=8 in each group

We first investigated of the activity levels of the JAK2 pathway by utilizing western blot technology. To measure the activation status of the JAK2 protein we measure the level of phosphorylation on the protein. It requires phosphorylation at specific sites to be active and we use an antibody formulated specifically for these sites. On day 7 there was a decrease in activated JAK2 (Figure 5A) in the vascular smooth muscle cells isolated from the thoracic aorta. However, at day 28 there is an increase in activated JAK 2 in the vascular smooth muscle cells isolated from the thoracic aorta (Figure 5B). These data suggest that activation levels of JAK2 on day 7 and day 28 are different. This may be due to initial compensation mechanisms to try to compensate for the increased blood pressure and then vascular remodeling which requires JAK2 activation to compensate structurally for the increased pressure. The walls of the blood vessel need to remodel and become thicker to withstand the increased pressure. If the walls do not increase their structural strength then they will rupture. This is a leading cause of the internal hemorrhaging associated with strokes.

We next tested the hypothesis that SHP-1 would be activated to a lesser extent in renal-wrap hypertensive rats when compared to control normotensive rats. This hypothesis was tested using 3 renal-wrap rats and 3 control rats. Due to limited supply of rat aortic tissue, we were unable to test this hypothesis on more rats. We examined the serine phosphorylation of Serine site 591 on SHP-1, which is phosphorylated when the phosphatase is inactive (Figure 6). Our data gives us a P value of 0.09. Although this is not a significant P value (not <0.05), we will repeat these studies again.

Figure 5A

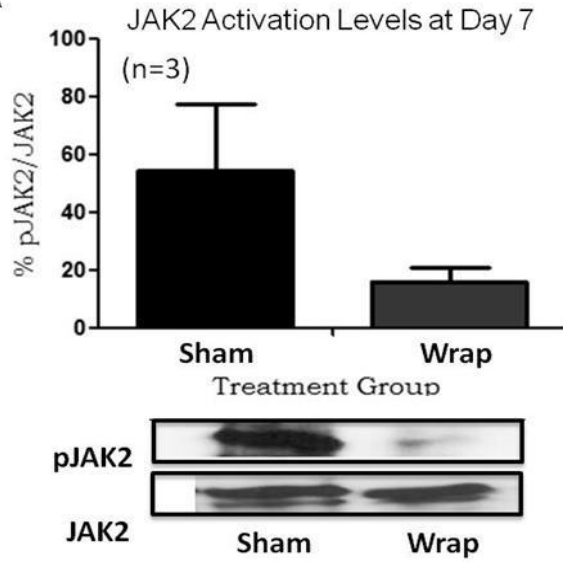


Figure 5B

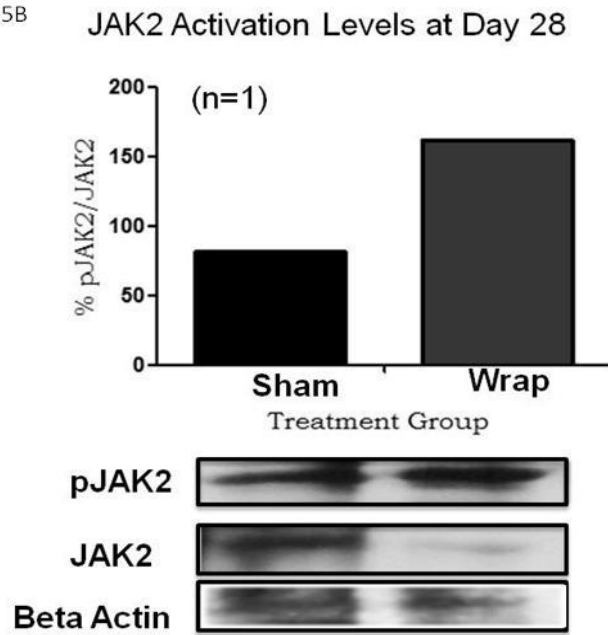


Figure 5. Activation levels of JAK2 at day 7 and 28 in aortic samples from hypertensive and normotensive rats.

