

**Modulation of Angiotensin II-Induced Renal Vascular Responses by  
PP-Fold Peptides**

by

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University of Pittsburgh

2006

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**Modulation of Angiotensin II-Induced Renal Vascular Responses by  
PP-Fold Peptides**

**John H. Dubinon Ph.D**

**University of Pittsburgh, 2006**

Earlier studies indicate that  $G_i$  mediates enhanced renovascular responses to Ang II in SHR. The potentiation of Ang II by the  $G_i$  pathway is blocked by pretreatment with pertussis toxin, an inhibitor of  $G_i$ . The  $G_i$  pathway is also activated by receptors for PP-fold peptides; NPY, PYY, and PYY<sub>3-36</sub>. **Therefore, we hypothesize that in genetically predisposed models of hypertension PP-fold peptides augment renovascular responses to endogenous Ang II.**

Our study shows that LPNPY, an analogue of NPY selective for the  $Y_1$  receptor, potentiates Ang II responses in SHR, but not WKY, kidneys *in vitro*. LPNPY's ability to potentiate Ang II renovascular responses is dependent on the  $Y_1$  receptor and an intact  $G_i$  pathway. The renal expression of  $Y_1$  receptors is similar in SHR versus WKY.

Our study also demonstrates that PYY<sub>3-36</sub>, selective for the  $Y_2$  receptor, potentiates renovascular responses to Ang II in SHR, but not WKY, *in vitro*. PYY<sub>3-36</sub> is dependent on an intact  $Y_2$ - $G_i$  pathway, and the  $Y_2$  receptor is similarly expressed in the kidney of both strains.

In comparing the PP-fold peptides, PYY is the most efficacious at potentiating Ang II-induced renovascular responses. Lower levels of these peptides have little effect on renal vasculature. Yet, these peptides are released with other G<sub>i</sub> coupled agonists, namely NE that acts on  $\alpha_2$ -adrenoceptors. We observe a significant enhancement of Ang II-induced renal vasoconstriction with low level combinations of UK 14,304, an  $\alpha_2$ -adrenoceptor agonist, and PYY/NPY.

We demonstrate, in SHR, that nerve stimulation potentiates renal vasoconstrictive responses to Ang II. This interaction is dependent on an intact Y<sub>1</sub>-G<sub>i</sub> pathway suggesting that NPY plays a predominate role in increasing renal vascular responses.

PYY is a more potent agonist at augmenting renal vascular responses than is PYY<sub>3-36</sub>. Blockade of the conversion of PYY to PYY<sub>3-36</sub> via a DPPIV inhibitor, P32/98, results in an increase in MABP in SHR. We also demonstrate that this effect is dependent on the Y<sub>1</sub> receptor pathway. **This project demonstrates that PP-fold peptides may play a role in the etiology of genetic hypertension. This project is significant because it suggests a link between a high fat diet, sympathetic activation, and hypertension in a genetically susceptible animal.**

## FORWARD

The Lord has blessed me abundantly. It is because of Him that this work was possible. In His awesome power, He sent many angels to my aid, and this is my opportunity to thank them for their role in this dissertation. He began this work through the lives of John H. Dubinion Sr. and Diane B. Dubinion. My father and mother, who I'm indebted to for life, raised me with love and understanding while giving me as many opportunities as they could find and provide. Although my father is no longer with us, I'm certain that he is my number one fan in heaven. My mother continues to be my ultimate role model in life. Her spirit and fortitude inspire me to be the best person I can be, and I believe it is because of her that I've accomplished many successes in my young life. Through my mother and father, God gave me Steve and Shawna. While my mother and father are the foundation of my life, my brother and sister act as the backbone. They're own successes and continued support give me the strength to continue to reach goal's that are unimaginable. THANK YOU for being my role models and support. I LOVE YOU more than you can imagine.

I was also blessed to be born into a warm and loving family. It is because of them that I'm a strong friendly caring successful black man. The Dubinion's: Granny(Betty), Poppy(John Henry), Uncle Reggie, Aunt Karen, Aunt Cookie, Uncle John, Little Reggie, Roy, Tre, and Janea. The Bolden's: Nana(Jean), Poppy(Jesse), Aunt Lori, Uncle Greg, Uncle Jeffrey, Garrett, Avery, Sydney. Thank you all for your continued support and encouragement. It is because of you that this work was sought and completed. I would like to especially thank you NANA because living with you this past three years not only lightened my load but allowed me to stay focused.

Science can be particularly difficult which is why I'm overwhelmingly thankful for the greatest laboratory mates I could ask for during my graduate career. First, Dr. Jackson, having you as a mentor has meant the world to me. You're an amazing scholar, and having the opportunity to see you work has been great. I'm especially thankful for your continued upliftment even in times when things looked bleak. As my career begins and develops, I plan to follow your huge footsteps, and thus I'll be continuing to bother you often. ☺ Second, Zaichuan Mi, thank you so much for keeping a smile on my face. You led me to believe that everything was going great even when things weren't working at all. It's because of you that failure never found its way into my spirit during my stay with the Jackson Lab. Lastly, thank you to Bill Herzer, Del Gillespie, Chong Zhu, Lefteris Zacharia, Prajakta Sonalker, Jimmy Porter, Tom Jones, Stevan Tofovic and the staff of the Center for Clinical Pharmacology.

During my graduate career, I also am thankful for the mentorship of Dr. Sandra Murray and Dr. Jack Yalowich. I'm also indebted to the scientific expertise of my dissertation committee. Thank you for your direction: Dr. Barchowsky, Dr. De Groat, Dr. Friedman, and Dr. Kleyman. I'd also like to say a special thank you to the staff of the department of Pharmacology; your smiling faces and quick help made paper work easy and fun.

Before I conclude, I'd also like to thank a host of friends for their support during this time: Paris(Jazz), Alicia(Lily), Dj, LaVern, Val, Christie, B. Berry, Rob, Binky, Jovan, Dr. JP, Kim, Ayana, B. Stuck, David, Travis, and my graduate school classmates John, Marjet, Lori, Julie, and Sole. To my little swimmers of the Three Rivers Aquatic Club, thank you for your smiles and stress relieving laughter. To my Art's Tavern Family, thank you for the support, encouragement, and entertainment.

I'd like to conclude with this excerpt from a novel by J. California Cooper entitled "Some People, Some Other Place":

*Choices. Some people sneer and laugh, calling people stupid when humans call on God, but they would do better thanking God that some humans believe in God. Think! What would the world be like if humans didn't know about loving their neighbor. And about the shalt not kill. All of the Ten Commandments, in fact. Take them from the world and all you have left are killing fields and hate. The world seems full of hate. Satan, the great propagandist, the very first liar, with his representatives that have lived on earth since Cain. He has seen to that! There is a fine line that separates sinners from Satan's representatives. One has a chance. The other is lost. Learn why, if you have opportunity.*

## PREFACE

Two articles have been published from the work of this dissertation:

Chapter 3: John H. Dubinion, Zaichuan Mi, Chongxue Zhu, Liping Gao, Edwin K. Jackson. Pancreatic Polypeptide-Fold Peptide Receptors and Angiotensin II–Induced Renal Vasoconstriction. *Hypertension*. 2006;47:545-551.

Chapters 4 and 5: John H. Dubinion, Zaichuan Mi, and Edwin K. Jackson. Role of Renal Sympathetic Nerves in Regulating Renovascular Responses to Angiotensin II in Spontaneously Hypertensive Rats. *J Pharmacol Exp Ther.* 2006;317:1330–1336.



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## ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
Ang II	Angiotensin II
ARB	Angiotensin Receptor Blocker
AT <sub>1/2</sub> R	Angiotensin II Receptor Type I or Type II
BB	Beta Blocker
BIBP	BIBP3226, Y <sub>1</sub> antagonist
BIIE	BIIE0246, Y <sub>2</sub> antagonist
cAMP	Cyclic Adenosine Monophosphate
CCB	Calcium Channel Blocker
DBP	Diastolic Blood Pressure
DPPIV	Dipeptidyl Peptidase IV
G <sub>i</sub>	Inhibitor G Protein
GI	Gastrointestinal Tract
GLPI	Glucagon-Like Protein I
GPCR	G Protein Coupled Receptor
HR	Heart Rate
LPNPY	[Leu31,Pro34] Neuropeptide Y
MABP	Mean Arterial Blood Pressure
NE	Norepinephrine
NPY	Neuropeptide Y
PGMV	Preglomerular Microvessels

PLC	Phospholipase C
PP	Pancreatic Polypeptide
PT	Pertussis Toxin
PYY	Peptide YY (PYY <sub>1-36</sub> )
PYY <sub>3-36</sub>	Truncated Peptide YY
RAS	Renin-Angiotensin System
RBF	Renal Blood Flow
RNS	Renal Nerve Stimulation
RVR	Renal Vascular Resistance
SBP	Systolic Blood Pressure
SHR	Spontaneous Hypertensive Rat
SNS	Sympathetic Nervous System
UK	UK14,304, $\alpha_2$ -adrenoreceptor agonist
WKY	Wistar Kyoto Rat
Y <sub>1/2</sub> R	Y <sub>1</sub> or Y <sub>2</sub> Receptor



## **CHAPTER 1**

# **Introduction**

### **1.1 Hypertension**

Hypertension is a major, worldwide problem. The American Heart Association has published recent estimates that in the United States 1 in 3 adults has high blood pressure, yet most hypertensive adults are unaware of their high blood pressure.[1] This disease, labeled as a “silent killer”, leads to serious complications; including stroke, heart failure, renal disease, and ultimately death. There are two subsets of hypertensive patients; essential hypertensives and secondary hypertensives. While physicians are capable of pinpointing the origin of high blood pressure in individuals that have secondary hypertension, the pathophysiology of essential hypertension remains unclear and is due to a combination of environmental and genetic factors. Although lifestyle modifications in hypertensive patients, such as weight loss, sodium intake reduction, physical activity, and lowering of alcohol consumption, lower blood pressure and have become vital parts in the management of hypertension, patient compliance is problematic.[2] Therefore, a major effort is underway to establish the fundamental causes of hypertension and to develop permanent treatments.

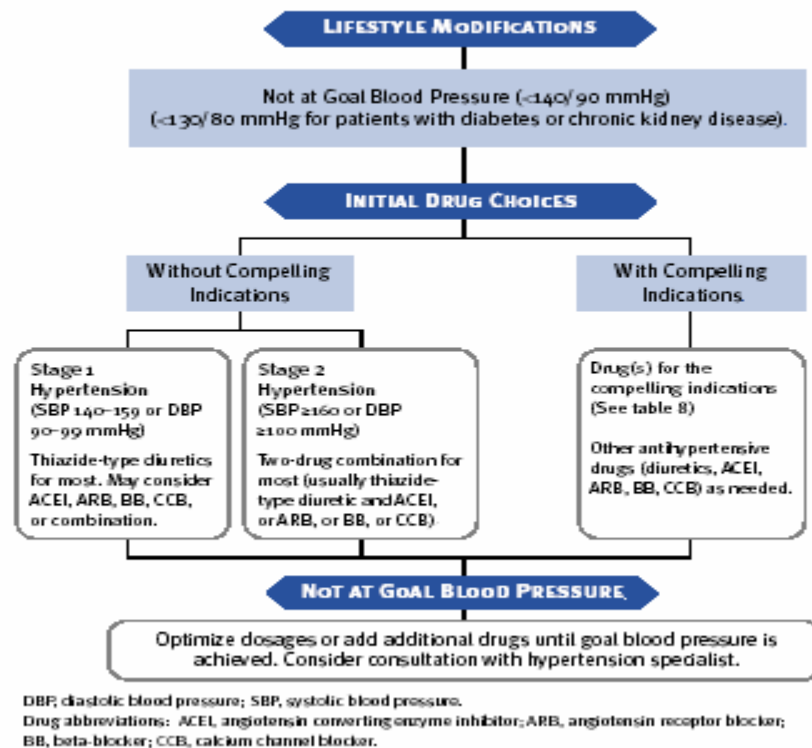
Researchers in the field of hypertension have suggested the theories of an overactive renin-angiotensin system (RAS) and/or an overactive sympathetic nervous system (SNS) as the leading instigators in the development of essential hypertension.[2] To date, the most effective pharmacological treatment of the disease has been through the disruption of the RAS because of its primary role in regulating blood pressure. Normally, juxtaglomerular cells within the kidney

release renin when signals of low blood pressure, low salt content or beta adrenergic activation occur. Renin in turn catalyzes the conversion of angiotensinogen to angiotensin I, and then angiotensin I is rapidly converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE) released from endothelial cells within the kidney. Ang II is the active protein in the RAS, and is responsible for raising arterial blood pressure through primarily the angiotensin type I receptor (AT<sub>1</sub>R) by increasing total peripheral resistance and inhibiting excretion of sodium and water by the kidneys.[3]

Elucidation of RAS has resulted in the development of several classes of drugs that regulate blood pressure in individuals with hypertension: ACE inhibitors block the conversion of Ang I to Ang II; Angiotensin receptor blockers interfere with Ang II communication with its receptor; Beta-blockers inhibit beta-adrenergic activation of renin release; Calcium channel blockers and diuretics increase the excretion of sodium by the kidneys, thus counteracting some of the effects of Ang II. While these drugs have been proven to lower blood pressure, many hypertensives have to take a combination of these drugs to achieve a healthy blood pressure.

The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure has released an algorithm for the treatment of hypertension, figure 1.1. While this figure demonstrates an effective method in treating high blood pressure, many individuals go untreated. In most cases, lifestyle modifications prove to be too difficult for most patients to comply, and the physician then prescribes pharmacological control. However, some patients lack the ability to comply with taking their medication as prescribed, and/or patients with fixed incomes do not have the means to finance the medications needed to effectively treat their high blood pressure. Therefore, only a minority of hypertensives are adequately treated. Clearly, a better

Figure 1. Algorithm for treatment of hypertension



**FIGURE 1.1**

An algorithm for treatment of hypertension published in the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure by the National Institutes of Health.[2]

understanding of the development and maintenance of the disease is needed in order to better treat this wide-spread and devastating disease.

### **1.1.A The Spontaneously Hypertensive Rat**

To experimentally approach the pathophysiology of hypertension, researchers have created a number of animal models of high blood pressure. The spontaneously hypertensive rat (SHR), a genetic model of hypertension, is one such animal model. These rats were developed by selective inbreeding of Wistar Kyoto Rats (WKY) with increased blood pressure by researchers at the Kyoto School of Medicine, Okamoto, Japan in the 1950s.[4,5] In many respects, SHR resemble high blood pressure in the human population (for example, spontaneous elevation of blood pressure with age, sensitivity to antihypertensive drugs effective in humans, and involvement of the RAS and sympathetic nervous system in the development and maintenance of hypertension). Consequently, the SHR is the most studied hypertensive model with close to 13,000 papers to date being published on research with SHR. Even though research on these animals has revealed a vast amount of information, the precise cause of hypertension in these animals is still unknown.

Importantly, the development of hypertension in SHR is dependent on an intact RAS.[4,5] In this regard, treatment of SHR with captopril, an ACE inhibitor, lowers blood pressure to similar levels as its WKY normotensive counterpart. The normalization of blood pressure by blockade of RAS is prolonged even following the removal of drugs that inhibit the RAS pathway suggesting the possibility of an acute preventive pharmacological method.[5] Moreover, transplantation studies reveal that, in addition to the RAS, the SHR kidney is pivotal to the pathophysiology of hypertension in the SHR.[6,7] When normotensive WKY animals are transplanted with an SHR kidney they become hypertensive while the reverse is also true. The

renal sympathetic nervous system also appears to importantly contribute to the pathophysiology of hypertension in SHR. In support of this latter concept, chronic denervation of the SHR kidney both delays the development of hypertension and attenuates the maximum increase in blood pressure in SHR[8-11]. Thus there appears to be a co-involvement of the RAS, the sympathetic nervous system and the kidney in SHR hypertension.

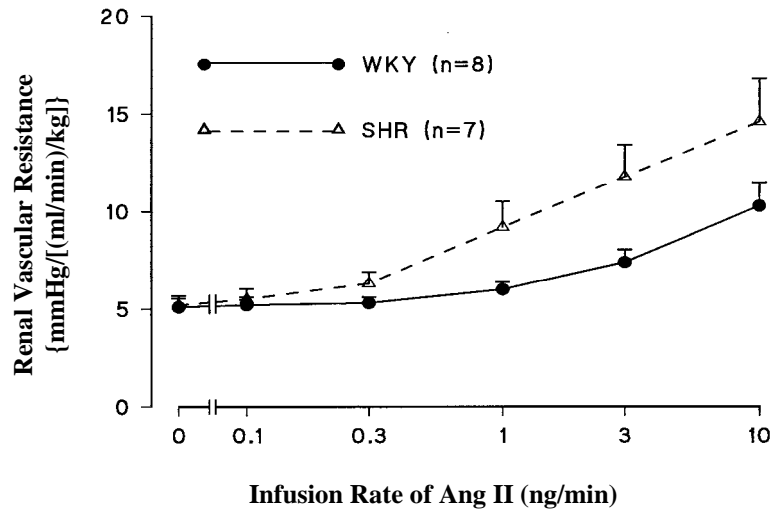
Many studies have been performed in search of a possible explanation for the co-involvement of the RAS and the kidney in SHR hypertension. In this regard, studies do not support an increased expression of renal Ang II receptors or increased levels of circulating or renal Ang II; also, SHR do not have altered renal Ang II degradation rates.[12-15] However, SHR do exhibit increased renovascular responses to Ang II as shown in figure 1.2[16,17], and this appears to be the explanation for the co-involvement of the RAS and the kidney in SHR hypertension.

### **1.1.B Pathophysiology Of Hypertension In The SHR**

Previous research in the Jackson laboratory indicates that the inhibitory G protein ( $G_i$ ) mediates in part the enhanced renovascular response to Ang II in SHR. In this regard, pertussis toxin (PT), an inhibitor of  $G_i$ , abolishes the increased renovascular response to Ang II in SHR as shown in figure 1.3.[18,19] Additionally, figure 1.4a/b, reveals that activation of the  $\alpha_2$ -adrenoreceptor via an  $\alpha_2$ -adrenoreceptor agonist, UK14,304, which is  $G_i$ -coupled, results in potentiation of renovascular responses to Ang II in SHR (b) but not WKY (a). However, pretreatment of SHR with PT blocks the potentiation of Ang II-induced renovasconstriction due to UK14,304.[12,20] Yet, there are many  $G_i$ -coupled receptors, and these previous results suggest the possibility of other agonists causing a potentiation of Ang II-induced renovascular tone in a genetic susceptible model.

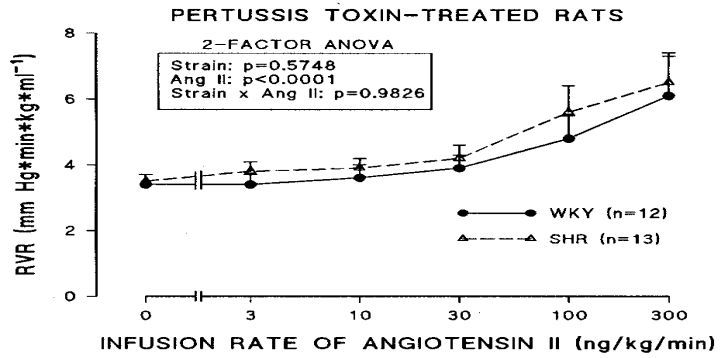
### **1.1.C Hypothesis**

$Y_1$  and  $Y_2$  receptors, PP-fold peptide receptors, are expressed in the kidney and known to activate the  $G_i$ -pathway.[21,22,23] Thus, we hypothesize that these  $G_i$  coupled receptors also have the ability to potentiate Ang II-induced renal responses in the appropriate genetic milieu. In way of background, Section B will review the PP-fold peptides and their receptors.



**FIGURE 1.2**

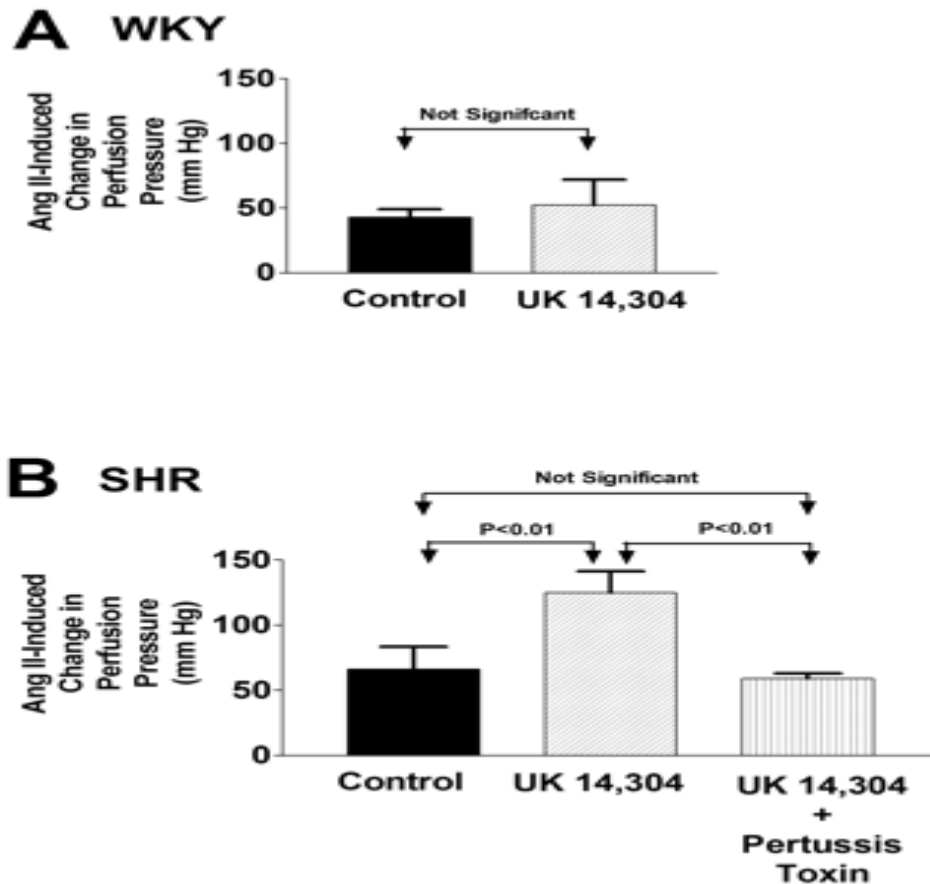
Absolute change in renal vascular resistance following infusion of Ang II of increasing concentrations (0.1, 0.3, 1, 3, 10 nmoles/min) in WKY (circle) and in SHR (triangle). [16]



**FIGURE 1.3**

Absolute change in renal vascular resistance following infusion of Ang II of increasing concentrations (0.1, 0.3, 1, 3, 10 nmoles/min) in pertussis toxin pretreated WKY (circle) and SHR (triangle).[18]





**FIGURE 1.4**

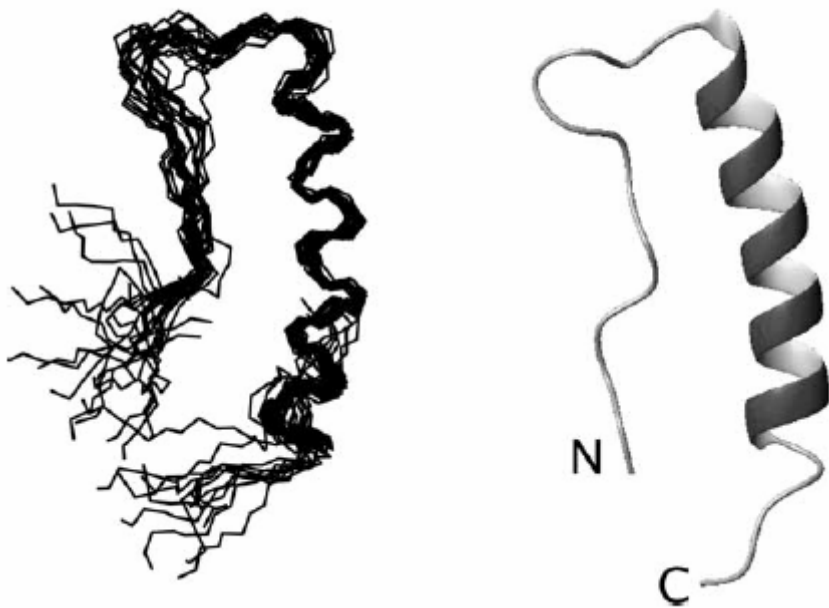
Changes in perfusion pressure induced by angiotensin II (10 nM) in control WKY kidneys (panel A), UK-14,304 (10 nM)-treated WKY kidneys (panel A), control SHR kidneys (panel B), UK-14,304-treated SHR kidneys (panel B), and UK-14,304-treated kidneys removed from SHRs pretreated 3 to 4 days earlier with 30  $\mu$ g/kg pertussis toxin (panel B). Values represent means  $\pm$  S.E.M. from six animals.[20]

## 1.2 PP-Fold Peptides And Their Receptors

The PP-fold peptide family is made up of neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP). These peptides are all 36 amino acids long with an amidated carboxy-terminus, and share a common hairpin-like three dimensional structure, labeled the PP-fold. While each peptide has a different origin, these peptides are all found in the bloodstream and act via multiple G-protein coupled receptors (GPCR). By use of x-ray crystallography and nuclear magnetic resonance, the general structure of these peptides has been elucidated. Figure 1.5 is a structural representation of PYY, which has a similar profile to NPY. [24] Amino acid residues 1-8 form a type II proline helix followed by a loop. Residues 15-32 form an  $\alpha$ -helix, and the four most carboxy-terminal residues are in a flexible loop conformation.[25] The PP-fold peptides are known agonists for the Y receptor family that is  $G_i$  coupled.