SHP-1 Serine Phosphorylation at Day 7

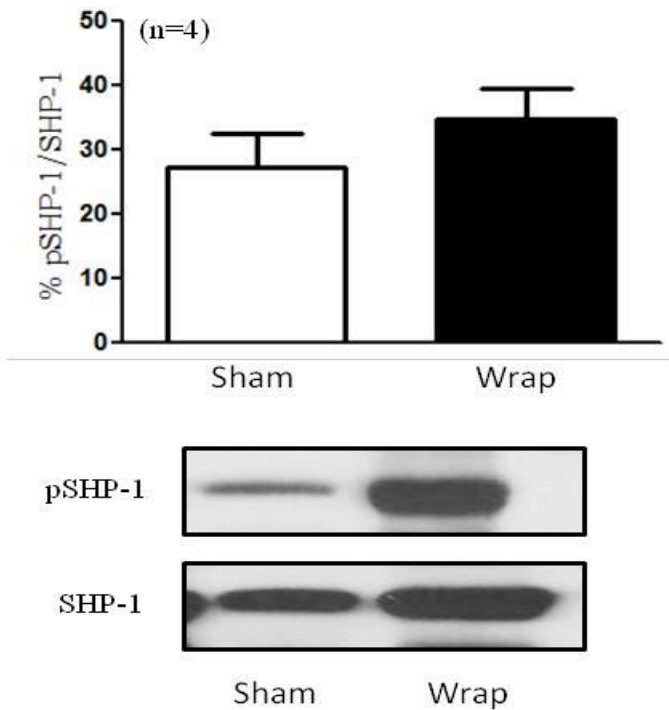


Figure 6. SHP-1 serine phosphorylation levels in control and renal-wrap hypertensive rats. Day 7

This trend of SHP-1 serine phosphorylation being greater in hypertensive rats as compared to normotensive rats suggests to us that SHP-1 inactivation precedes the increase in JAK2 activation that we see in the later stages of established hypertension at day 28. Further experimentation will need to be done to confirm this hypothesis, however. Future studies will also require us to test the tyrosine phosphorylation of SHP-1, as this is a marker of activated SHP-1 phosphatase activity. The ratio of inactive to active SHP-1 may be critically important to understand regulation of this protein and would be highly beneficial in better understand it's involvement in hypertension.

The next step taken was to investigate STAT3. STAT3 is the next step in the JAK2 signaling pathway and has been shown in previous studies to be important for maintaining increased levels of blood pressure. To determine if STAT3 is activated in the onset of hypertension (7 days post renal-wrap), we utilized western blotting techniques and measured the

ratio of phosphorylated STAT3 to total amount of STAT3 protein in both renal-wrap and control models. We found that STAT3 does not seem to be activated in 7-day renal-wrap rats (Figure 7). Considering that STAT3 is one of the traditional binding partner of JAK2, STAT3 activation may serve as an indirect way of measuring JAK2 activation.

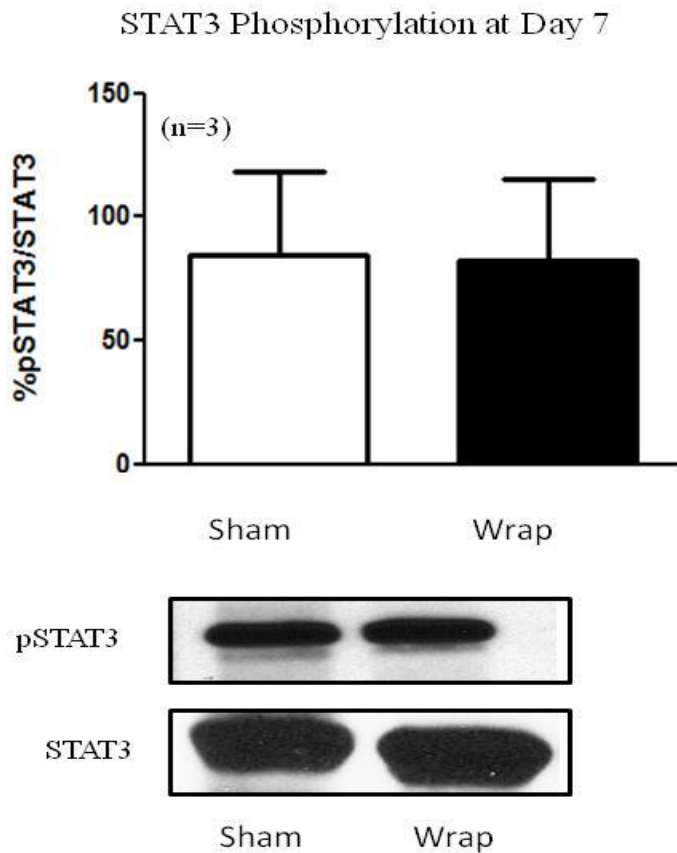


Figure 7. STAT3 is not enhanced in the 7 day renal-wrap model of hypertension.

To determine if the tissues were utilizing the “healthy” NO generating pathways of PI3-kinase we tested for the phosphorylated levels of AKT. We did not find a significant difference in the amount of activated AKT in renal-wrap samples as compared to the sham samples (Figure 8). This is consistent with our hypothesis that once PI3-kinase is activated it may be utilizing another mechanism beside AKT to create hypertensive effects. It is therefore possible that PI3-kinase may be activating another signaling pathway such as L-type calcium channels to create its hypertensive effects.

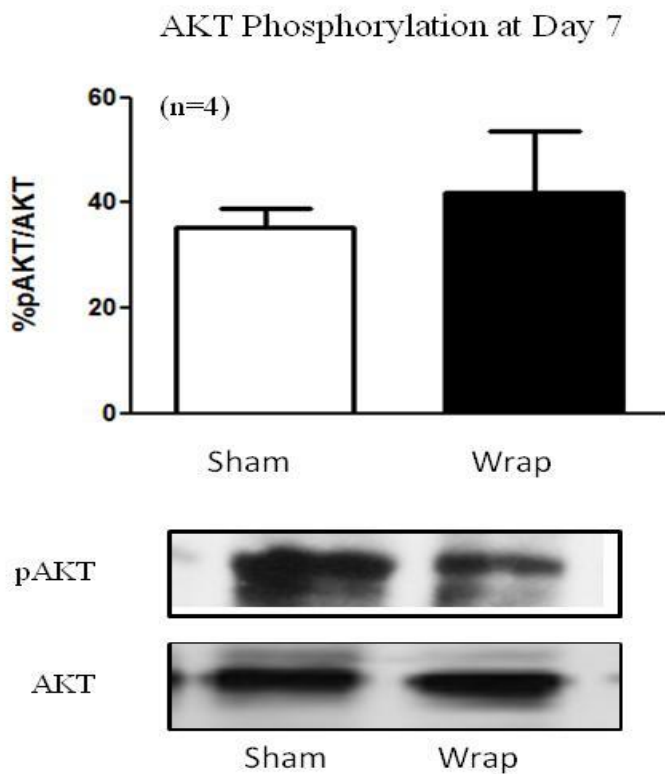


Figure 8. AKT activation is not enhanced in the renal-wrap model of hypertension.

Vascular Contractility Studies

To see if there is a difference in the vascular contractility between 7-day renal-wrap rats and 28-day renal-wrap rats, we utilized Myograph technology. Acetylcholine (ACH) is a potent vasodilator of blood vessels. ACH stimulates the release of the vasodilator nitric oxide (NO). NO diffuses from the endothelium to the vascular smooth muscle cells where it causes relaxation. When blood vessels lose their ability to relax following contraction as a consequence of endothelial damage, they are said to have endothelial dysfunction. To test the functional integrity of the endothelial cells in renal wrap hypertension, we tested endothelium-dependent relaxation of their thoracic aorta with ACH. Aortas were harvested from both 7-day and 28-day renal-wrap and control rats. Once the aortas were cut into small segments and hung on the Myograph machine. The rings were then washed with PSS, and contracted with 50 uL of 3M KCl to contract the tissues for to test for endothelial dysfunction. Once the aortic rings achieved a stable contraction, endothelial function was determined by the amount of relaxation achieved after adding 50 uL of 10^{-3} M ACH to the bath (final concentration 10^{-6} M). In the hypertensive treatment groups we found that there is a significant decrease in relaxation ability compared to the normotensive control group at both time points (Figure 9). These results were expected, and show that in renal-wrap hypertension, there is in fact endothelial dysfunction. We did not expect to see such dramatic endothelial cell dysfunction as early as 7 days of hypertension. These data suggest that damage occurs very early in the disease pathology and clinical control recommendation may need to be altered to be more aggressive in reducing blood pressure sooner.