### 1.2.A NPY Review

NPY was isolated within the porcine brain in the early 1980s by a method that captures peptides with an amidated-carboxy terminus. [26] Generated from a 97 amino acid precursor, NPY is widely distributed throughout the brain and the periphery.[25,26] NPY is synthesized in the nerve cell body and co-stored with noradrenaline in large dense-cored vesicles. The vesicles are then transported via axonal transport to sympathetic nerve terminals, where the molar ratio of noradrenaline:NPY has been estimated to be about 150:1.[27] NPY is subsequently co-released with sympathetic activation and an influx of calcium into the sympathetic nerve terminal. This effect is increased following  $\alpha$ -adrenoceptor blockade,  $\beta$ -adrenoreceptor stimulation, or Ang II receptor activation.[28-30] The release of NPY is also affected by other hormones involved in energy homeostasis. Ghrelin increases NPY mRNA while leptin inhibits NPY release.[25]



**FIGURE 1.5**

Structure of PYY in solution. Left: ensemble of 20 lowest energy structures superimposed over backbone atoms of residues 17–31 and 5–7. Right: secondary structure of PYY of a representative conformer.[24]

NPY, conserved among most species, is a potent orexigenic peptide leading to an increase in food intake and a decrease in energy expenditure. Within the vasculature system, NPY has been demonstrated as vasoconstrictor as well as stimulator of smooth muscle proliferation.[31] However, centrally administered NPY has alternating effects that include a decrease in food intake and an increase in energy expenditure. Central NPY also reduces arterial blood pressure.[25-31] NPY levels vary in several diseases that may be linked to hypertension. In this respect, several cardiovascular dysfunctions and tumors have been associated with increased NPY plasma levels, and NPY levels are altered in conditions that involve energy balance, such as diabetes.[25]

### **1.2.B NPY Receptor (Y Receptor) Review**

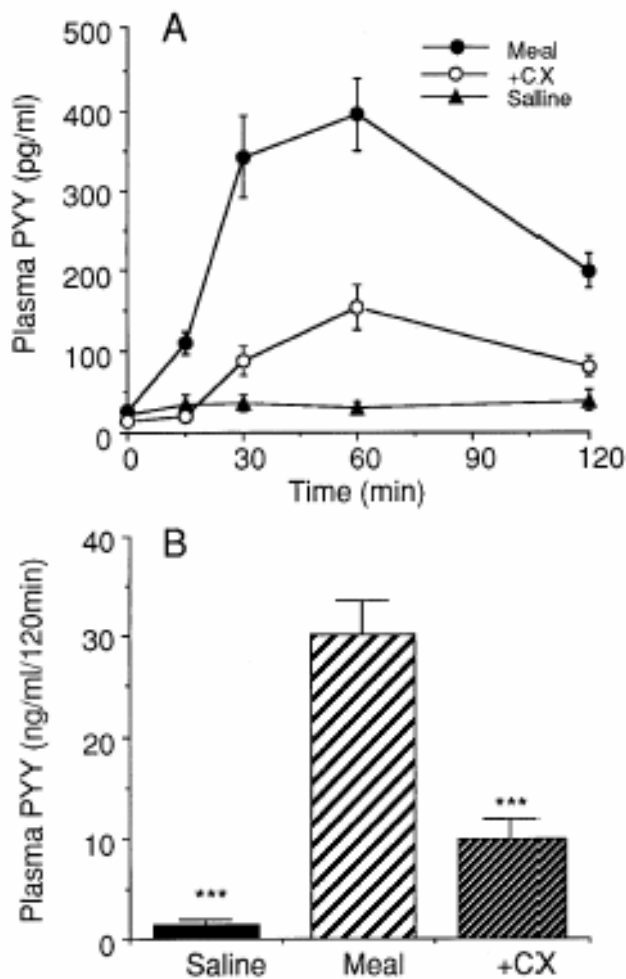
In the late 1980s, NPY postjunctional and prejunctional receptors were postulated, and named  $Y_1$  and  $Y_2$  receptors, respectively ( $Y_1R/Y_2R$ ).[32,33] Since that time, the Y receptor family has grown to at least four subtypes of GPCRs belonging to the rhodopsin-like superfamily of receptors ( $Y_1, Y_2, Y_4, Y_5$ ). NPY binds with high affinity to  $Y_1, Y_2$  and  $Y_5$  receptors, but not  $Y_4$  receptors [25], whereas  $Y_3$  receptors most likely do not exist[33].  $Y_5$  receptors are expressed predominantly in the central nervous system, not the kidney, and it has been suggested that mainly  $Y_1$  and  $Y_2$  are responsible for vascular control.[31,34,35] The  $Y_1$  receptor is located mostly postjunctionally, and is thought to mediate its vasoconstrictive activity by coupling through the cyclic adenosine monophosphate (cAMP)/phospholipase C pathway. On the other hand, the  $Y_2$  is located mostly prejunctionally and has the ability to act as a negative feedback inhibitor decreasing the release of NPY. Although this information seems to suggest an opposing effect with the activation of these receptors, these receptors vary in their action and location.

The  $Y_2$  receptor can cause vasoconstriction, and  $Y_1$  receptors have been found presynaptically.[31] Therefore, because  $Y_1$  and  $Y_2$  receptors coupled to  $G_i$ [33], exist in the kidney and are stimulated by NPY, it is conceivable that the co-involvement of the RAS, sympathetic nervous system and kidney in SHR hypertension is mediated in part by activation of  $Y_1$  and/or  $Y_2$  receptors leading to a potentiation of Ang II-induced renal vasoconstriction in the SHR kidney.

### **1.2.C PYY Review**

Similarly to NPY, PYY was first isolated using a method that captures peptides with an amidated carboxy terminus, but this regulatory peptide was found in the porcine intestine.[26] PYY has tyrosines at both ends of its 36 amino acid sequence and thus its name was derived from this finding. PYY is synthesized and released from endocrine L-cells of the terminal small bowel, caecum, colon, and rectum.[25, 36-48] Researchers have demonstrated that PYY is released from the gastrointestinal tract (GI) in response to meals, and that plasma levels increase approximately 4-fold after 30 minutes of a meal as shown in figure 1.6.[49] While the removal of the ileum and colon result in the loss of PYY release, researchers have observed that direct exposure of PYY-releasing cells to fat leads to the release of the peptide.[45,47] It has been observed that PYY levels begin to increase as early as 15 minutes following a high-fat meal and that these levels remain elevated for more than 6 hours. Researchers have suggested that the release of PYY is therefore regulated by more than direct exposure of fat, but also by some neuronal pathway. The finding of PYY within neurons further corroborates this idea.[43,44,47,49]

PYY is abundant in human blood, and passes through the blood brain barrier via



**FIGURE 1.6**

Peptide YY (*PYY*) release by an intraduodenal meal (3 ml of a semi-liquid diet containing 21 kJ provided as 57% carbohydrate, 13% lipid and 30% protein) in normal rats (*Meal*,  $n = 18$  rats) and in caecocolonectomized rats (*+CX*,  $n = 9$ ). In a third group of 5 rats, saline was administered instead of the meal as a control (*Saline*). **A** Time course of plasma *PYY*. **B** The 120 min integrated *PYY* responses over the basal level. Mean  $\pm$  SEM; \*\*\*  $P < 0.001$  versus Meal [49]

transmembrane diffusion.[48] In this respect, PYY has demonstrated similar activity as other peptides, such as leptin, ghrelin, and NPY, involved in gut-brain communication. Thusly, PYY can affect appetite and influence neuronal activity at the arcuate nucleus.[34] Within the GI, PYY has demonstrated the ability to inhibit gall bladder secretion, gut motility, and pancreatic secretion. PYY has also been shown to inhibit fluid and electrolyte secretion in the intestinal tract functioning as an “ileal brake.”[25,42,49] PYY has also been observed as a vasoconstrictive agent.

Like NPY, PYY acts via the Y receptor family. In this respect, PYY has demonstrated similar affinity to Y<sub>1</sub> and Y<sub>2</sub> receptors as NPY. Table 1.1 compares the affinities of agonists for the receptors.[25]

#### **1.2.D PYY<sub>3-36</sub> Review**

PYY containing endocytes also release a truncated form of PYY, where amino acids 1 and 2 have been removed. Although both PYY peptides are released from the gastrointestinal tract postprandially in direct proportion to meal size, the truncated PYY levels increase more in response to food.[42-46] PYY<sub>3-36</sub> has high affinity for the Y<sub>2</sub> receptor, but has no affinity for the Y<sub>1</sub> receptor. This change in affinity would suggest that PYY<sub>3-36</sub> has opposing effects compared to its full length counterpart. Indeed, researchers have observed that PYY<sub>3-36</sub> is an inhibitor of food intake. It is believed that PYY<sub>3-36</sub> elicits this function by blockade of NPY release through the negative feedback of the Y<sub>2</sub> receptor.[42,46] The blockade of NPY by PYY<sub>3-36</sub> results in activation of adjacent pro-opiomelanocortin neurons which are similar in action to leptin.[39] These effects have suggested PYY<sub>3-36</sub> as a potential candidate in both weight and glucose control.[50]

Table I. Cloned NPY Receptors with Ligand-Binding Profiles

Receptor	Ligand-binding profile	Cloned from (Ref.)
Y1	NPY = PYY = [Leu <sup>27</sup> , Pro <sup>34</sup> ]NPY > NPY2-36 > NPY3-36 ≥ PP > NPY13-36	Human (88, 89) Rat (97, 297) Mouse (298) Guinea pig (299) Dog (105) Pig (105, 139) Rhesus monkey (300) Xenopus laevis (190) Chicken (189)
Y2	NPY ≥ NPY2-36 = NPY3-36 = NPY13-36 >> [Leu <sup>27</sup> , Pro <sup>34</sup> ]NPY	Human (134–136, 205) Rat (204, 301) Mouse (302) Guinea pig (138) Pig (105, 139) Cow Accession no: AAB40600 Rhesus monkey (300) Chicken (140)
Y4	PP > PYY ≥ NPY > NPY2-36	Human (90, 95, 154) Rat (152, 154) Mouse (303) Guinea pig (155) Pig (139) Chicken (304)
Y5	NPY = PYY = NPY2-36 > hPP > [D-Trp <sup>22</sup> ]NPY > NPY13-36 > rPP	Human (91, 164) Rat (91, 164) Mouse (165, 166) Guinea pig (168) Pig (139) Dog (166) Rhesus monkey (300) Chicken (189)
y6*	1) NPY = PYY = [Leu <sup>27</sup> , Pro <sup>34</sup> ]NPY >> PP 2) PP > [Leu <sup>27</sup> , Pro <sup>34</sup> ]NPY > NPY = PYY	Human (183, 184, 186) Mouse (184, 185) Rabbit (183) Guinea pig (188) Pig (139)

\* Two different labs cloned the mouse y6 receptor but reported very different binding profiles: 1) according to (185) and 2) according to (184). No pharmacology is available for the truncated human, guinea pig, and pig y6 receptors.

Table 1.1

Cloned Y receptors and affinity profiles for each form.[25]



### **1.2.E DPPIV Review**

Although both PYY constructs are released from L-cells within the GI, it is thought that PYY is the proform, whereas PYY<sub>3-36</sub> has been labeled the active form. The conversion of PYY to PYY<sub>3-36</sub> is created via an aminopeptidase, dipeptyl dipeptidase IV (DPPIV).[51-56] DPPIV has gained a lot of attention lately because of its proposed role in glucose homeostasis. The 110-150 kDa protein is ubiquitously expressed as a homodimeric membrane-bound protein that can be shed into a soluble form.[55] The highest concentrations have been found on the proximal tubules in the kidney and epithelial cells of the small intestines. Although researchers have demonstrated many peptides cleaved by DPPIV, DPPIV inhibitors have taken the highlight of DPPIV research because of their ability to lower blood glucose. Glucagon-like protein I (GLPI) stimulates insulin secretion and biosynthesis while inhibiting the release of glucagon. GLPI is a substrate for DPPIV and is cleaved to a truncated non-active form.[51-54] Therefore, inhibition of DPPIV would lead to increases in GLP and decreases in blood glucose that could prove beneficial in type 2 diabetes. On the other hand, as mentioned previously DPPIV has demonstrated activating ability with PYY that would lead to a construct that would work primarily through the Y<sub>2</sub> receptor. Thus, DPPIV may be a factor in determining which Y receptor is activated.

### **1.3 Detailed Hypothesis And Objectives**

The focus of this project is the ability of PP-fold peptides to augment Ang II-induced renovascular responses in SHR and WKY kidneys. The rationale for this focus is that 1) the G<sub>i</sub> pathway appears to augment renovascular responses to Ang II in SHR, but not WKY, and 2) receptors for PP-fold peptides appear to activate the G<sub>i</sub> pathway. The significance of this project

is that 1) NPY is released from renal sympathetic nerves in response to sympathetic activation, and 2) PYY and PYY<sub>3-36</sub> are released from the intestines in response to a meal. Therefore our **HYPOTHESIS** is that in genetically predisposed individuals (i.e. individuals with “SHR-like” kidneys), PP-fold peptides acting on PP-fold receptors augment renovascular responses to endogenous Ang II and contribute to the etiology of hypertension.

In this respect, the following **OBJECTIVES** were undertaken:

- 1) Determine whether Y<sub>1</sub> receptor activation potentiates renal vasoconstriction in SHR or WKY kidneys (chapter 3)
- 2) Determine whether Y<sub>2</sub> receptor activation potentiates renal vasoconstriction in SHR or WKY kidneys (chapter 3)
- 3) Compare the effects of the naturally occurring PP-fold peptides in their ability to augment renovascular responses to Ang II in SHR kidneys (chapter 4)
- 4) Determine whether the activation of the  $\alpha_2$ -adrenoreceptor adds to any augmentation of Ang II induced renovascular responses by the PP-fold peptides in SHR (chapter 4)
- 5) Determine whether endogenous PP-fold peptides released during renal nerve stimulation regulate renovascular responses to Ang II in SHR or WKY kidneys (chapter 5)
- 6) Determine whether blockade of PYY conversion to PYY<sub>3-36</sub> via DPPIV has an effect on arterial blood pressure in SHR or WKY (chapter 6)

## **CHAPTER 2**

# **EXPERIMENTAL METHODS**

### **2.1 Drugs.**

Ang II, NPY, PYY (sometimes referred to as PYY<sub>1-36</sub>), PYY<sub>3-36</sub>, [Leu<sup>31</sup>,Pro<sup>34</sup>]-neuropeptide Y (LPNPY), aldosterone, hydrocortisone, captopril and UK14,304 were obtained from Sigma (St. Louis, MO). BIBP3226 and BIIE0246 were obtained from Tocris (Ellisville, MO).

### **2.2 Animals.**

Studies utilized adult (14-16 weeks-of-age) male SHR or WKY obtained from Taconic Farms (Germantown, NY). The Institutional Animal Care and Use Committee approved all procedures. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### **2.3 Experiments In Isolated, Perfused Kidneys.**

SHR and WKY were anesthetized with Inactin (90 mg/kg, i.p.), and the left kidney was isolated and perfused with Tyrode's solution using a Hugo Sachs Elektronik-Harvard Apparatus GmbH (March-Hugstetten, Germany) kidney perfusion system as previously described[15]. Briefly, all branches of the left renal artery and vein were ligated. A PE-50 cannula was placed into the left renal artery, and a PE-90 cannula was placed into the left renal vein. The left kidney

was removed, attached to the perfusion system and allowed to stabilize for an hour before the experimental protocol. Kidneys were perfused (single pass mode) at a constant flow (5 ml/min), and perfusion pressure was monitored with a pressure transducer.

### **2.3.A Protocol 1**

Ang II (Sigma) was infused at increasing doses to provide nominal concentrations in the perfusate of 0.3, 1, or 3 nmoles/L. Each dose of Ang II was infused for two minutes, and the perfusion pressure was allowed to return to basal levels over the next five minutes before initiating the next higher dose of Ang II. After the highest dose of Ang II, and following a rest period of 15 minutes, LPNPY (Sigma) was infused into the kidney to provide a concentration in the perfusate of 10 nmoles/L. Ten minutes into the infusion of LPNPY, the kidney was restimulated with Ang II. The response to Ang II was taken as the change in perfusion pressure during the Ang II infusion and was calculated as the perfusion pressure recorded at the end of the infusion of Ang II minus the basal perfusion pressure recorded just before the Ang II infusion. Time control experiments demonstrated that concentration-response curves to Ang II were stable over the duration of the experiment.

### **2.3.B Protocol 2**

Ang II was infused to achieve a concentration in the perfusate of 0.3 nmoles/L. After 10 minutes, the infusion of Ang II was stopped, and the perfusion pressure was allowed to return to basal levels over the next 10 minutes, then LPNPY or PYY<sub>3-36</sub> was infused into the kidney to provide a final concentration in the perfusate of 6 nmoles/L. Twenty minutes into the infusion of LPNPY or PYY<sub>3-36</sub>, the kidney was restimulated with Ang II for 10 minutes. Changes in

perfusion pressure in response to Ang II were calculated as described above. Some of these experiments were conducted in kidneys obtained from SHR pretreated with pertussis toxin. Also, some of these experiments were conducted in kidneys in which BIBP3226 or BIIE0246 was infused into the perfusate to provide a concentration in the perfusate of 1  $\mu$ mole/L. The infusions of BIBP3226 or BIIE0246 were initiated at the beginning of the one-hour rest period and continued until the end of the protocol.

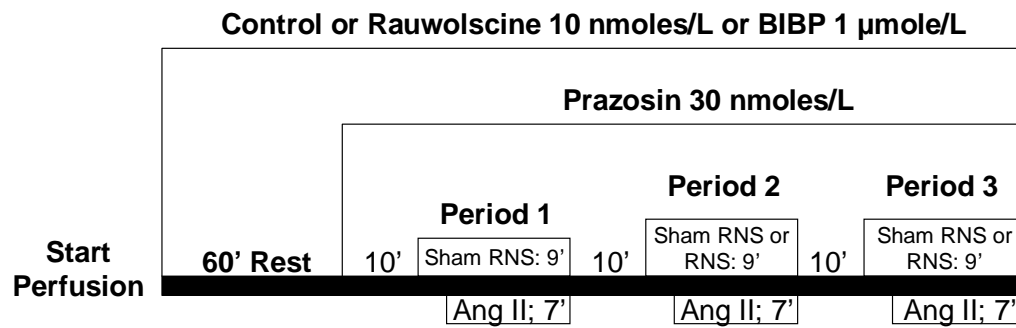
### **2.3.C Protocol 3**

After the stabilization period, Ang II was infused to achieve a nominal concentration in the perfusate of 0.3 nmoles/L. After 10 minutes, the infusion of Ang II was stopped, and the perfusion pressure was allowed to return to basal levels over the next 10 minutes. Then the following agents were infused to achieve the indicated nominal concentration in the perfusate: NPY (0.1 nmoles/L), NPY (1 nmole/L), NPY (6 nmoles/L), PYY<sub>1-36</sub> (0.1 nmoles/L), PYY<sub>1-36</sub> (1 nmole/L), PYY<sub>1-36</sub> (6 nmoles/L), PYY<sub>3-36</sub> (0.1 nmoles/L), PYY<sub>3-36</sub> (1 nmoles/L), PYY<sub>3-36</sub> (6 nmoles/L), UK14,304 (10 nmoles/L), NPY (1 nmole/L) + UK14,304 (10 nmoles/L), PYY<sub>1-36</sub> (1 nmole/L) + UK14,304 (10 nmoles/L), PYY<sub>3-36</sub> (1 nmole/L) + UK14,304 (10 nmoles/L), NPY (6 nmoles/L) + BIBP3226 (1  $\mu$ mole/L), PYY<sub>1-36</sub> (6 nmoles/L) + BIBP3226 (1  $\mu$ mole/L) or PYY<sub>3-36</sub> (6 nmoles/L) + BIIE0426 (1  $\mu$ mole/L). In the case of those kidneys treated with BIBP3226 or BIIE0226, these antagonists were infused beginning from the outset of perfusion (i.e., during the stabilization period). Because a given kidney received only one treatment, this protocol represented 16 distinct groups with 6 to 7 kidneys per group (total of 98 kidney perfusion experiments). Twenty minutes into the treatments, the kidney was restimulated with Ang II for 10 minutes. By using only a low concentration of Ang II and by limiting the experiment to two

challenges with Ang II, tachyphylaxis to Ang II was avoided and responses to Ang II were stable. The response to Ang II was taken as the change in perfusion pressure during the Ang II infusion and was calculated as the perfusion pressure recorded at the end of the infusion of Ang II minus the basal perfusion pressure recorded just before the Ang II infusion.

#### **2.3.D Protocol 4**

Experiments were conducted in 66 isolated, perfused rat kidneys using the protocol outlined in figure 2.1. Immediately after initiating perfusion of the kidney, a platinum bipolar electrode was placed around the renal artery for renal nerve stimulation (RNS). The electrode was connected to a Grass stimulator (model SD9E; Grass Instruments, Quincy, MA). After a 60 minute rest period, prazosin (30 nmoles/L) was added to the perfusate to block  $\alpha_1$ -adrenoceptors so that RNS would not cause direct vasoconstriction and increase basal vascular tone. This was necessary because changes in basal vascular tone might non-specifically elevate responses to Ang II. Ten minutes after adding prazosin to the perfusate, RNS was simulated by going through the motions of activating the stimulator while not actually activating the stimulator (sham RNS). Two minutes into the sham RNS, Ang II was infused into the renal artery for seven minutes to provide a final concentration of 100 pmoles/L, which is a physiological level of Ang II in SHR as recently determined by capillary electrophoresis[57]. This first response to Ang II was designated period 1. At the end of the Ang II infusion, the kidney was allowed a rest period of 10 minutes, then the kidney was subjected to either sham RNS or RNS (biphasic, 5 Hz, 1 millisecond pulse duration, 35 volts) for 9 minutes. This frequency of RNS is well within the physiological range[21]. Two minutes into the sham RNS or RNS, Ang II was infused once again for seven minutes to provide a final concentration of 100 pmoles/L. This second response



**FIGURE 2.1**

Overview of the experimental protocol. RNS, periarterial renal nerve stimulation; Ang II, angiotensin II.

to Ang II was designated period 2. Next, the periarterial electrodes were repositioned, and after another 10-minute rest period, a third response to Ang II was obtained in the absence (sham RNS) or presence (RNS) of renal sympathetic activation (period 3). Kidneys were randomly assigned to receive either sham RNS during periods 2 and 3 or active RNS during periods 2 and 3.

In some experiments, either rauwolscine (10 nmoles/L, a highly selective  $\alpha_2$ -adrenoceptor antagonist [58]) or BIBP3226 (1  $\mu$ mole/L, a highly selective  $Y_1$  receptor antagonist [25]) was added to the perfusate at the beginning of kidney perfusion. Also, some kidneys were removed from rats that had been pretreated three days earlier with an iv injection of pertussis toxin (30  $\mu$ g/kg) to block  $G_i$  proteins as previously described by us [18]. Kidneys were randomly assigned to either no inhibitor, rauwolscine, or BIBP3226.

## **2.4 Experiments In Kidney *In Vivo*.**

### **2.4.A Protocol 1**

SHR (n=31) and WKY (n=31) were anesthetized with Inactin (90 mg/kg, i.p.), and animals were prepared as previously described [59]. Briefly, cannulas were inserted into the trachea, jugular vein and carotid artery. A digital blood pressure analyzer measured MABP via the carotid artery cannula. To remove the influence of endogenous catecholamines on  $\alpha_2$ -adrenoceptors, both adrenal glands were removed and the left kidney was denervated. To replace loss of adrenal steroids, aldosterone and hydrocortisone were infused. A transit-time flow probe was positioned around the left renal artery to monitor renal blood flow (RBF). A 32-gauge needle connected to a catheter was placed into the renal artery, and animals were given a bolus



injection of captopril (30 mg/kg) to remove the influence of endogenous Ang II and a bolus of saline to improve hemodynamic stability. After one hour, SHR and WKY received an intrarenal infusion of either vehicle (control; n=7), UK14,304 (0.3 µg/kg per min; n=7), LPNPY (1 µg/kg/min; n=7) or UK14,304 + LPNPY (n=10). Twenty minutes later while continuing the treatments, RBF and MABP were recorded just before and during the last minute of a five-minute intrarenal infusion of Ang II (10 ng/kg per minute). Renovascular resistance (RVR) was calculated by dividing RBF per gram kidney weight into the MABP.

#### **2.4.B Protocol 2**

SHR (n=26) and WKY (n=12) were anesthetized with Inactin (90 mg/kg, i.p.), and animals were prepared as previously described [59]. Briefly, cannulas were inserted into the trachea, jugular vein and carotid artery. A digital blood pressure analyzer measured MABP via the carotid artery cannula. After one hour, SHR and WKY received an intravenous infusion via the jugular vein of either vehicle, P32/98 (0.3, 1, 3 mg/kg), or P32/98 plus BIBP3226 (40 µg/kg). MABP were recorded just before and during the last minute of a ten-minute interval.

#### **2.5 RT-PCR For Y<sub>1</sub> Or Y<sub>2</sub> Receptor mRNA.**

Y<sub>1</sub> or Y<sub>2</sub> receptor mRNA was obtained from SHR and WKY preglomerular microvessels (PGMV) and whole kidneys and measured as previously described for other receptors [15]. Briefly, PGMVs were obtained by iron oxide injection into the renal artery followed by magnetic retrieval of PGMVs. Total RNA was isolated from PGMVs or whole kidney with TRIzol reagent. RNA was reverse transcribed and amplified using a Titanium One-Step RT-PCR Kit.

For the Y<sub>1</sub> receptor, the forward primer was 5'-CTGATCGTGAACCTCTCCTTCT-3' and the reverse primer was 5'-GTCGTGTAAGACAGCCTGTGAG-3'. For the Y<sub>2</sub> receptor, the forward primer was 5'-GGTCTGGCAGTACAAGTGTCC-3' and the reverse primer was 5'-GGTCGTTTTGTGCCTTCGCTG-3'. Each PCR cycle consisted of denaturing at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 60 seconds. RT-PCR products were separated on a 1.2% agarose gel and gels were stained with ethidium bromide.

## **2.6 Western Blotting For Y<sub>1</sub> Or Y<sub>2</sub> Receptor Protein.**

Y<sub>1</sub> or Y<sub>2</sub> receptor protein was obtained from PGMVs and whole kidneys and measured as previously described for other receptors [15]. Briefly, protein samples from whole kidneys and PGMVs were loaded onto a 7.5% acrylamide gel and subjected to SDS-PAGE using the Bio RAD mini-gel system. Proteins were electroblotted onto PVDF membranes. Membranes were blocked with 5% milk then incubated with primary antibody to either the Y<sub>1</sub> or Y<sub>2</sub> receptor (Sigma). After washing, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody. Membranes were then exposed to films, and the signals were detected by a Supersignal Substrate kit. Band densities were quantitatively measured using Scion Image software.

## **2.7 Statistical Analysis.**

Data were analyzed by paired or unpaired two-tailed Student's t-test or one-factor or two-factor analysis of variance, as appropriate. The Fisher's Least Significant Difference (LSD) test was used for post-hoc analyses if a significant analysis of variance was obtained. The criterion of significance was P<0.05. All data are presented as means ± SEM.

## CHAPTER 3

### PP-Fold Peptides And Ang II-Induced Renal Vasoconstriction

#### 3.1 Introduction

There appears to be a co-involvement of the RAS, the sympathetic nervous system, and the kidney in SHR hypertension. Our previous research indicates that  $G_i$  mediates the enhanced renovascular response to Ang II in SHR as demonstrated in figure 1.2. Activation of renal sympathetic nerves leads to the release of NPY, and consumption of a high fat diet results in the release of PYY<sub>1-36</sub>/PYY<sub>3-36</sub>. NPY and PYY<sub>1-36</sub>/PYY<sub>3-36</sub> bind Y receptors found within the renal vasculature.[25] It is conceivable that the coinvolvement of the RAS, sympathetic nervous system, and the kidney in SHR hypertension is partially mediated by activation of Y receptors leading to a potentiation of Ang II-induced renal vasoconstriction in the SHR kidney.

The purpose of the present study was to test the hypothesis that  $Y_1$  and/or  $Y_2$  receptor activation potentiates renal vascular responses to Ang II selectively in SHR kidneys by a mechanism involving inhibitory G proteins. In this regard, we examined the effects of LPNPY, a highly selective  $Y_1$  agonist, and PYY<sub>3-36</sub>, a highly selective  $Y_2$  agonist, on renovascular responses to Ang II in SHR and WKY kidneys *in vitro* in the absence and presence of a highly selective  $Y_1$  antagonist BIBP3226, a highly selective  $Y_2$  antagonist BIIE0246, or pertussis toxin to inhibit G proteins.

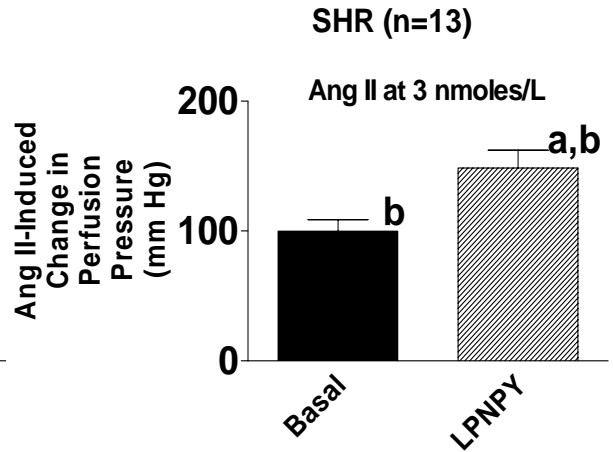
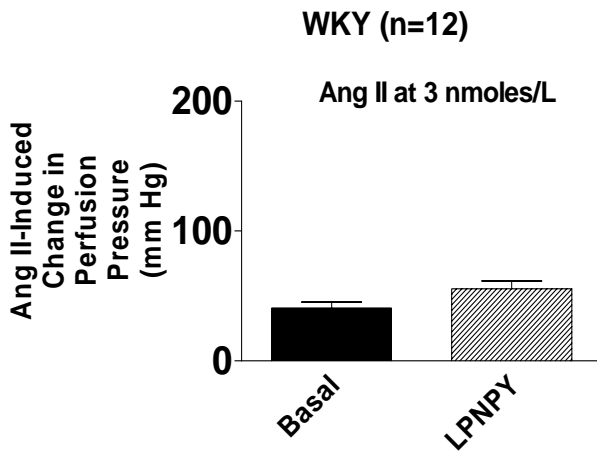
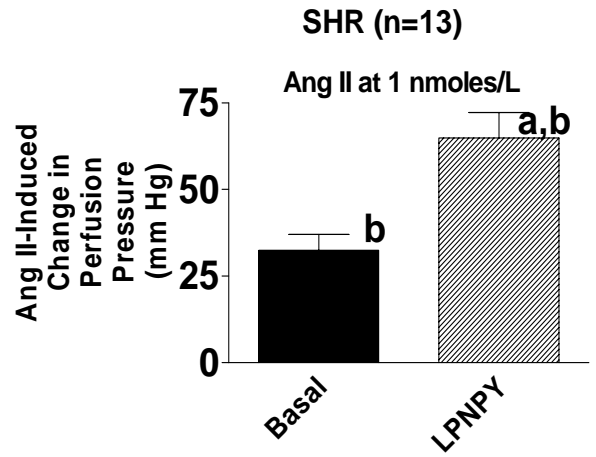
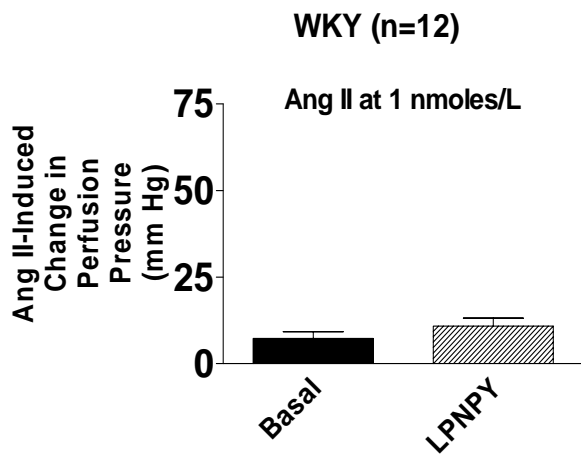
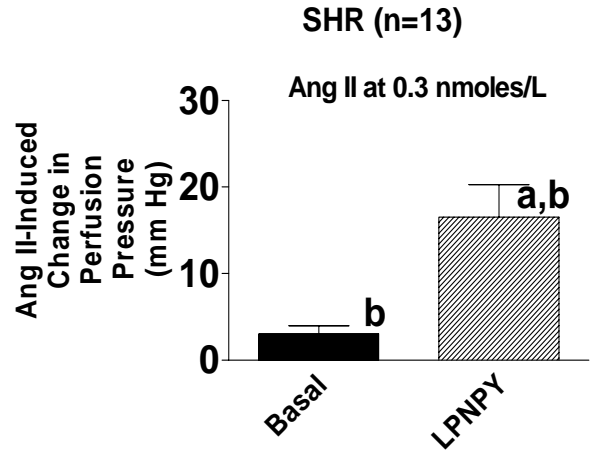
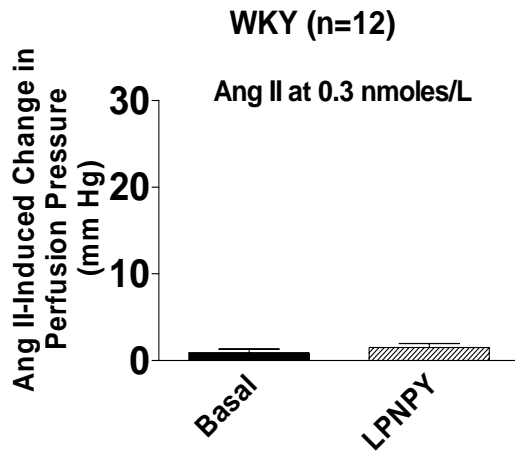
#### 3.2 Results

**Basal Renal Perfusion Pressures.** In this study, baseline renal perfusion pressures were similar ( $60 \pm 3$  and  $52 \pm 2$  mm Hg) in WKY and SHR kidneys, respectively, and were not affected by any of the various treatments or combinations of treatments.

**Experiments in Isolated, Perfused Kidneys with LPNPY.** As shown in the left panel of figure 3.1, Ang II caused a significant concentration-dependent (0.3, 1 and 3 nmoles/L) increase in renal perfusion pressure in WKY. Ang II also caused a significant concentration-dependent increase in renal perfusion pressure in SHR kidneys (figure 3.1, right panel). Ang II-induced increases in renal perfusion pressure were significantly greater in SHR compared with WKY kidneys (right versus left panels in figure 3.1). In SHR kidneys, and at all concentrations of Ang II, LPNPY significantly potentiated Ang II-induced increases in renal perfusion pressure (figure 3.1, left panel). In contrast, in WKY kidneys, LPNPY did not augment Ang II-induced increases in renal perfusion pressure regardless of the concentration of Ang II (figures 3.1, right panel). Two-factor analysis of variance demonstrated a significant interaction between rat strain and LPNPY on Ang II-induced changes in perfusion pressure ( $P=0.003$ ,  $P<0.001$  and  $P=0.004$  for 0.3, 1 and 3 nmoles/L Ang II, respectively).

### **FIGURE 3.1**

Absolute change in perfusion pressure of isolated, perfused kidneys following infusion of Ang II (final concentration of 0.3, 1, and 3 nmoles/L, respectively) in the absence (Basal) and presence of LPNPY (final concentration of 10 nmoles/L) in WKY (left panel) and SHR (right panel) kidneys. “a” indicates significantly different (Fisher’s LSD test) within strain comparing without and with LPNPY. “b” indicates significantly different (Fisher’s LSD test) between strains at same level of LPNPY. Values are means  $\pm$  SEM.



In a separate set of experiments, in naïve SHR kidneys LPNPY again markedly and significantly potentiated the ability of Ang II (0.3 nmoles/L) to increase renal perfusion pressure (figure 3.2A). The ability of LPNPY to potentiate Ang II-induced renal vasoconstriction was abrogated by BIBP3226 (figure 3.2B,D) and pertussis toxin (figure 3.2C,D). Moreover, Ang II-induced renal vasoconstriction was significantly attenuated by BIBP3226 and pertussis toxin even in the absence of LPNPY (compare basal responses to Ang II in A and B/C graphs of figure 3.2).

***RT-PCR for Y<sub>1</sub> Receptor mRNA and Western Blotting for Y<sub>1</sub> Receptor Protein.*** As shown in Figure 3.3, mRNA and protein expression for Y<sub>1</sub> receptors was detected in whole kidney and PGMVs from both WKY and SHR. However, the expression of Y<sub>1</sub>-receptor mRNA and protein when normalized to beta-actin and quantified by densitometry did not differ between WKY and SHR tissues (Figure 3.4).

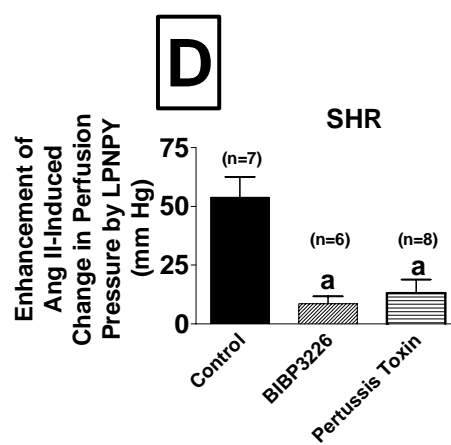
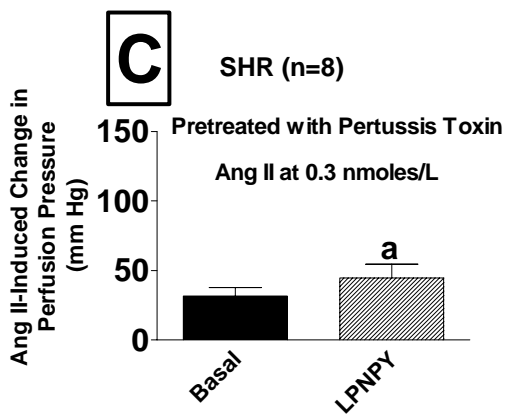
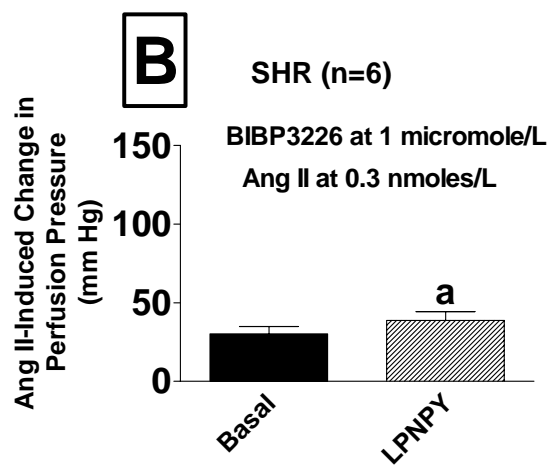
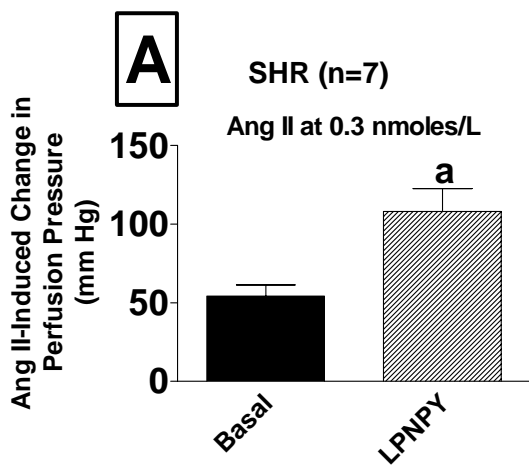
***Experiments in Isolated, Perfused Kidneys with PYY<sub>3-36</sub>.*** As shown in figure 3.5A, PYY<sub>3-36</sub> caused a very small, but statistically significant and reproducible, potentiation of Ang II-induced renal vasoconstriction (P=0.0014). However, in WKY, PYY<sub>3-36</sub> did not significantly alter Ang II-induced renal vasoconstriction (data not shown). The difference in Ang II-induced renovascular changes in SHR versus WKY was statistically significant (P= 0.0017).

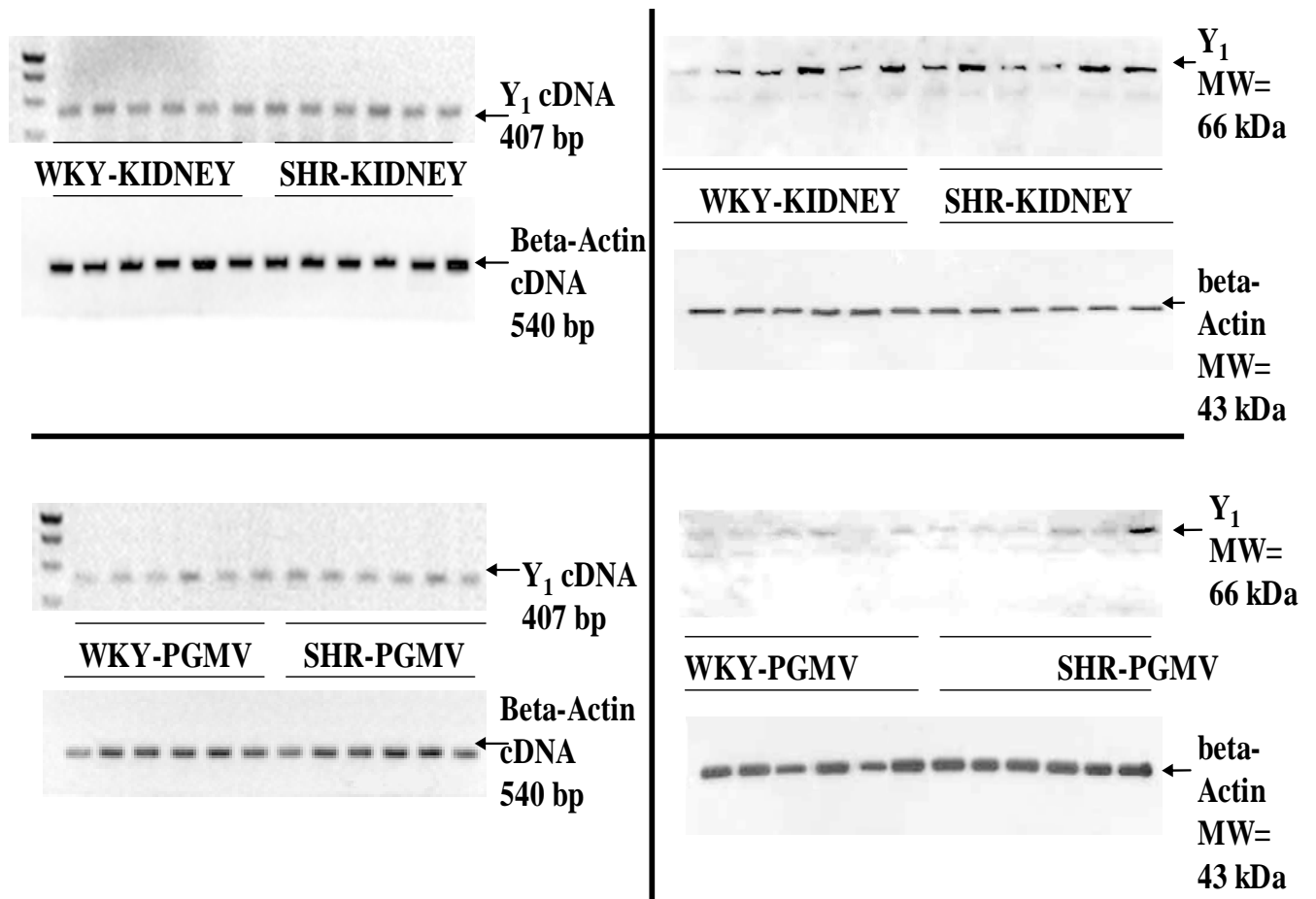
As shown in figures 3.5B and 3.5C, respectively, BIIE0246 and pertussis toxin completely blocked PYY<sub>3-36</sub>-induced potentiation of Ang II-induced changes in perfusion pressure. Consistent with our previous experiments, Ang II-induced renal vasoconstriction was

### **FIGURE 3.2**

Absolute change in perfusion pressure of isolated, perfused kidneys after infusion of Ang II (final concentration in the perfusate of 0.3 nmol/L) in the absence (Basal) and presence of LPNPY (final concentration in the perfusate of 6 nmol/L) in untreated SHR kidneys (A), in SHR kidneys pretreated with BIBP3226 (1  $\mu$ mol/L; B), and in kidneys obtained from SHR pretreated with intravenous pertussis toxin 3 days before removing the kidney for perfusion (C). D illustrates the enhancement of Ang II–induced changes in perfusion pressure by LPNPY in control, BIBP3226-treated and pertussis toxin–treated kidneys. In A, B, and C, “a” indicates significant difference (Student t test) comparing without and with LPNPY. In D, “a” indicates significant difference between BIBP3226 and control and pertussis toxin and control (Fisher LSD test). Values are mean  $\pm$  SEM.