Figure 9A

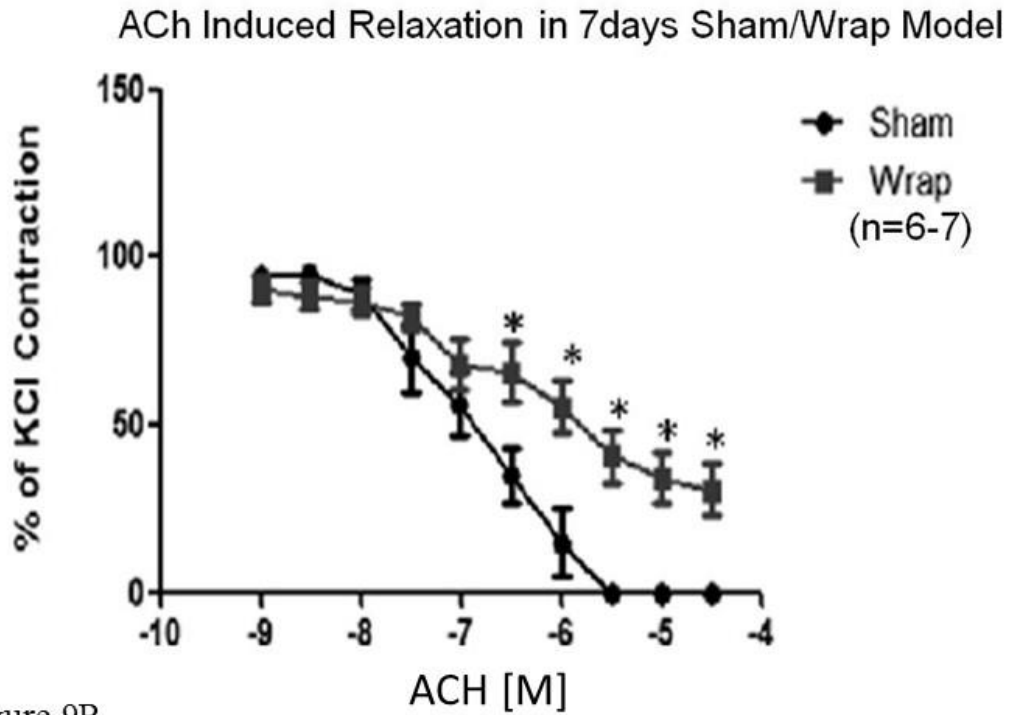


Figure 9B

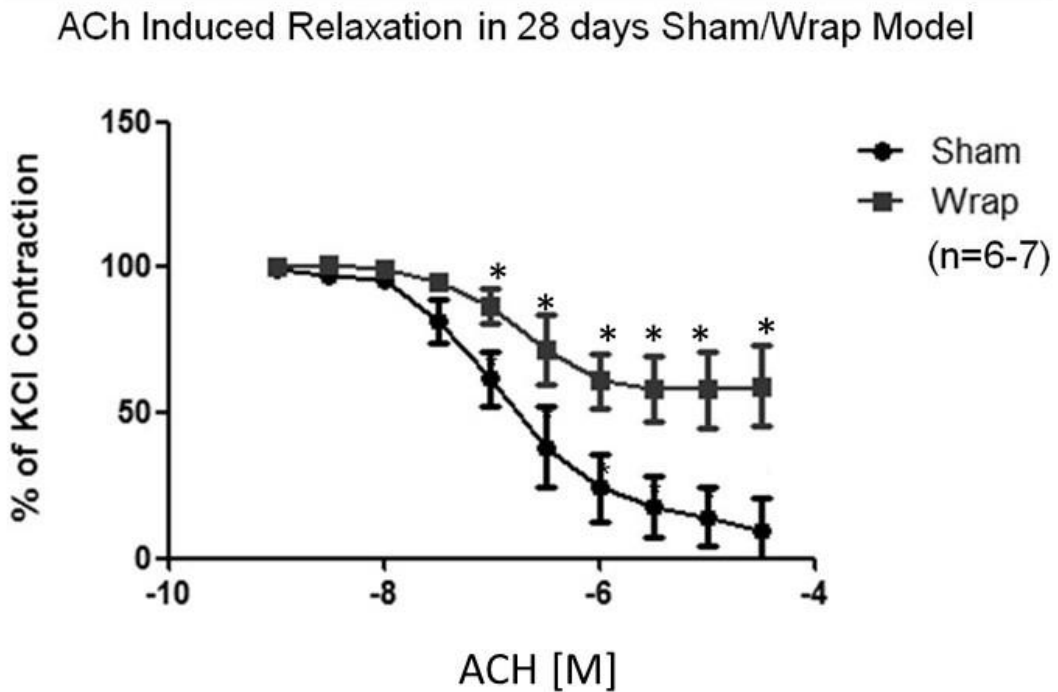


Figure 9. Achetylcholine-induced relaxation in thoracic aorta from control and renal- wrap hypertensive rats. Top:Day 7; Bottom: Day 28

Hypertension has been associated with alterations in the vasculature, and in particular, has been associated with enhanced contractions in the diseased tissues. To investigate the difference in contractility in 7-day vs. 28-day renal-wrap models, we subjected aortic rings from each treatment group to phenylephrine (PE)-induced contraction. PE is an alpha1 adrenergic receptor agonist that mimics the natural ligand of Norepinephrine which is produced by the sympathetic nervous system. While the 7-day renal-wrap models showed to contract less than the controls, we found that the 28-day renal-wrap models show a significantly greater amount of contractility compared to the control group. These data (Figure 10) suggest to us that between 7-day and 28-day models of hypertension there are changes occurring in the vasculature that make the 28-day model of hypertension more sensitive to contraction. One of these changes may be the enhanced activation of the JAK2 pathway.