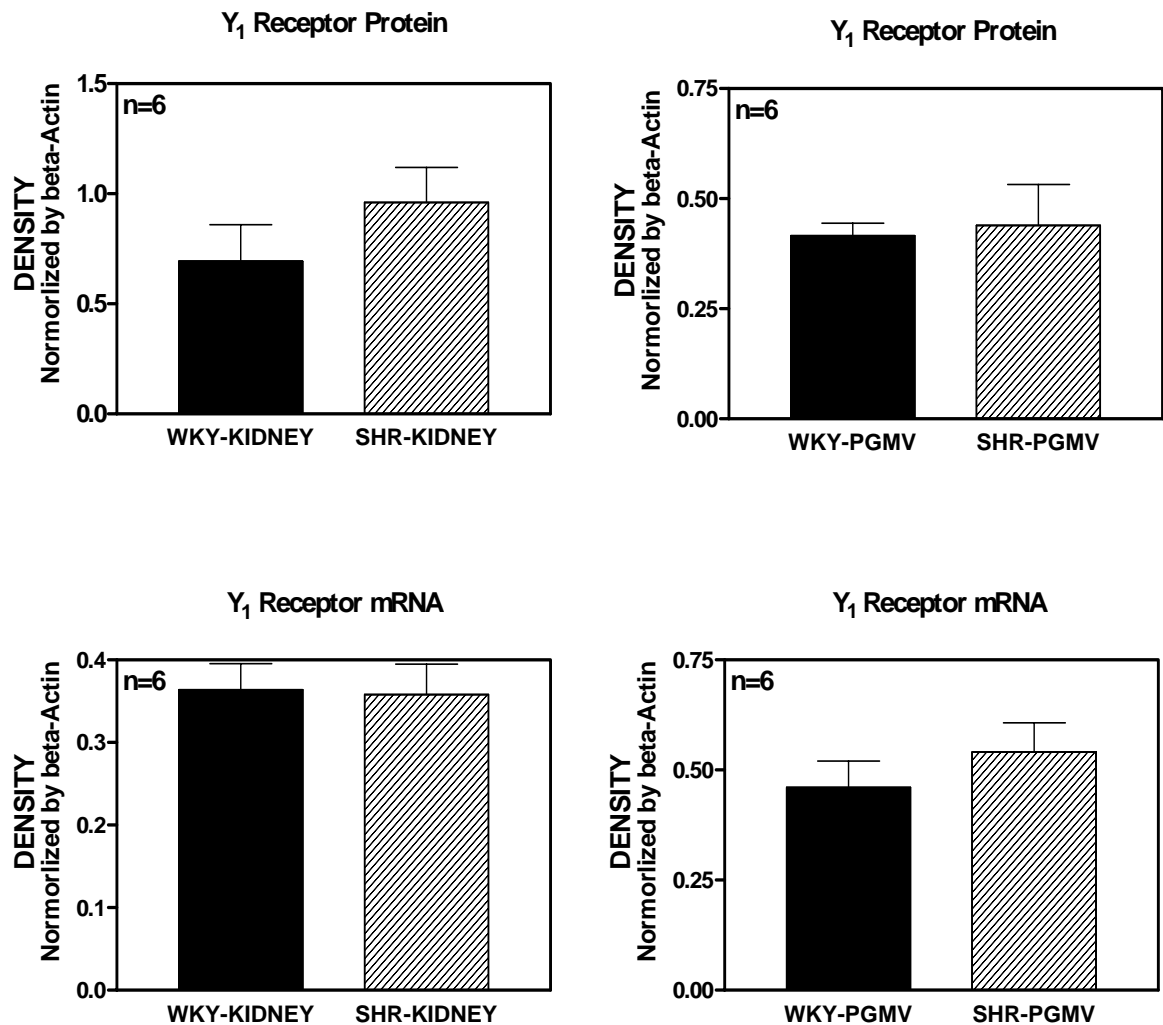






**FIGURE 3.3**

RT-PCR products for Y<sub>1</sub> receptor mRNA and beta-actin mRNA in WKY and SHR kidneys (top panel left) and in preglomerular microvessels (PGMV; bottom panel left). Western blot analysis of Y<sub>1</sub> receptor and beta-actin in WKY and SHR kidneys (top panel right) and preglomerular microvessels (PGMV; bottom panel right).



**FIGURE 3.4**

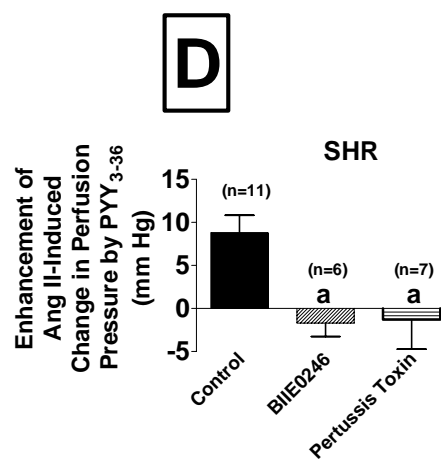
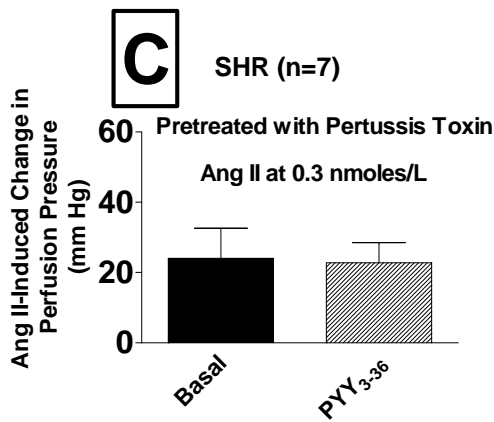
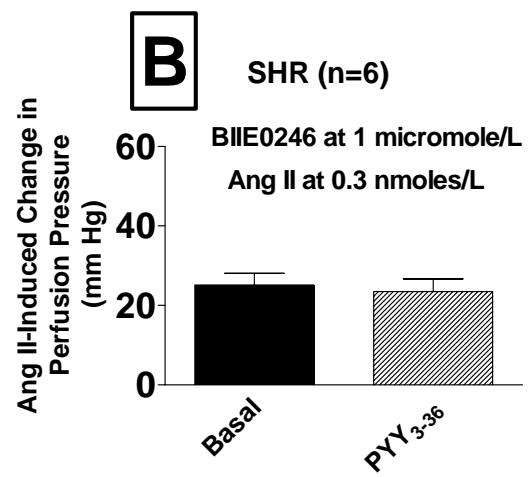
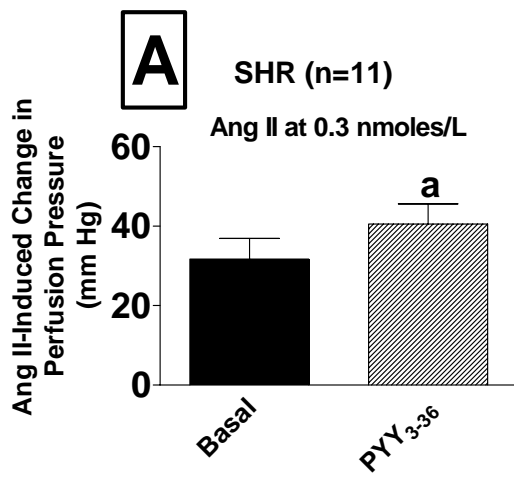
Top panels: Densitometry results (normalized to beta-actin signal) from Western blot analysis of Y<sub>1</sub> receptor mRNA in WKY versus SHR kidneys (left panel) and preglomerular microvessels (PGMV; right panel). Bottom panels: Densitometry results (normalized to beta-actin signal) from RT-PCR products for Y<sub>1</sub> receptor mRNA in WKY versus SHR kidneys (left panel) and preglomerular microvessels (PGMV; right panel).

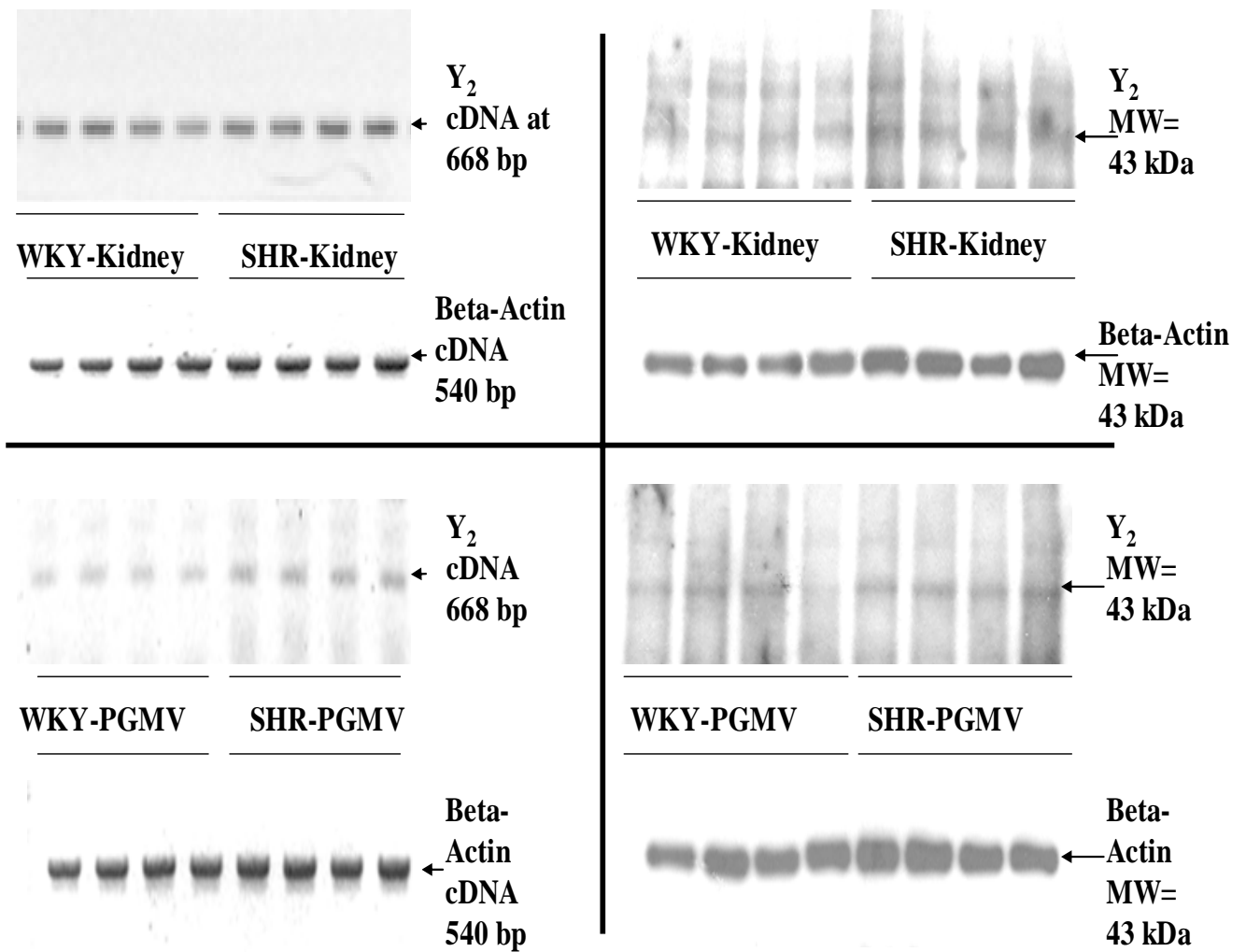
significantly attenuated by pertussis toxin even in the absence of PYY<sub>3-36</sub> (compare basal responses to Ang II in figures 3.5A versus 3.5C).

***RT-PCR for Y<sub>2</sub> Receptor mRNA and Western Blotting for Y<sub>2</sub> Receptor Protein.*** As shown in figure 3.6, mRNA and protein expression for Y<sub>2</sub> receptors was detected in whole kidney and PGMVs from both WKY and SHR. However, the expression of Y<sub>2</sub> receptor mRNA and protein when normalized to beta-actin and quantified by densitometry did not differ between WKY and SHR tissues (data not shown).

### FIGURE 3.5

Absolute change in perfusion pressure of isolated, perfused kidneys after infusion of Ang II (final concentration in the perfusate of 0.3 nmol/L) in the absence (Basal) and presence of PYY<sub>3-36</sub> (final concentration in the perfusate of 6 nmol/L) in untreated SHR kidneys (A), in SHR kidneys pretreated with BIIE0246 (1 μmol/L; B), and in kidneys obtained from SHRs pretreated with intravenous pertussis toxin 3 days before removing the kidney for perfusion (C). D illustrates the enhancement of Ang II–induced changes in perfusion pressure by PYY<sub>3-36</sub> in control, BIIE0246-treated, and pertussis toxin–treated kidneys. In A, B, and C, “a” indicates significant difference (Student t test) comparing without and with PYY<sub>3-36</sub>. In D, “a” indicates significant difference between BIIE0246 and control and pertussis toxin and control (Fisher LSD test). Values are mean ± SEM.





**FIGURE 3.6**

RT-PCR products for  $Y_2$  receptor mRNA and beta-actin mRNA in WKY and SHR kidneys (top panel right) and in preglomerular microvessels (PGMV; bottom panel right). Western blot analysis of  $Y_2$  receptor and beta-actin in WKY and SHR kidneys (top panel left) and preglomerular microvessels (PGMV; bottom panel left).

### 3.3 Summary

The results in chapter three focus on the role of the  $Y_1$  and  $Y_2$  receptors' ability to augment Ang II induced renal vasoconstriction. In this respect LPNPY, a highly selective  $Y_1$  receptor agonist, greatly enhances the renovascular responses to Ang II in the SHR kidney, yet does not potentiate responses to Ang II in the WKY kidney. Moreover, our findings demonstrate that this effect is dependent on an intact  $Y_1$ - $G_i$  pathway. In this regard, we observe two findings: First, BIBP3226, a highly selective  $Y_1$  receptor antagonist, blocks the ability of LPNPY to enhance renovascular responses to Ang II, and second, pertussis toxin, a toxin that ADP-ribosylates  $G_i$  rendering it inactive, blocks the ability of LPNPY to enhance renovascular responses to Ang II.

The results of the present study also indicate that  $Y_2$  receptor activation (via PYY<sub>3-36</sub>) also enhances renal vasoconstrictor responses to Ang II in SHR but not WKY. While this effect is much smaller compared to the effect observed from  $Y_1$  receptor activation, the observed potentiation is also dependent on an intact  $Y_2$ - $G_i$  pathway. In this regard, BIIE0246, a highly selective  $Y_2$  antagonist, completely abrogates the ability of PYY<sub>3-36</sub> to enhance Ang II-induced renal vasoconstriction. Similarly, pertussis toxin abolishes the effect observed with  $Y_2$  activation alone.



## CHAPTER 4

### Naturally-Occurring PP-Fold Peptides Enhance Angiotensin II-Induced Renal Vasoconstriction: Interaction With $\alpha_2$ -Adrenoceptors

#### 4.1 Introduction

Our recent study (chapter 3) indicates that the selective, synthetic  $Y_1$ -receptor agonist LPNPY greatly enhances Ang II-induced changes in renovascular resistance in isolated, perfused kidneys from SHR, but not in kidneys from normotensive WKY rats. Moreover, this interaction is abolished by the selective  $Y_1$ -receptor antagonist BIBP3226 and is mediated by the  $G_i$  signal transduction pathway. These findings would suggest that perhaps naturally-occurring, endogenous  $Y_1$ -receptor agonists facilitate Ang II-induced renal vasoconstriction in genetic hypertension.

The only known naturally-occurring, endogenous agonists of  $Y_1$  receptors are NPY and PYY.[25] NPY is released within the kidney from renal sympathetic nerves directly into the neuroeffector junction between renal sympathetic varicosities and renal vascular smooth muscle cells, and therefore would have ready access to renovascular smooth muscle cells responsive to Ang II.[21] Although not released from sympathetic varicosities, PYY is secreted into the systemic circulation from intestinal enterocytes in response to food intake.[25] Thus PYY would also be expected to circulate to the kidney and be available to activate  $Y_1$  receptors in the renal microcirculation.

The above discussion supports the hypothesis that both NPY and PYY potentially could play a role with regard to enhancing Ang II-induced renal vasoconstriction in genetic hypertension. However, the effects of NPY and PYY on Ang II-induced renal vasoconstriction

in kidneys from genetically hypertensive animals are unknown. Therefore, the primary goal of this chapter was to determine and compare the effects of NPY and PYY on renovascular responses to Ang II in isolated, perfused kidneys obtained from SHR, and to determine the role of  $Y_1$  receptors in the effects of NPY and PYY on Ang II renovascular responses.

As mentioned in the introduction, PYY is cleaved by DPPIV to PYY<sub>3-36</sub>, which is another naturally-occurring PP-fold peptide.[36,37] Unlike NPY and PYY which bind to and activate  $Y_1$ ,  $Y_2$  and  $Y_5$  receptors, PYY<sub>3-36</sub> is selective for  $Y_2$  receptors.[25] Our previous results indicate that  $Y_2$ -receptor activation only slightly potentiates Ang II-induced renal vasoconstriction in kidneys from genetically hypertensive animals, and not at all in kidneys from normotensive rats. This suggests that intestinal processing of PYY to PYY<sub>3-36</sub> may importantly determine the effects of the PYY system on renovascular responses to Ang II in kidneys from genetically hypertensive rats (further explored in chapter 6). However, a direct comparison between PYY<sub>3-36</sub> and PYY on renovascular response to Ang II in kidneys from genetically hypertensive rats is lacking. Therefore the second objective of this chapter was to determine and compare the effects of PYY<sub>3-36</sub> and PYY on renovascular responses to Ang II in isolated, perfused kidneys obtained from spontaneously hypertensive rats.

Catecholamines can activate  $\alpha_2$ -adrenoceptors, and like  $Y_1$  receptors,  $\alpha_2$ -adrenoceptors are also coupled to  $G_i$  proteins. Thus it is not surprising that  $\alpha_2$ -adrenoceptor activation, like  $Y_1$ -receptor activation, facilitates Ang II-induced renal vasoconstriction in the genetically hypertensive kidney as shown in figures 1.2a/b.[20,59,60] Because  $\alpha_2$ -adrenoceptors and  $Y_1$  receptors both couple to  $G_i$  proteins, it is conceivable that sub-threshold/near-threshold activation of  $\alpha_2$ -adrenoceptors synergize with PP-fold peptides with regard to enhancing renovascular responses to Ang II in kidneys from genetically hypertensive rats. Renal vascular

smooth muscle cells are bathed with catecholamines released from renal sympathetic nerves and are simultaneously under the influence of PP-fold peptides. Accordingly, a third objective of this chapter was to determine whether a sub-threshold concentration of the  $\alpha_2$ -adrenoceptor agonist UK14304 plus a sub-threshold concentration of either NPY, PYY or PYY<sub>3-36</sub> would enhance renovascular responses to Ang II in isolated, perfused kidneys obtained from spontaneously hypertensive rats.

Indeed, we did observe a synergy between UK14,304 and NPY and UK14,304 and PYY, but not UK14,304 and PYY<sub>3-36</sub>, suggesting that the synergy between the  $\alpha_2$ -adrenoceptor agonist and the PP-fold peptides was mediated via the Y<sub>1</sub> receptor. To confirm this, we also examined the interaction between a sub-threshold dose of UK14,304 and a sub-threshold dose of LPNPY (selective Y<sub>1</sub>-receptor agonist) on Ang II-induced renal vasoconstriction. This latter study was conducted *in vivo* to ensure that the synergy was not an artifact of the isolated, perfused kidney and was performed in kidneys from both SHR and WKY to determine whether the synergy was restricted to kidneys from genetically hypertensive rats.