Figure 10A

PE-Induced Contraction in 7 days Sham/Wrap Model

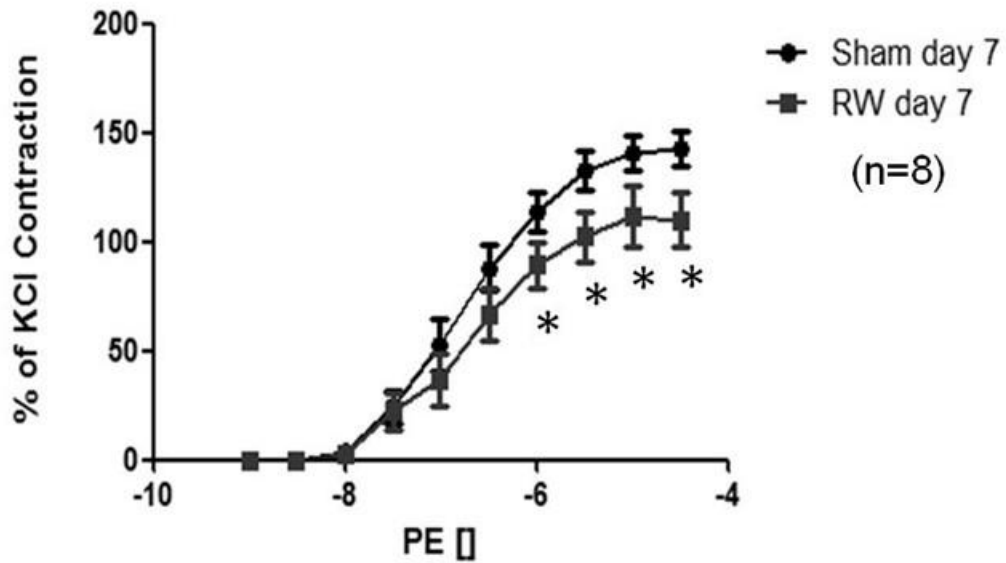


Figure 10B

PE-Induced Contraction in 28 days Sham/Wrap Model

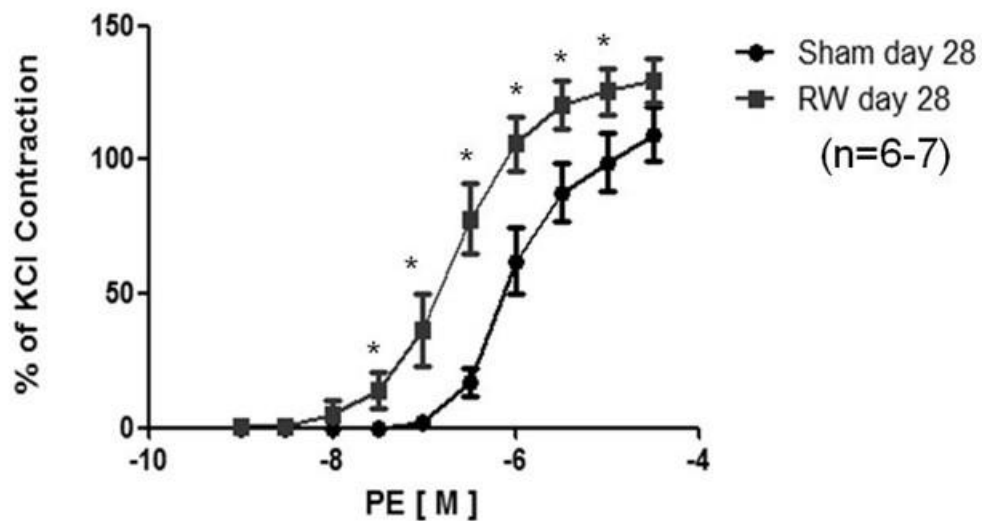


Figure 10. Phenylephrine-induced contraction in thoracic aorta from control and renal-wrap hypertensive rats. Top:Day 7; Bottom: Day 28

Hypertension is a serious disease that afflicts millions of people worldwide. Unfortunately, the specific intracellular signaling cascades that are involved in hypertension are poorly understood. Prior to this study, the JAK/STAT signaling cascade was believed to play a possible role in the development of hypertension. The exact role that this pathway plays in hypertension, however, needed to be further elucidated. In these experiments we utilized various techniques to gain a better understanding of how the JAK/STAT pathway and other signaling cascades such as PI3-kinase are involved in the etiology of hypertension. Our data suggests that JAK2 is greatly activated in chronic hypertension (28 days), and it is not found to be activated in the onset of hypertension (7 day). With this knowledge, the question then arises as to what changes occur between the onset and establishment of hypertension, and what pathways are being utilized prior to activation of the JAK/STAT pathway. In an attempt to answer this question we focused primarily on the onset of hypertension and the activation levels of SHP-1, AKT, and STAT3.