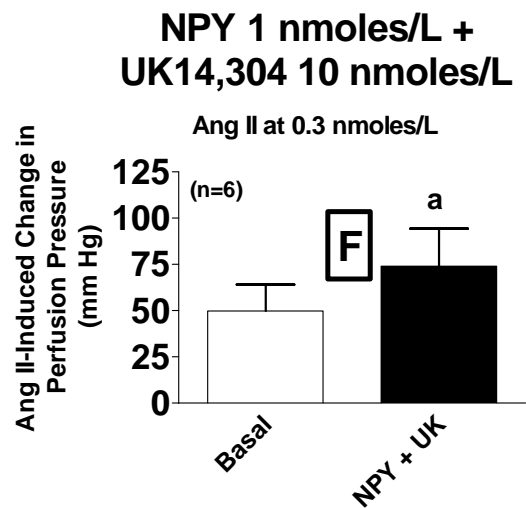
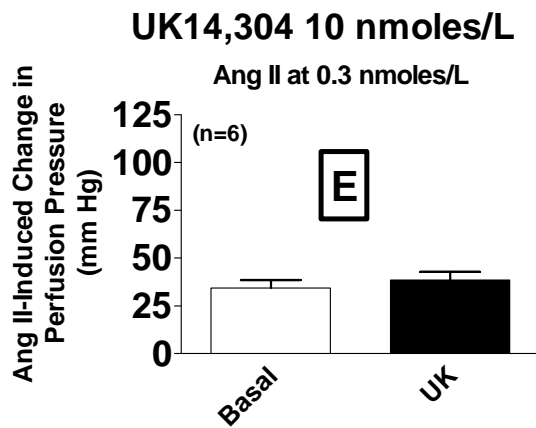
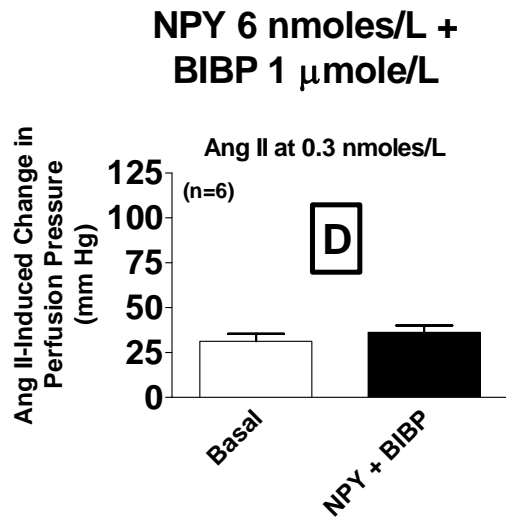
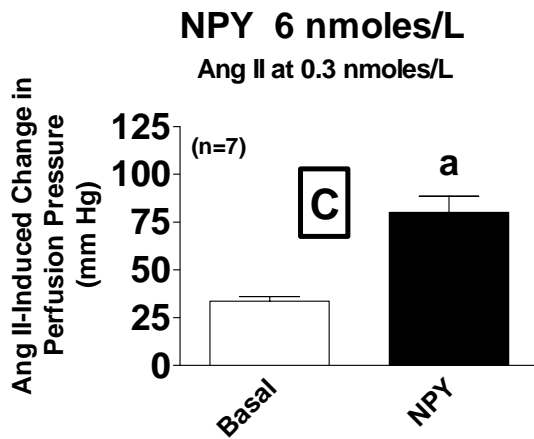
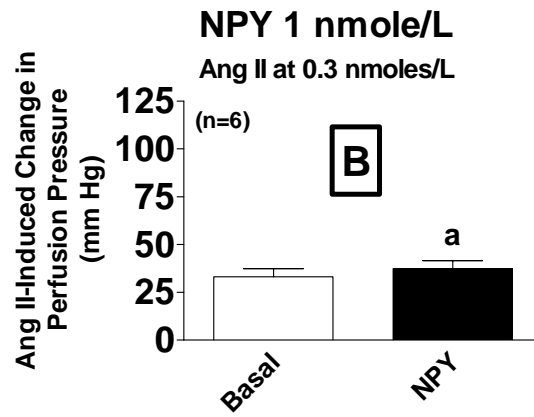
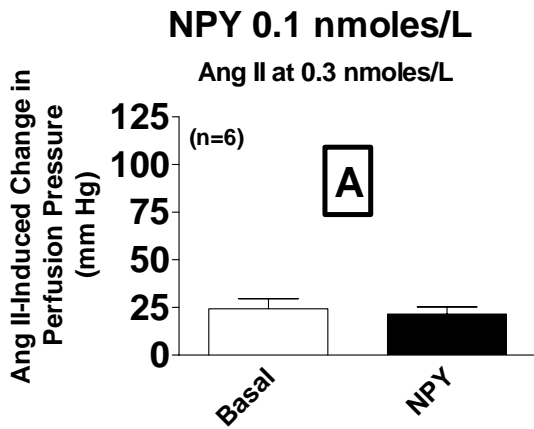
## 4.2 Results

*Experiments in Isolated, Perfused Kidneys.* The average baseline renal perfusion pressure in all 98 kidney perfusion experiments was  $54 \pm 2$  mm Hg, and was not affected by any of the various treatments or combinations of treatments. Although the perfusion flow rate was physiological (5 ml/min), baseline perfusion pressures were below the normal renal perfusion pressure *in vivo* because of the low viscosity of Tyrode's solution compared with whole blood. However, despite the low basal perfusion pressure, the kidneys were very responsive to Ang II. As shown in figure 4.1, Panel A, 0.1 nmoles/L of NPY did not potentiate renovascular responses to Ang II. Although 1 nmoles/L of NPY did significantly enhance renovascular responses to Ang II, this effect was barely detectable and represented a concentration of NPY that was low enough to only mildly effect Ang II responses (figure 4.1, Panel B). In contrast, 6 nmoles/L of NPY more than doubled the renovascular response to Ang II (figure 4.1, Panel C). Higher concentrations of NPY were not examined because although concentrations equal to or less than 6 nmoles/L did not change baseline perfusion pressure, higher concentrations did. As shown in figure 4.1, Panel D, in kidneys treated with BIBP3226, 6 nmoles/L of NPY only slightly augmented renovascular responses to Ang II, and this effect was not statistically significant. Although our previously published studies demonstrated that 10 nmoles/L of UK14,304 augmented renovascular responses to 10 nmoles/L of Ang II, as shown in figure 4.1, Panel E, 10 nmoles/L of UK14,304 did not enhance renovascular responses to a very low concentration of Ang II (0.3 nmoles/L). However, when a sub-threshold concentration of UK14,304 (10 nmoles/L) was combined with a near-threshold concentration of NPY (1 nmole/L) a significant

potentiation of the renovascular response to Ang II was observed. These results are represented differently in figure 4.2 which

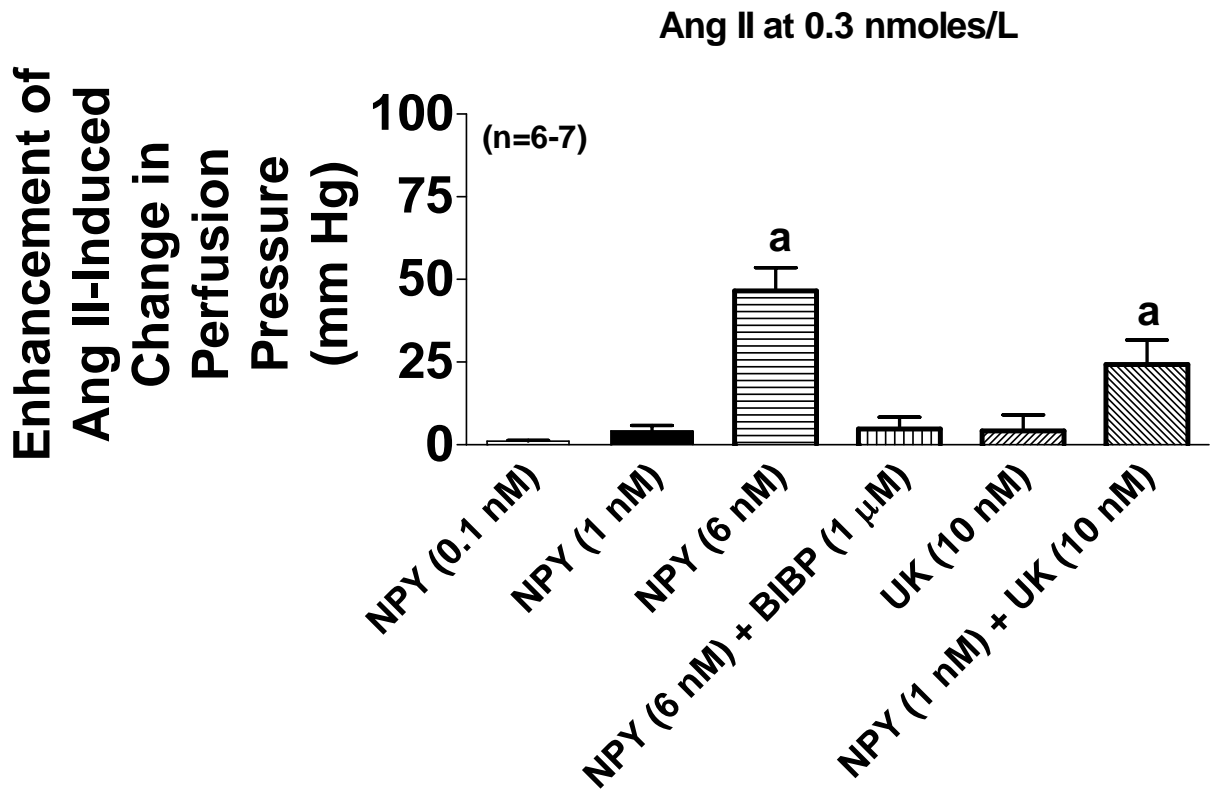
#### **FIGURE 4.1**

Bar graph illustrating the effects of neuropeptide Y (NPY) at 0.1 (Panel A), 1 (Panel B) and 6 (Panel C) nmoles/L on vasoconstrictor responses (change in perfusion pressure) to angiotensin II (Ang II; 0.3 nmoles/L) in isolated, perfused kidneys obtained from spontaneously hypertensive rats. Also shown are the effects of NPY at 6 nmoles/L in combination with the  $Y_1$ -receptor antagonist BIBP3226 (BIBP; 1  $\mu$ mole/L) (Panel D) and the effects of the  $\alpha_2$ -adrenoceptor agonist UK14,304 (10 nmoles/L) alone (Panel E) and in combination with 1 nmoles/L of NPY (Panel F). Values are means  $\pm$  SEM. <sup>a</sup>P<0.05 compared with basal (2-tailed paired Student's t-test).



illustrates the difference in response to Ang II before and after the various treatments. As indicated, the enhancement of Ang II-induced change in perfusion pressure was greater in kidneys treated with either 6 nmoles/L of NPY or 1 nmoles/L of NPY + 10 nmoles/L of UK14,304 compared with all other groups.

As shown in figure 4.3, Panel A, 0.1 nmoles/L of PYY<sub>1-36</sub> did not potentiate renovascular responses to Ang II. Although 1 nmoles/L of PYY<sub>1-36</sub> did significantly enhance renovascular responses to Ang II, this effect was barely detectable and represented a near-threshold concentration of PYY<sub>1-36</sub> (Figure 4.3, Panel B). In contrast, 6 nmoles/L of PYY<sub>1-36</sub> more than doubled the renovascular response to Ang II (Figure 4.3, Panel C). As with NPY, higher concentrations of PYY<sub>1-36</sub> were not examined because although concentrations equal to or less than 6 nmoles/L did not change baseline perfusion pressure, higher concentrations did. As shown in Figure 4.3, Panel D, in kidneys treated with BIBP3226, 6 nmoles/L of PYY<sub>1-36</sub> only slightly augmented renovascular responses to Ang II. When a sub-threshold concentration of UK14,304 (10 nmoles/L) was combined with a near-threshold concentration of PYY<sub>1-36</sub> (1 nmole/L) a potentiation of the renovascular response to Ang II was observed (figure 4.3, Panel F). These results are represented differently in figure 4.4 which illustrates the difference in response to Ang II before and after the various treatments. As indicated, the enhancement of Ang II-induced change in perfusion pressure was greater in kidneys treated with either 6 nmoles/L of PYY<sub>1-36</sub> or 1 nmoles/L of PYY<sub>1-36</sub> + 10 nmoles/L of UK14,304 compared with all other groups.



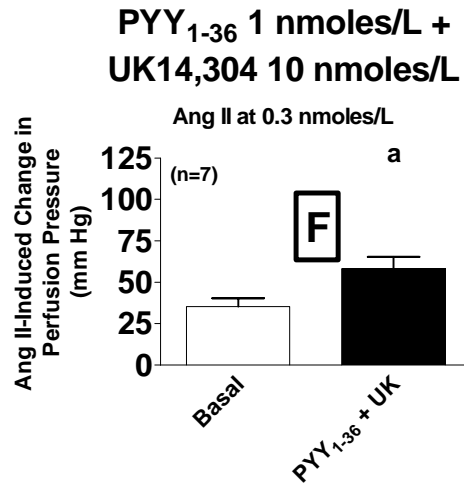
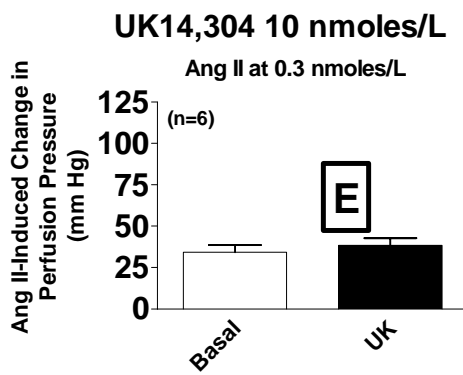
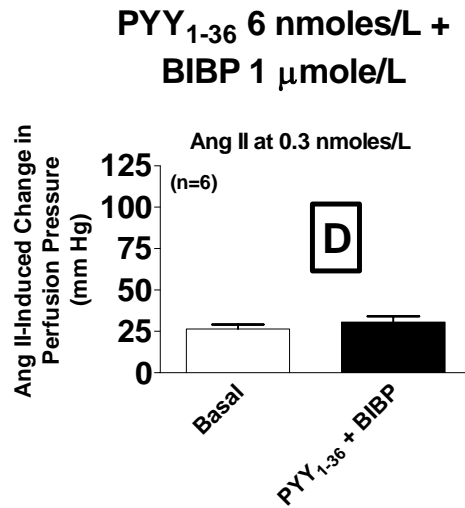
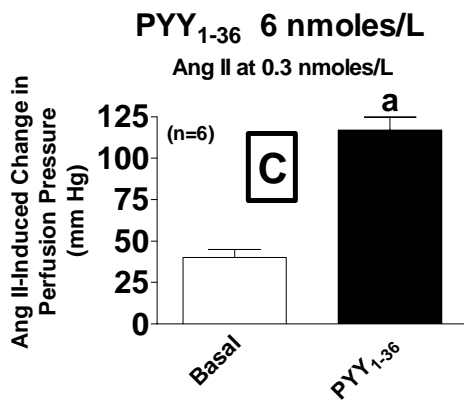
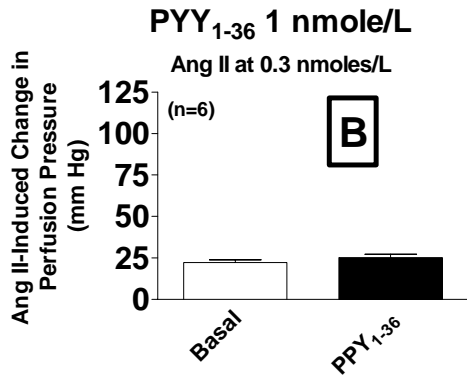
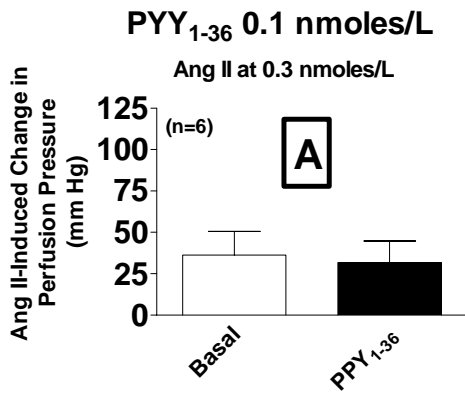
**FIGURE 4.2**

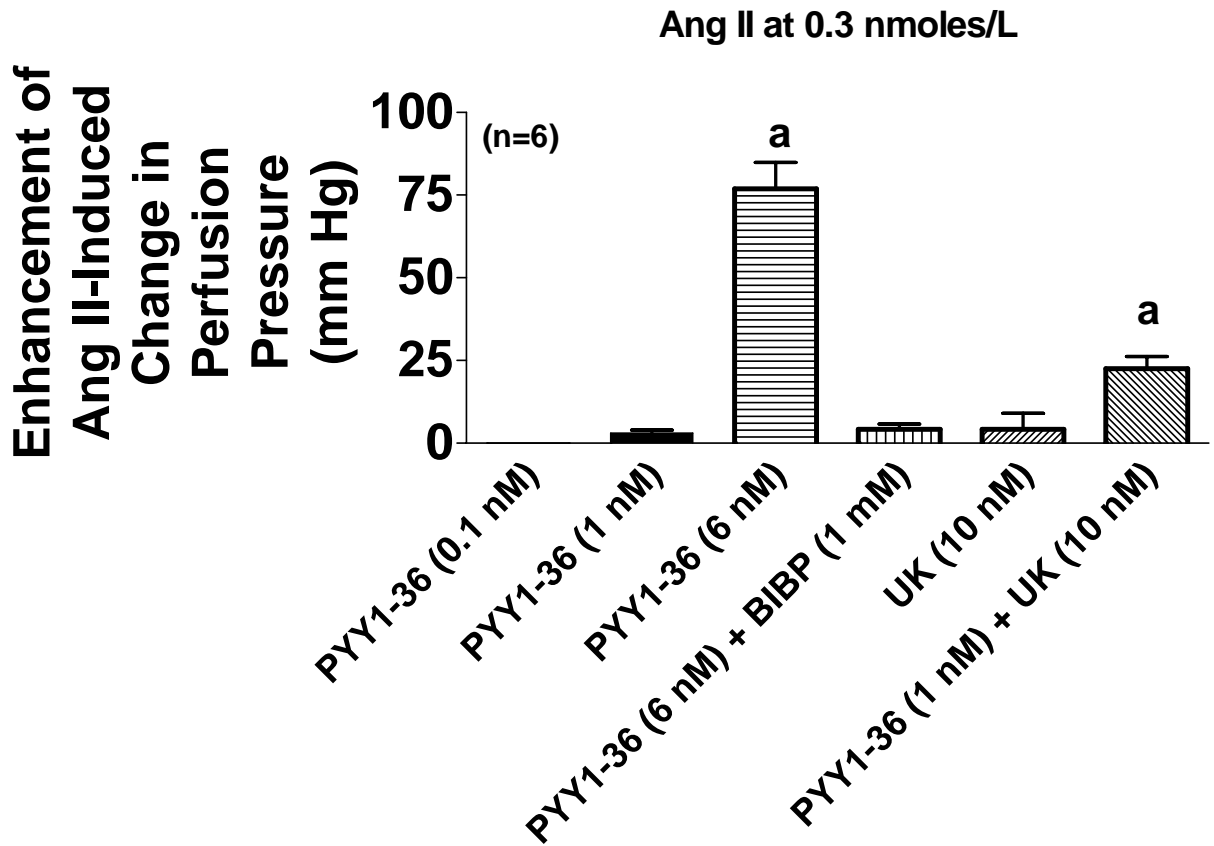
Enhancement of angiotensin II (Ang II)-induced change in perfusion pressure in isolated, perfused kidneys obtained from spontaneously hypertensive rats by 0.1, 1 and 6 nmoles/L of neuropeptide Y (NPY), by 6 nmoles/L of NPY in the presence of the  $Y_1$ -receptor antagonist BIBP3226 (BIBP; 1  $\mu$ mole/L), by the  $\alpha_2$ -adrenoceptor agonist UK14304 (10 nmoles/L) and by UK14,304 in combination with 1 nmoles/L of NPY. Values are means  $\pm$  SEM. <sup>a</sup>P<0.05 compared with basal (Fisher's least significant difference test).



### FIGURE 4.3

Bar graph illustrating the effects of peptide YY<sub>1-36</sub> (PYY<sub>1-36</sub>) at 0.1 (Panel A), 1 (Panel B) and 6 (Panel C) nmoles/L on vasoconstrictor responses (change in perfusion pressure) to angiotensin II (Ang II; 0.3 nmoles/L) in isolated, perfused kidneys obtained from spontaneously hypertensive rats. Also shown are the effects of PYY<sub>1-36</sub> at 6 nmoles/L in combination with the Y<sub>1</sub>-receptor antagonist BIBP3226 (BIBP; 1 μmole/L) (Panel D) and the effects of the α<sub>2</sub>-adrenoceptor agonist UK14,304 (10 nmoles/L) alone (Panel E) and in combination with 1 nmoles/L of PYY<sub>1-36</sub> (Panel F). Values are means ± SEM. <sup>a</sup>P<0.05 compared with basal (2-tailed paired Student's t-test).