These results demonstrated that STAT3 activation is not changed between Sham and Wrap animals in the 7-day renal-wrap model of hypertension. Considering that STAT3 is the traditional binding partner of JAK2, this finding is consistent with our findings that JAK2 is not activated in the onset of hypertension at 7 days. Whether or not this is true in established hypertension will be looked at in the future.

To determine the difference in vascular contractility between the onset of hypertension and established hypertension, we took aortic rings from each treatment group and examined the difference in induced relaxation and contraction. We found that while both of the treatment groups had a significant decrease in response to induced relaxation, only day 28 showed a higher

response to induced contraction. This data suggest that while both day 7 and day 28 treatment groups have undergone endothelial dysfunction, there is a definite change occurring between these three weeks that make the aortas in chronic hypertension to more susceptible to greater contractions. This finding is novel, and we plan to repeat these contractility studies utilizing other drugs to gain a better understanding of the exact changes that are occurring between the onset and established hypertension. The proposed studies will also help us to determine why these changes are occurring, as well.

Future experiments would look at the interactions of these pathways and the regulatory pathways not addressed in this proposal such as SHP-2, PTP-1B and SOCS. For these pathways to be highly activated there must be multiple regulatory pathways, which cease to function properly. These data do suggest that altered JAK2 function may be a future clinical treatment target as it was elevated at 28 days.

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