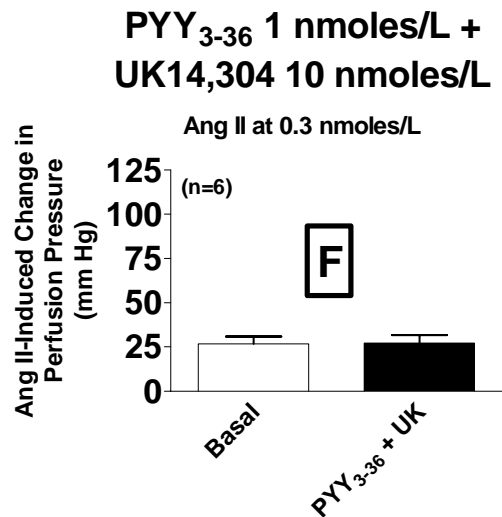
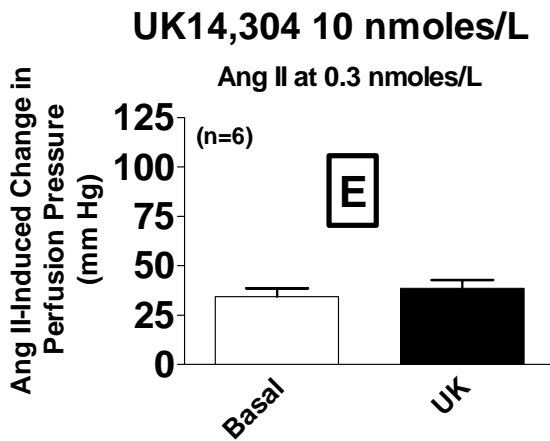
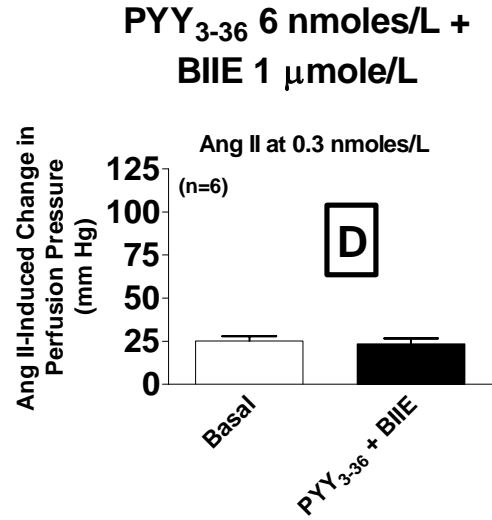
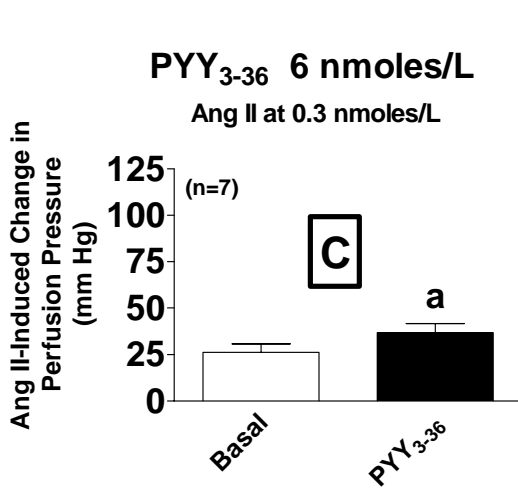
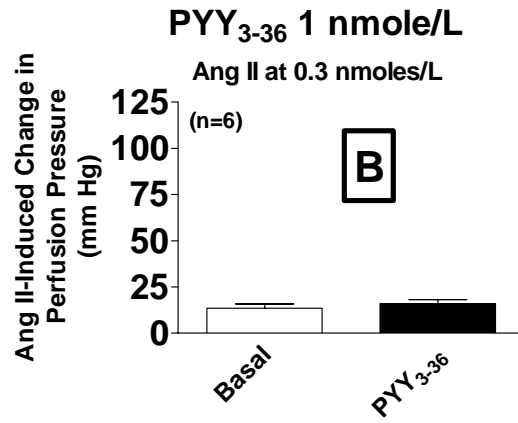
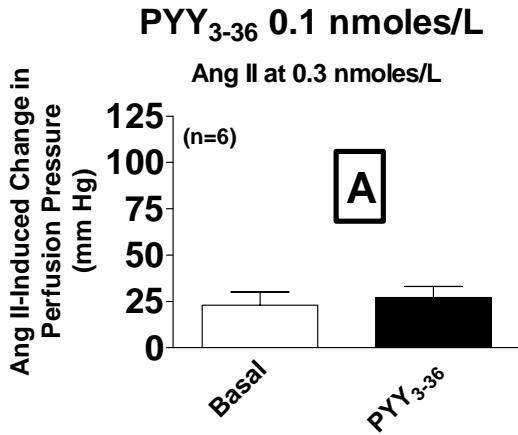
**FIGURE 4.4**

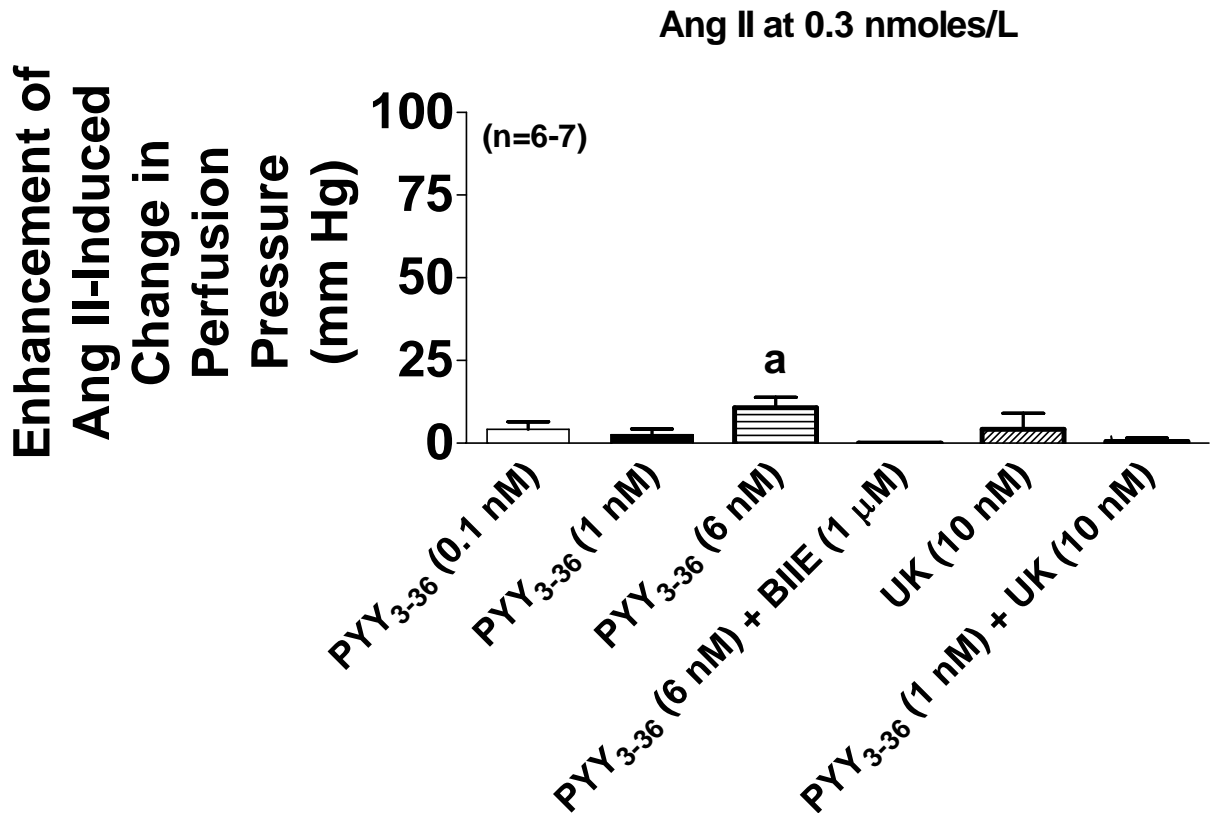
Enhancement of angiotensin II (Ang II)-induced change in perfusion pressure in isolated, perfused kidneys obtained from spontaneously hypertensive rats by 0.1, 1 and 6 nmoles/L of peptide YY<sub>1-36</sub> (PYY<sub>1-36</sub>), by 6 nmoles/L of PYY<sub>1-36</sub> in the presence of the Y<sub>1</sub>-receptor antagonist BIBP3226 (BIBP; 1 μmole/L), by the α<sub>2</sub>-adrenoceptor agonist UK14304 (10 nmoles/L) and by UK14,304 in combination with 1 nmoles/L of PYY<sub>1-36</sub>. Values are means ± SEM. <sup>a</sup>P<0.05 compared with basal (Fisher's least significant difference test).

As shown in figure 4.5, neither 0.1 (Panel A) nor 1 (Panel B) nmoles/L of PYY<sub>3-36</sub> potentiated renovascular responses to Ang II. Even 6 nmoles/L of PYY<sub>3-36</sub> only very slightly, but reproducibly, enhanced the renovascular response to Ang II (Figure 4.5, Panel C). As shown in figure 4.5, Panel D, treatment with BIIE0246 (1  $\mu$ mole/L) abolished the ability of PYY<sub>3-36</sub> (6 nmoles/L) to augment renovascular responses to Ang II. When a sub-threshold concentration of UK14,304 (10 nmoles/L) was combined with a sub-threshold concentration of PYY<sub>3-36</sub> (1 nmole/L) no potentiation of the renovascular response to Ang II was observed (figure 4.5, Panel F). These results are represented differently in figure 4.6 which illustrates the difference in response to Ang II before and after the various treatments. As indicated, the enhancement of Ang II-induced change in perfusion pressure was greater in kidneys treated with either 6 nmoles/L of PYY<sub>3-36</sub> compared with all other groups.

#### **FIGURE 4.5**

Bar graph illustrating the effects of peptide YY<sub>3-36</sub> (PYY<sub>3-36</sub>) at 0.1 (Panel A), 1 (Panel B) and 6 (Panel C) nmoles/L on vasoconstrictor responses (change in perfusion pressure) to angiotensin II (Ang II; 0.3 nmoles/L) in isolated, perfused kidneys obtained from spontaneously hypertensive rats. Also shown are the effects of PYY<sub>3-36</sub> at 6 nmoles/L in combination with the Y<sub>1</sub>-receptor antagonist BIIE0246 (BIIE; 1 μmole/L) (Panel D) and the effects of the α<sub>2</sub>-adrenoceptor agonist UK14,304 (10 nmoles/L) alone (Panel E) and in combination with 1 nmoles/L of PYY<sub>3-36</sub> (Panel F). Values are means ± SEM. <sup>a</sup>P<0.05 compared with basal (2-tailed paired Student's t-test).



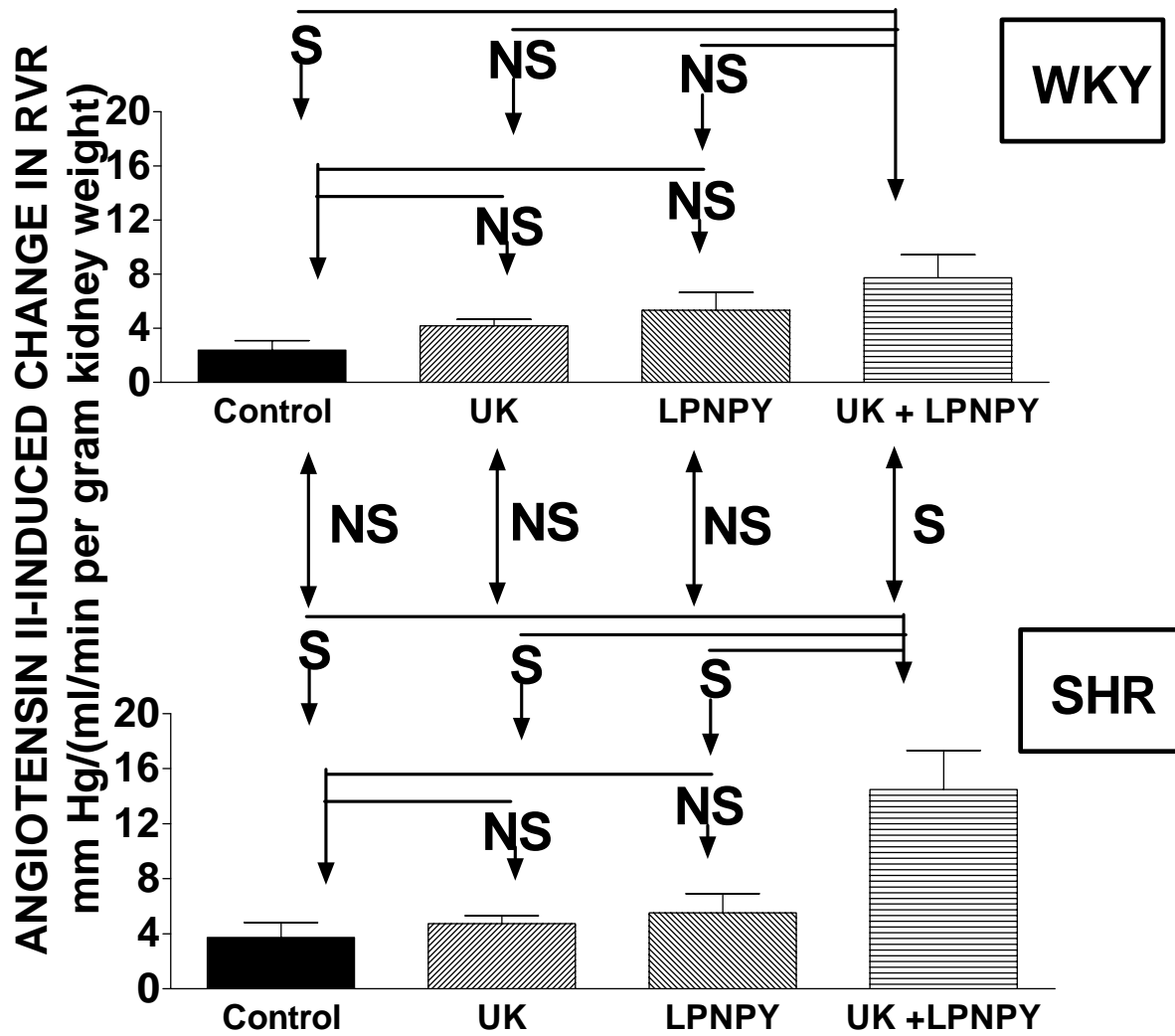


**FIGURE 4.6**

Enhancement of angiotensin II (Ang II)-induced change in perfusion pressure in isolated, perfused kidneys obtained from spontaneously hypertensive rats by 0.1, 1 and 6 nmoles/L of peptide YY<sub>3-36</sub> (PYY<sub>3-36</sub>), by 6 nmoles/L of PYY<sub>3-36</sub> in the presence of the Y<sub>2</sub>-receptor antagonist BIIE0246 (BIIE; 1 μmole/L), by the α<sub>2</sub>-adrenoceptor agonist UK14304 (10 nmoles/L) and by UK14,304 in combination with 1 nmoles/L of PYY<sub>3-36</sub>. Values are means ± SEM. <sup>a</sup>P<0.05 compared with basal (Fisher's least significant difference test).

***Experiments in Kidneys In Vivo.*** In WKY, baseline RVRs in control, UK14,304- treated, LPNPY-treated and UK14,304 plus LPNPY-treated rats were  $11 \pm 1$ ,  $11 \pm 1$ ,  $15 \pm 2$  and  $11 \pm 1$  mm Hg/(ml/min per gram kidney weight), respectively (no significant differences among four groups). In SHR, baseline RVRs in control, UK14,304-treated, LPNPY-treated and UK14,304 plus LPNPY-treated rats were  $16 \pm 2$ ,  $18 \pm 2$ ,  $18 \pm 4$  and  $22 \pm 3$  mm Hg/(ml/min per gram kidney weight), respectively (no significant differences among four groups). Baseline RVRs were greater in SHR compared with WKY ( $P < 0.0001$ ). Ang II increased RVR in both WKY and SHR kidneys (Figure 4.7). Due to careful selection of sub-threshold doses, treatment with UK14,304 by itself and treatment with LPNPY by itself did not enhance Ang II-induced changes in RVR. However, treatment with UK14,304 plus LPNPY significantly enhanced Ang II-induced increases in RVR in both WKY and SHR (Figure 4.7); however, the potentiation of Ang II-induced increases in RVR by UK 14,304 plus LPNPY was greater in SHR compared with WKY kidneys (Figure 4.7).





**FIGURE 4.7**

Absolute change in renal vascular resistance (RVR; n=7) *in vivo* following infusion of Ang II (10 ng/kg per minute) plus low doses of UK14,304 (UK; 0.3  $\mu$ g/kg/min), [Leu31,Pro34]-neuropeptide Y (LPNPY; 1  $\mu$ g/kg/min) or UK14,304 plus LPNPY (UK+LPNPY). All statistically significant differences are noted with S (Fisher's LSD test). Values are means  $\pm$  SEM.

### 4.3 Summary

The present study, chapter four, illustrates the potential of endogenous PP-fold peptides to potentiate renovascular responses in genetic hypertension. Although all PP-fold peptides were investigated, PYY<sub>3-36</sub> demonstrated little to no ability to alter renovascular responses at concentrations closer to physiological relevance. PYY<sub>3-36</sub> also revealed no potential to synergize with alternative agonist for G<sub>i</sub>, namely UK14,304. Alternatively, *in vitro* in our perfusion system, the threshold concentration of naturally occurring PP-fold Y<sub>1</sub>-receptor agonists for enhancing the responses to Ang II is equal to or less than 1 nmole/L. This concentration would be considered an upper limit for the *in vivo* threshold concentration because small peptides commonly bind tightly to various surfaces. Thus it is likely that in the transit from the infusion catheter, through the warming coil and through the bubble trap of our kidney perfusion system that these low concentrations of peptides were rendered even lower by binding of the peptides to surfaces. The actual concentrations reaching the kidney were most likely much lower. In general, sympathetic nerve terminals release NPY directly onto vascular smooth muscle, and thus it seems likely that such concentrations (1 nmole/L) would be achieved locally with elevated renal sympathetic tone; however, direct evidence for this concept is not available. In rats, systemic levels of PYY can reach 0.1 nmoles/L to 0.25 nmoles/L.[43] This concentration is only 10 to 20% of the *in vitro* threshold concentration required for potentiation after a few minutes of exposure, it is conceivable that over longer periods of time, even these lower concentrations could potentiate responses to Ang II in kidneys from genetically-susceptible animals and human beings.

In relation to the above idea, the present study further shows that low-level stimulation of  $\alpha_2$ -adrenoceptor synergizes with threshold concentrations of NPY and PYY to potentiate

renovascular responses to Ang II in isolated, perfused kidneys from SHR. These findings are significant because the renal vasculature is simultaneously under the influence of circulating PP-fold peptides and catecholamines released from renal sympathetic nerves. In order to demonstrate that these findings are not due to an artifact of our *in vitro* system, we also show that selective Y<sub>1</sub> receptor activation with LPNPY and selective α<sub>2</sub>-adrenoceptor stimulation with UK 14,304 leads to a robust renovascular response to Ang II in kidneys from genetically hypertensive animals compared to normotensive animals.

## CHAPTER 5

# Modulation of Angiotensin II-Induced Renal Vasoconstriction by Renal Sympathetic Nerves: Role of $Y_1$ -Receptor/ $G_i$ -Protein Pathway

### 5.1 Introduction

Our recent studies reveal yet another potential mode of interaction between the renal sympathetic nervous system and the renin-angiotensin system, particularly in animals with genetic hypertension. In this regard, chapter 4 demonstrates that activation of renal vascular  $\alpha_2$ -adrenoceptors with UK 14,304 or renal vascular  $Y_1$  receptors with either exogenous NPY or exogenous PYY<sub>1-36</sub> enhances Ang II-induced increases in renal vascular resistance in kidneys from SHR but not in kidneys from WKY[12,59,60,62]. Our previous studies also demonstrate that inhibiting the  $G_i$  signal transduction pathway with pertussis toxin blocks the ability of UK 14,304 and NPY to augment Ang II-induced renal vasoconstriction in SHR kidneys, indicating that in SHR kidneys both  $\alpha_2$ -adrenoceptors and  $Y_1$  receptors modulate Ang II-induced renal vasoconstriction via the  $G_i$  signal transduction pathway [12,60]. Moreover, our studies in chapter four show that in SHR kidneys very low concentrations of UK 14,304, NPY or PYY<sub>1-36</sub>, that do not per se enhance renal vascular responses to Ang II, potentiate Ang II-induced renal vasoconstriction when combined (i.e., UK 14,304 + NPY or UK 14,304 + PYY<sub>1-36</sub>). This suggests a synergy between agonists of the  $G_i$  pathway so that low concentrations of distinct ligands acting on their cognate receptors can augment Ang II-induced renal vasoconstriction in SHR kidneys by their combined effects of the  $G_i$  signal transduction pathway.

The importance of the aforementioned observations is that renal sympathetic nerves co-release an  $\alpha_2$ -adrenoceptor agonist (i.e., norepinephrine) and a  $Y_1$  receptor agonist (i.e., NPY) [21]. Therefore, it is conceivable that endogenous neurotransmitters released from renal sympathetic nerves modulate, either separately or synergistically, Ang II-induced renal vasoconstriction in SHR kidneys by activating the  $G_i$  pathway. The purpose of this chapter was to test this hypothesis.

## 5.2 Results

***Basal Renal Perfusion Pressures.*** As shown in Table 5.1, MABPs were significantly higher in SHR compared to WKY, and pretreatment with pertussis toxin significantly reduced MABP in SHR. Heart rates and basal renal perfusion pressures, either before or after prazosin, were similar among all ten groups. Sham RNS did not affect renal perfusion pressure, and actual RNS did not affect renal perfusion pressure in the presence of prazosin because prazosin blocked  $\alpha_1$ -adrenoceptor-induced vasoconstriction.

***Renal Nerve Stimulation in Isolated, Perfused Kidneys of WKY.*** Figure 5.1 demonstrates the lack of effect of RNS on renovascular responses to Ang II in WKY kidneys. During P1, a physiological concentration of Ang II (100 pmoles/L) increased renal perfusion pressure by  $1.6 \pm 0.2$  mm Hg in WKY kidneys. This response to Ang II did not change during P2 or P3 in either the sham RNS group or the actual RNS group.

***Renal Nerve Stimulation in Isolated, Perfused Kidneys of SHR.*** Figure 5.2 illustrates the ability of RNS to enhance renovascular response to Ang II in SHR kidneys. During P1, Ang II (100 pmoles/L) increased renal perfusion pressure by  $8.6 \pm 0.7$  mm Hg in SHR kidneys. This response to Ang II did not change during P2 or P3 in the sham RNS group; however, in the actual RNS group, the response to Ang II was increased to  $135 \pm 9\%$  and  $177 \pm 26\%$  (normalized to the P1 response) ( $P < 0.05$ ).

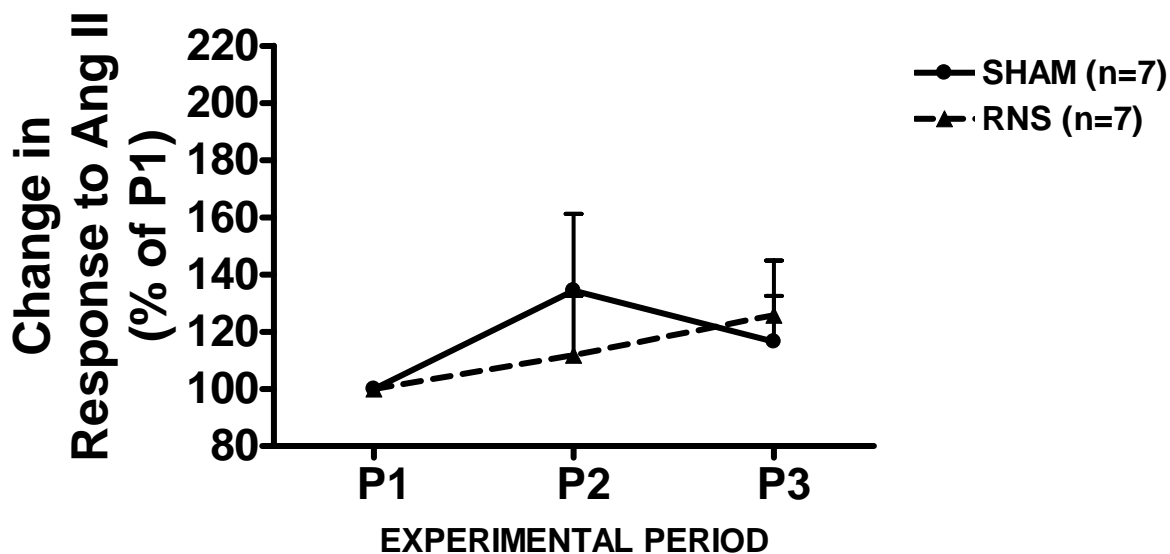
**Table 5.1: Mean arterial blood pressures and renal perfusion pressures.**

	MABP (mm Hg) Before Kidney Isolation	RENAL PERFUSION PRESSURE (mm Hg) AFTER KIDNEY ISOLATION						
		Before Prazosin	During Prazosin					
			Period 1		Period 2		Period 3	
			Basal	Sham RNS	Basal	Sham or Actual RNS	Basal	Sham or Actual RNS
WKY SHAM	<sup>a</sup> 121 2	49 4	49 4	49 5	46 4	46 4	43 3	43 3
WKY RNS	<sup>a</sup> 125 4	57 7	54 6	54 6	52 5	51 5	50 3	50 3
SHR SHAM	206 3	57 3	55 3	53 3	49 2	49 2	47 2	47 2
SHR RNS	203 5	51 2	50 2	50 2	47 2	47 2	48 2	49 3
SHR - RAU SHAM	206 6	56 4	53 4	53 4	52 5	51 5	51 5	51 5
SHR - RAU RNS	205 3	49 1	47 1	45 1	43 1	43 1	42 1	42 1
SHR - BIBP SHAM	199 5	55 3	51 3	50 3	46 2	46 2	45 2	44 2
SHR - BIBP RNS	197 4	57 4	52 2	51 2	46 2	46 2	45 2	45 2
SHR - PT SHAM	<sup>b</sup> 152 6	54 3	50 2	49 2	44 2	43 2	44 3	43 3
SHR - PT RNS	<sup>b</sup> 137 10	49 3	48 3	47 3	44 4	44 4	43 4	42 4

MABP, mean arterial blood pressure; RAU, rauwolscine; BIBP, BIBP3226; PT, pertussis toxin.

<sup>a</sup>Indicates significantly different than SHR; <sup>b</sup>Indicates significantly different than SHR not treated with PT. Values are means and standard errors for 7 kidneys in all groups except the PT groups in which n=5.

## WKY KIDNEY

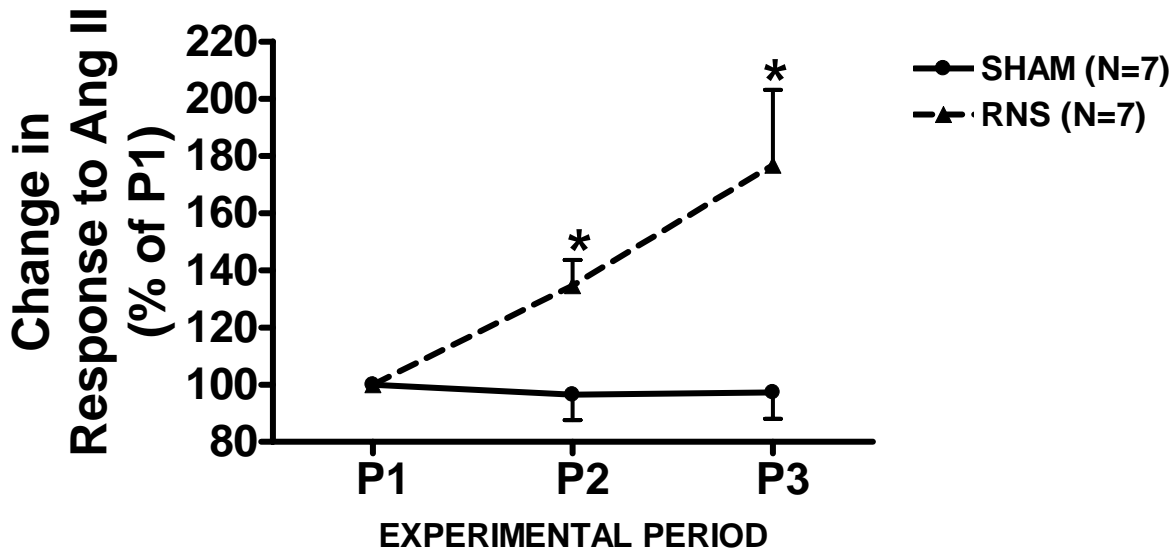


**FIGURE 5.1**

Lack of effect of periarterial renal nerve stimulation (RNS) or sham RNS (SHAM) on vasoconstrictor responses (change in renal perfusion pressure in a constant flow system) to angiotensin II (Ang II; 100 pmoles/L) in isolated, perfused kidneys obtained from normotensive Wistar-Kyoto (WKY) rats. The responses to Ang II during the second (P2) and third (P3) experimental periods were normalized to the responses during the control first period (P1). Values are means  $\pm$  SEM.



## SHR KIDNEY



**FIGURE 5.2**

Periarterial renal nerve stimulation (RNS) enhances vasoconstrictor responses (change in renal perfusion pressure in a constant flow system) to angiotensin II (Ang II; 100 pmoles/L) in isolated, perfused kidneys obtained from spontaneously hypertensive rats (SHR). The responses to Ang II during the second (P2) and third (P3) experimental periods were normalized to the responses during the control first period (P1). Some kidneys were exposed only to sham RNS (SHAM). Values are means  $\pm$  SEM. \* Indicates  $P < 0.05$  (unpaired Student's t-test) for RNS versus SHAM at the indicated experimental period.

Figure 5.3 shows that the ability of RNS to enhance renovascular response to Ang II in SHR kidneys is not blocked by rauwolscine, a highly potent and selective  $\alpha_2$ -adrenoceptor antagonist. In kidneys pretreated with rauwolscine, during P1, Ang II (100 pmoles/L) increased renal perfusion pressure by  $6.3 \pm 0.7$  mm Hg in SHR kidneys. This response to Ang II did not change during P2 or P3 in the sham RNS group; however, in the actual RNS group, the response to Ang II was increased to  $132 \pm 9\%$  and  $147 \pm 7\%$  (normalized to the P1 response) ( $P < 0.05$ ).

Figure 5.4 demonstrates that the ability of RNS to enhance renovascular response to Ang II in SHR kidneys is completely abrogated by BIBP3226, a highly selective and potent  $Y_1$ -receptor antagonist. In kidneys pretreated with BIBP3226, during P1, Ang II (100 pmoles/L) increased renal perfusion pressure by  $7.6 \pm 0.7$  mm Hg in SHR kidneys. This response to Ang II did not change during P2 or P3 in either the sham RNS group or the actual RNS group.

Figure 5.5 demonstrates that the ability of RNS to enhance renovascular response to Ang II in SHR kidneys is completely abrogated by pertussis toxin, a toxin that ADP ribosylates  $G_i$  proteins and thereby inhibits signaling via  $G_i$  proteins. In kidneys obtained from SHR pretreated with pertussis toxin, during P1, Ang II (100 pmoles/L) increased renal perfusion pressure by  $7.6 \pm 0.7$  mm Hg in SHR kidneys. This response to Ang II did not change during P2 or P3 in either the sham RNS group or the actual RNS group.

## SHR KIDNEY RAUWOLSCINE-PRETREATED

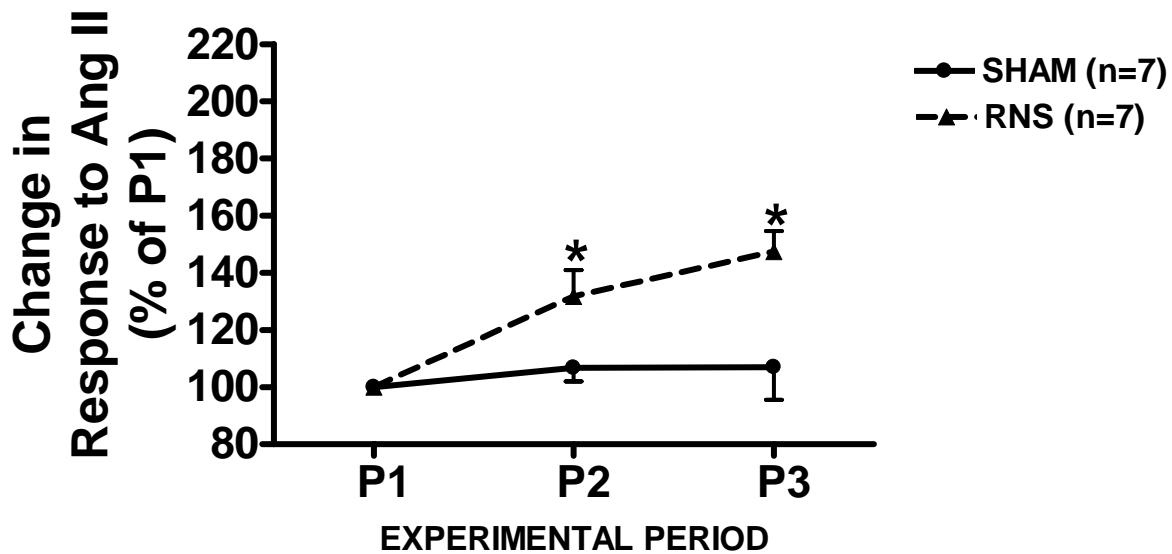


FIGURE 5.3

Periarterial renal nerve stimulation (RNS) enhances vasoconstrictor responses (change in renal perfusion pressure in a constant flow system) to angiotensin II (Ang II; 100 pmoles/L) in isolated, perfused kidneys obtained from spontaneously hypertensive rats (SHR) and that were pretreated with the  $\alpha_2$ -adrenoceptor antagonist rauwolscine (10 nmoles/L). The responses to Ang II during the second (P2) and third (P3) experimental periods were normalized to the responses during the control first period (P1). Some kidneys were exposed only to sham RNS (SHAM). Values are means  $\pm$  SEM. \* Indicates  $P < 0.05$  (unpaired Student's t-test) for RNS versus SHAM at the indicated experimental period.

## SHR KIDNEY BIBP3226-PRETREATED

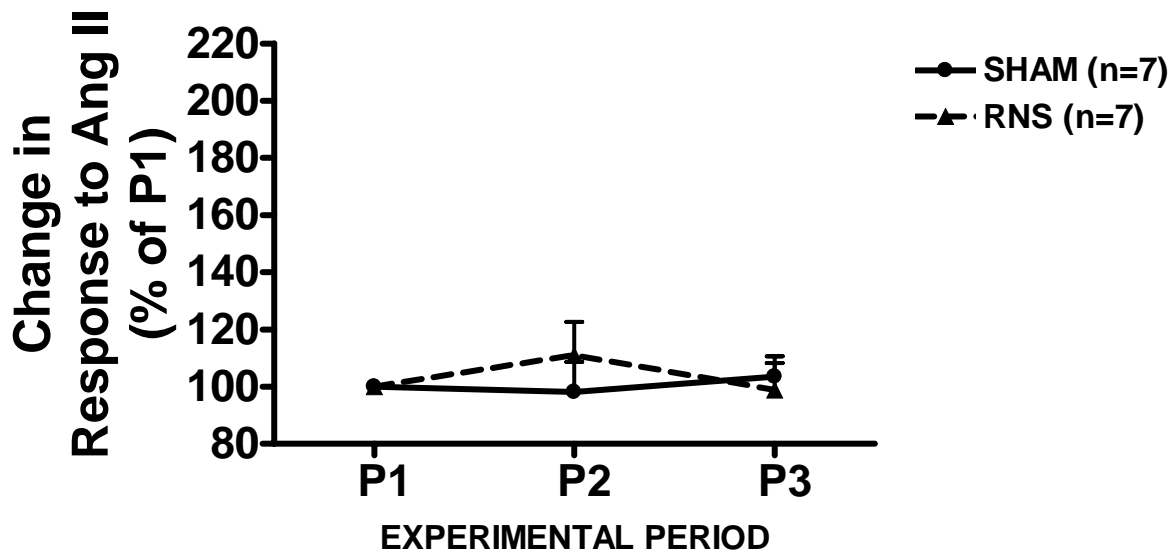


FIGURE 5.4

Periarterial renal nerve stimulation (RNS) does not enhance vasoconstrictor responses (change in renal perfusion pressure in a constant flow system) to angiotensin II (Ang II; 100 pmoles/L) in isolated, perfused kidneys obtained from spontaneously hypertensive rats (SHR) and that were pretreated with the  $Y_1$ -adrenoceptor antagonist BIBP3226 (1  $\mu$ moles/L). The responses to Ang II during the second (P2) and third (P3) experimental periods were normalized to the responses during the control first period (P1). Some kidneys were exposed only to sham RNS (SHAM). Values are means  $\pm$  SEM.

## SHR KIDNEY PERTUSSIS TOXIN-PRETREATED

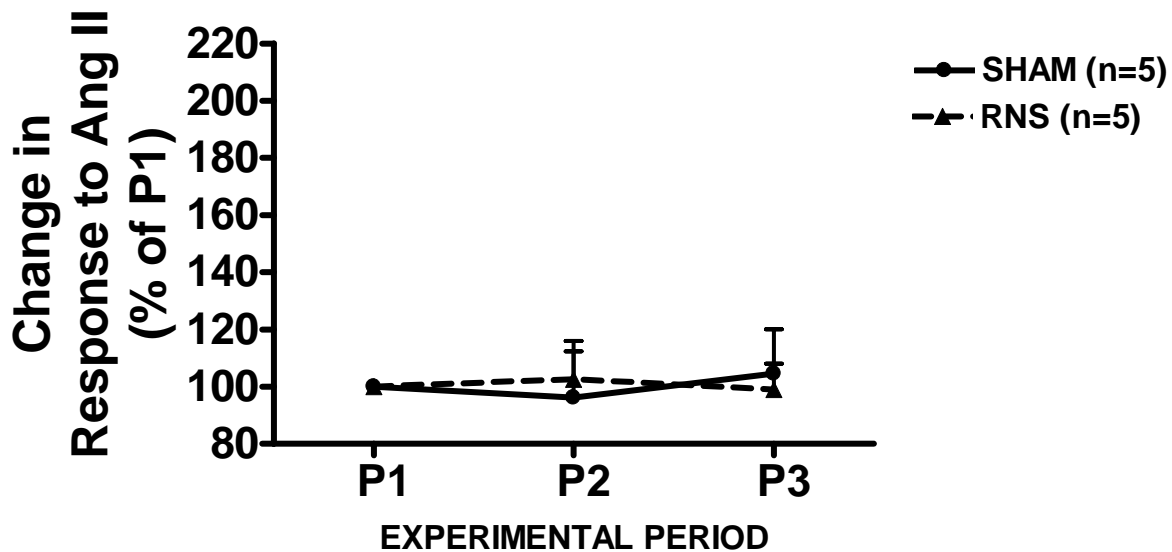


FIGURE 5.5

Periarterial renal nerve stimulation (RNS) does not enhance vasoconstrictor responses (change in renal perfusion pressure in a constant flow system) to angiotensin II (Ang II; 100 pmoles/L) in isolated, perfused kidneys obtained from spontaneously hypertensive rats (SHR) and that were pretreated three days prior to the perfusion experiment with an intravenous injection of pertussis toxin (30  $\mu\text{g}/\text{kg}$ ). The responses to Ang II during the second (P2) and third (P3) experimental periods were normalized to the responses during the control first period (P1). Some kidneys were exposed only to sham RNS (SHAM). Values are means  $\pm$  SEM.

### 5.3 Summary

Our results in chapter five demonstrate the natural role of the PP-fold  $Y_1$  peptide receptors ability to augment renal response in the SHR. In this respect, our findings reveal that a physiological level (5 Hz) of stimulation of the renal periarterial nerves, results in a near doubling of the renovascular response to a physiological level (100 pmoles/L) of Ang II in kidneys from SHR. In contrast, in kidneys from WKY rats, periarterial nerve stimulation does not significantly alter vasoconstrictor responses to Ang II. Although periarterial nerve stimulation results in the release of multiple neurotransmitters, namely catecholamines and NPY, specific antagonists were used to specify which released neurotransmitters were most likely altering renal vascular responses to Ang II in SHR. The results of the present study show that blockade of  $G_i$  with pertussis toxin abolishes the ability of renal sympathetic nerve stimulation to enhance renovascular responses to Ang II in SHR kidneys. This finding indicates that renal sympathetic nerve stimulation augments renovascular responses to Ang II in SHR kidneys via a  $G_i$  signal transduction pathway, similarly as our previous results.

Our previous studies, chapter 4, show that activation of the  $G_i$  pathway by agonists of  $\alpha_2$ -adrenoceptors or  $Y_1$  receptors or by co-activation of both  $\alpha_2$ -adrenoceptors and  $Y_1$  receptors potentiates renovascular response to Ang II in SHR kidneys. Because renal sympathetic nerves release both norepinephrine and NPY, which activate  $\alpha_2$ -adrenoceptors and  $Y_1$  receptors, respectively, we anticipated that the ability of renal sympathetic nerve stimulation to augment renovascular responses to Ang II would be attenuated somewhat by both rauwolscine (an  $\alpha_2$ -adrenoceptor antagonist) and BIBP 3226 (a  $Y_1$  receptor antagonist), but not completely by either. In contrast to our expectations, rauwolscine did not significantly affect the ability of renal

sympathetic nerve stimulation to augment renovascular responses to Ang II, whereas BIBP3226 completely abrogated stimulation-induced enhancement of Ang II responses.

## CHAPTER 6

# Blockade Of PYY Conversion To PYY<sub>3-36</sub> Via Dipeptidyl Peptidase IV Increases Arterial Blood Pressure in SHR

### 6.1 Introduction

PYY<sub>1-36</sub> is cleaved by dipeptidyl peptidase IV to PYY<sub>3-36</sub>. However, unlike NPY and PYY<sub>1-36</sub> that bind to and activate Y<sub>1</sub> receptors, PYY<sub>3-36</sub> is highly selective for Y<sub>2</sub> receptors.[25] Previous results described in chapter three indicate that Y<sub>2</sub>-receptor activation only slightly potentiates Ang II-induced renal vasoconstriction in kidneys from genetically hypertensive animals. This finding reveals the possibility that the catalytic conversion of PYY to PYY<sub>3-36</sub> may play a role in protecting against constant exposure of Y<sub>1</sub> receptor activation which demonstrated the ability to significantly potentiate renovascular responses to Ang II. This idea was further confirmed and extended in chapter four by the observations that compared to either NPY or PYY<sub>1-36</sub>, PYY<sub>3-36</sub> has much less ability to enhance renovascular responses to Ang II. In addition, PYY<sub>3-36</sub> had no ability to synergize with an alternative G<sub>i</sub> agonist, namely UK14,304, to augment renal vascular responses. The implication of these finding is that conversion of PYY<sub>1-36</sub> to PYY<sub>3-36</sub> may determine the net effects of the PYY on the cardiovascular and renal systems in genetically hypertensive rats.

Inhibitors of dipeptidyl peptidase IV are being developed for the treatment of type 2 diabetes, as mentioned in the introduction.[51-55]. This treatment may beneficially decrease blood glucose via GLP activation, but this type of treatment would also be expected to prevent the proteolytic cleavage of PYY<sub>1-36</sub> to PYY<sub>3-36</sub>. It is, thus, conceivable that in some patients,



inhibitors of dipeptidyl peptidase IV could adversely affect renal function and arterial blood pressure by raising PYY<sub>1-36</sub> levels. Chapter six focuses on the overall effects of inhibition of dipeptidyl peptidase IV on arterial blood pressure.

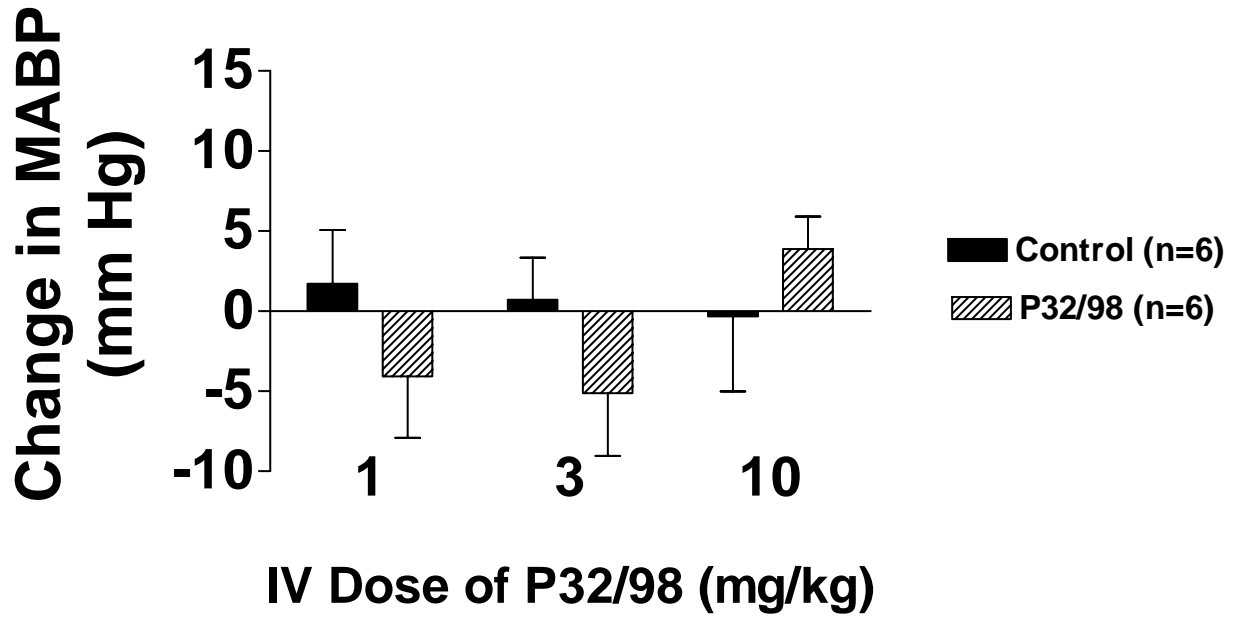
## 6.2 Results

***Basal Mean Arterial Blood Pressures.*** MABP were significantly higher in SHR compared to WKY animals following a one-hour rest period and an injection of captopril to remove increased Ang II due to surgical stress. Heart rates were comparable among both groups.

***In Vivo Experiments Monitoring MABP Following Blockade of DPPIV.*** Figure 6.1 demonstrates WKY's lack of response to P32/98, a commercially available DPPIV inhibitor. Time controls reveal that saline, the P32/98 vehicle, did not significantly alter the MABP in WKY animals, and if anything, reveal that each iv injection resulted in a smaller response where the last injection had no response. Injections of increasing concentrations of P32/98 (1, 3, 10 mg/kg) had no significant effect on WKY HR (data not shown).

Figure 6.2 reveals a significant response to P32/98 by SHR. Time controls show no significant response to the saline vehicle. In contrast, SHR significantly increased

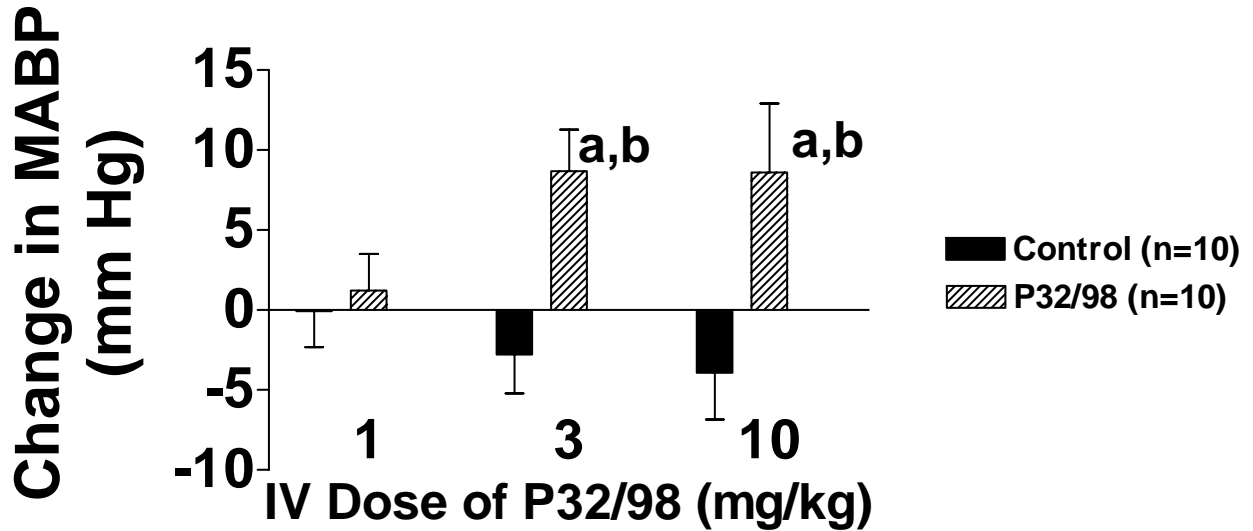
## P32/98 on MABP in WKY



**FIGURE 6.1**

Change in mean arterial blood pressure (n=6) *in vivo* following infusion of P32/98 (1, 3, & 10 mg/kg per minute) or saline control in WKY.

## P32/98 on MABP in SHR



ANOVA: P32/98 x Dose:  $P=0.0011$

<sup>a</sup> $P<0.05$  vs. Time/Vehicle Control

<sup>b</sup> $P<0.05$  vs. 1 mg/kg

**FIGURE 6.2**

Change in mean arterial blood pressure (n=10) *in vivo* following infusion of P32/98 (1, 3, & 10 mg/kg per minute) or saline control in WKY. All statistically significant differences are noted with an <sup>a</sup>(vs. time control) or <sup>b</sup>(vs. smallest dose). Values are means  $\pm$  SEM.

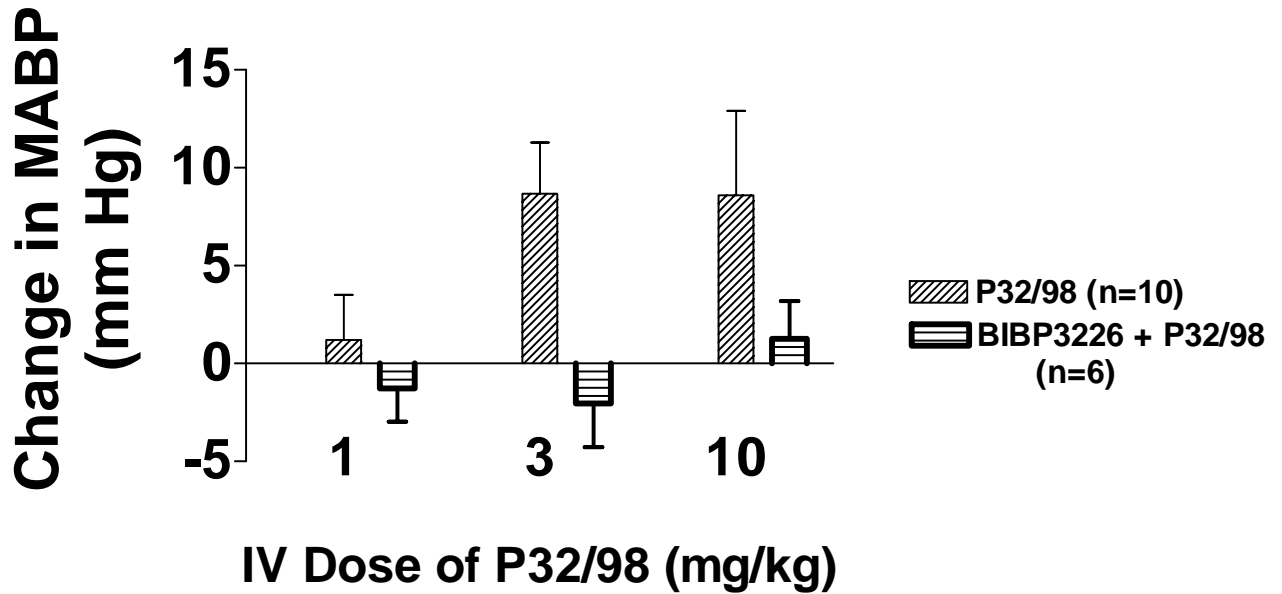
MABP about  $8.6 \pm 2.6$  mm Hg at 3 mg/kg P32/98. At 10 mg/kg, P32/98 also had a similar significant increase in MABP of  $8.6 \pm 4.3$  mm Hg.

Figure 6.3 demonstrates the extent to which P32/98 is capable of altering MABP in SHR. In this respect, pretreatment with BIBP3226 (40  $\mu$ g/kg) blocks P32/98's ability to potentiate MABP in SHR. At 3 mg/kg P32/98, where an 8.6 mm Hg increase in MABP was observed in SHR, BIBP pretreatment leads to a 2.1 mm Hg decrease in MABP. At the higher concentration of P32/98 (10 mg/kg), BIBP pretreatment resulted in a 1.3 mm Hg increase which is markedly lower than the previously mentioned increase in MABP in the absence of BIBP.

### **6.3 Summary**

Our results in chapter six reveal the role of dipeptidyl peptidase IV to protect against acute increases in blood pressure in SHR. Previous chapters reveal that physiological activation of the  $Y_1$ - $G_i$  signaling pathway cause a significant increase in renal vascular resistance in SHR while activation of the  $Y_2$ - $G_i$  has much less of a response. DPPIV is responsible for the conversion of PYY to PYY<sub>3-36</sub>. [25,51-55] As mentioned earlier, PYY has an affinity for primarily the  $Y_1$  receptor, and PYY<sub>3-36</sub> has affinity only for the  $Y_2$  receptor. [25] Therefore, we hypothesized that lack of DPPIV would lead to increases in PYY that would in turn lead to an increase in renal vascular tone, and perhaps arterial blood pressure. Our results begin to confirm our hypothesis. In this regard, P32/98, an inhibitor of DPPIV, causes a significant increase in mean arterial blood pressure in SHR, but not in WKY animals. Furthermore, pretreatment with BIBP3226 to block the  $Y_1$ - $G_i$  signaling pathway abrogates the increase in mean arterial blood pressure observed in the presence of P32/98.

## P32/98 on MABP in BIBP3226 treated SHR



**FIGURE 6.2**

Change in mean arterial blood pressure (n=10) *in vivo* following infusion of P32/98 (1, 3, & 10 mg/kg per minute) or P32/98 + BIBP3226 (40  $\mu$ g/kg)(n=6)in SHR. Values are means  $\pm$  SEM.

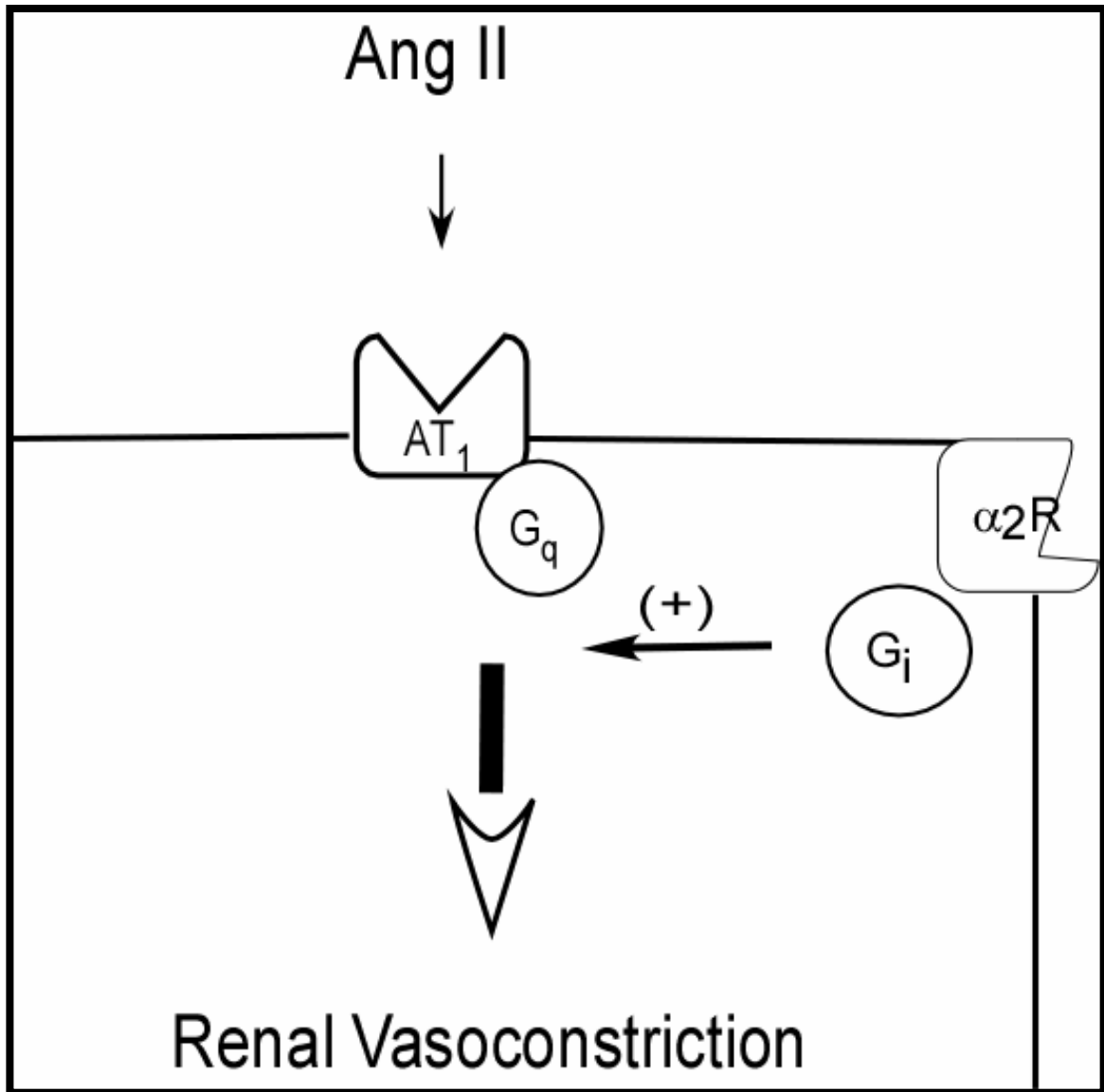
## CHAPTER 7

### DISCUSSION

#### 7.1 $Y_1$ And $Y_2$ As Regulators Of Ang II Induced Renal Vasoconstriction

The PP-fold family of peptides consists of four members, NPY, PYY, PYY<sub>3-36</sub> and pancreatic polypeptide (PP). Four active PP-fold peptide receptors have been cloned in primate species, namely  $Y_1$ ,  $Y_2$ ,  $Y_4$  and  $Y_5$  [25,34]. Although, as confirmed in chapter three,  $Y_1$  and  $Y_2$  receptors are expressed in the kidney,  $Y_4$  and  $Y_5$  receptors are expressed predominantly in the intestines and brain [25], not the kidney. Therefore, in chapter three, we focused on the role of  $Y_1$  and  $Y_2$  receptors as regulators of Ang II-induced renal vasoconstriction.

Previous research demonstrated that SHR respond more to Ang II than WKY without increased Ang II receptors, Ang II concentrations, or decreased Ang II degradation. Furthermore, the observed increase in response to Ang II by SHR can be blocked via an inhibitor of  $G_i$ , and potentiated by agonists for  $G_i$ . Figure 7.1 is a schematic of view of Ang II signaling known at the onset of this project, and as the chapter progresses, this figure will be expanded to describe further the findings within this work.



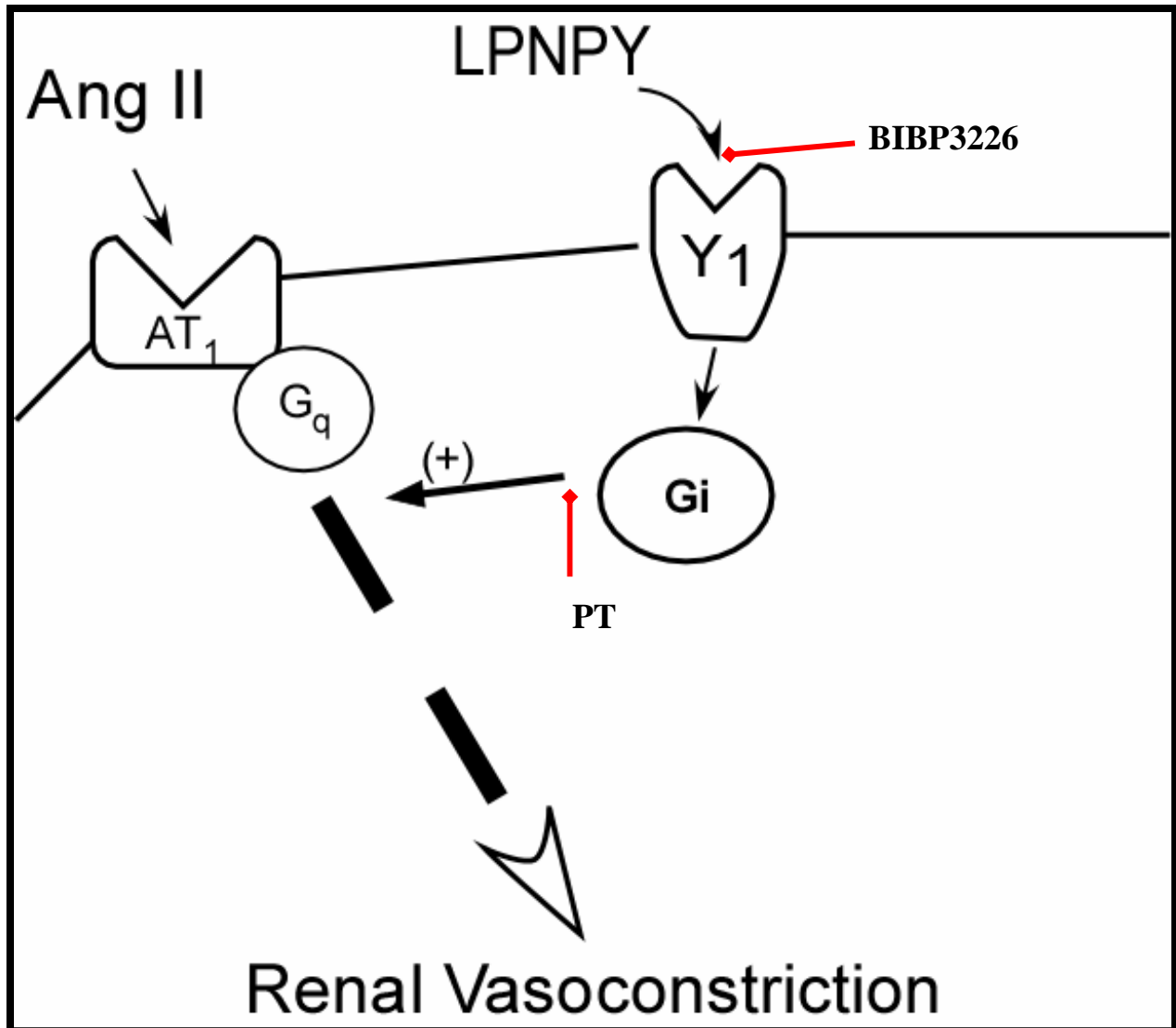
**FIGURE 7.1**

This is a diagram of the known signaling at the start of this project. Ang II signaling is more active in the SHR. Blockade of the G<sub>i</sub> pathway via pertussis toxin normalizes Ang II signaling in SHR. Activation of the α<sub>2</sub>-adrenoreceptor-G<sub>i</sub> pathway potentiates Ang II signaling in the SHR but not WKY.

The results of chapter three strongly support the concept that  $Y_1$  receptors enhance renal vasoconstrictor responses to Ang II and that the potentiation of Ang II responses by  $Y_1$  receptors is greater in SHR compared with WKY rats. In this regard, our results, represented in figure 7.2, showed:

- 1) LPNPY, a highly selective  $Y_1$ -receptor agonist, greatly enhances the renovascular response to Ang II in the SHR kidney, yet does not potentiate responses to Ang II in the WKY kidney.
- 2) BIBP3226, a highly selective  $Y_1$  receptor antagonist, blocks the ability of LPNPY to enhance renovascular responses to Ang II. This finding confirms that the effects of LPNPY are mediated mostly by the  $Y_1$  receptor.
- 3) Pertussis toxin, a potent blocker of the  $G_i$ , blocks the ability of LPNPY to enhance renovascular responses to Ang II. This finding confirms that the effects of LPNPY are mediated by the  $G_i$  pathway as well.





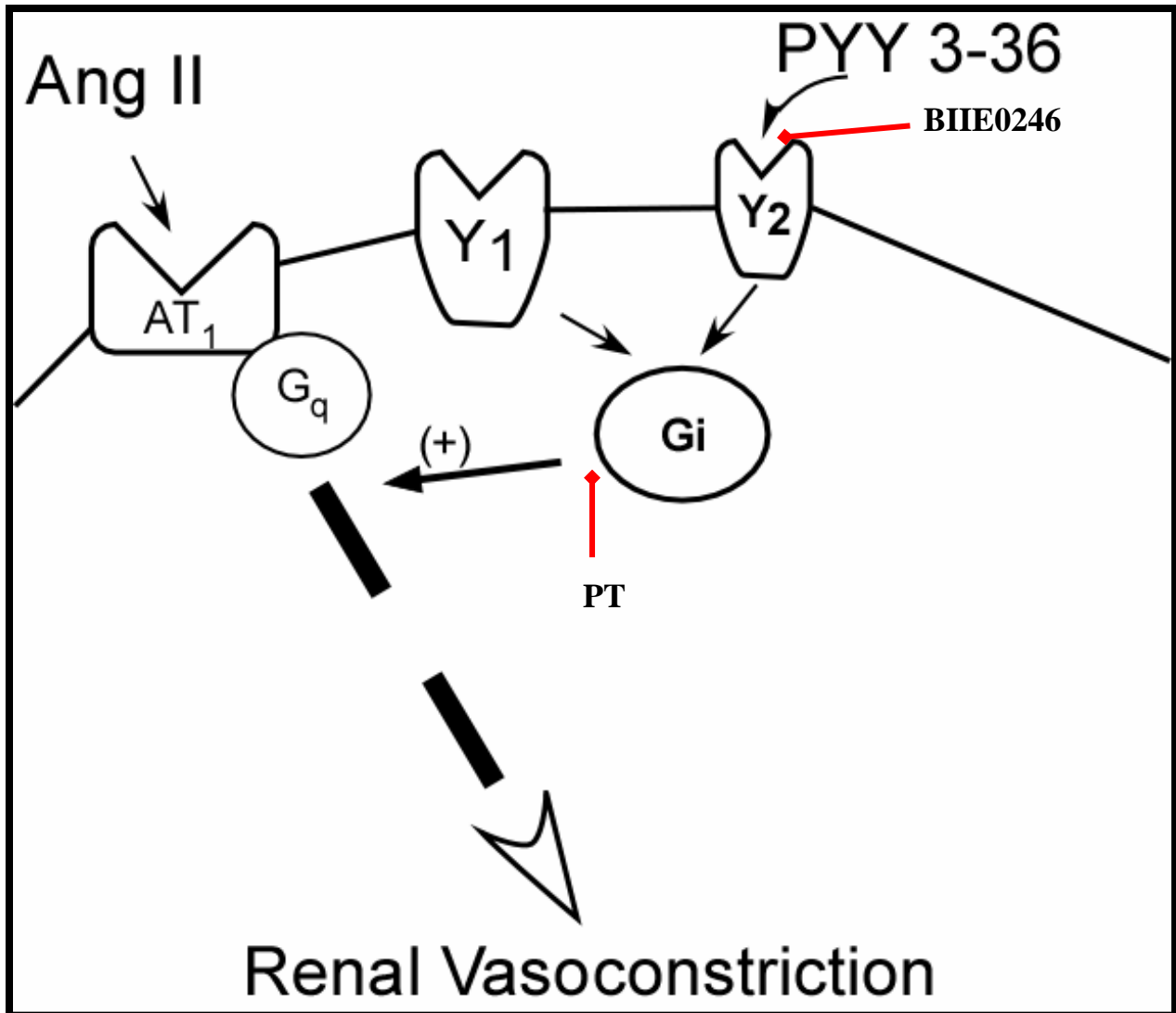
**FIGURE 7.2**

This is a diagram of our primary results. Ang II signaling is more active in the SHR. Activation of the Y<sub>1</sub>-G<sub>i</sub> pathway via LPNPY, a selective Y<sub>1</sub> agonist, potentiates Ang II signaling in the SHR but not WKY. BIBP3226 and PT block the LPNPY induced potentiation of Ang II signaling in SHR.

The results of chapter three also indicate that Y<sub>2</sub> receptors enhance –albeit only slightly– renal vasoconstrictor responses to Ang II and that the potentiation of Ang II responses by Y<sub>2</sub> receptors is greater in SHR compared with WKY rats. In this regard, as represented in figure 7.3:

- 1) PYY<sub>3-36</sub>, a highly selective Y<sub>2</sub>-receptor agonist, slightly enhances the renovascular response to Ang II in the SHR kidney, yet does not potentiate responses to Ang II in the WKY kidney.
- 2) BIIE0246, a highly selective Y<sub>2</sub>-receptor antagonist, completely abrogates the ability of PYY<sub>3-36</sub> to enhance Ang II-induced renal vasoconstriction. This effect confirms that the effects of PYY<sub>3-36</sub> are mediated by the Y<sub>2</sub> receptor and no other PP-fold peptide receptors.
- 3) Pertussis toxin, a potent blocker of the G<sub>i</sub>, blocks the ability of PYY<sub>3-36</sub> to enhance renovascular responses to Ang II. This finding confirms that the effects of PYY<sub>3-36</sub> are mediated by the G<sub>i</sub> pathway as well.

The findings of chapter three have important implications regarding the pathophysiology of genetic hypertension. Renal sympathetic nerves release two major neurotransmitters, norepinephrine and NPY [21]. Norepinephrine causes direct vasoconstriction by  $\alpha_1$ -adrenoceptors that reside within the neuroeffector junction [21]. Importantly, NPY can bind to and activate Y<sub>1</sub> and Y<sub>2</sub> receptors [25,34]. Our findings indicate, therefore, that an important contributing cause to genetic hypertension could be co-release of NPY from renal sympathetic nerves with subsequent activation of postjunctional Y<sub>1</sub> receptors, and perhaps to a much lesser extent Y<sub>2</sub> receptors, leading to significant potentiation of the renal vasoconstrictor responses to



**FIGURE 7.3**

This is a diagram of our secondary results. Ang II signaling is more active in the SHR. Activation of the Y<sub>2</sub>- G<sub>i</sub> pathway via PYY<sub>3-36</sub>, a selective Y<sub>2</sub> agonist, potentiates Ang II signaling in the SHR but not WKY. BIIIE0246 and PT block the PYY<sub>3-36</sub> induced potentiation of Ang II signaling in SHR.

Ang II. The fact that acute blockade of  $Y_1$  receptors does not lower arterial blood pressure in SHR [63] does not disprove this hypothesis. Acute blockade of the  $Y_1$  receptor would result in an increase in  $Y_2$  receptor activation which also has the ability to potentiate Ang II responses in SHR as demonstrated in chapter 3. Chronic treatment, on the other hand, would result in a decrease in NPY release by the negative feedback of the prejunctional  $Y_2$  receptor. Thusly, we suspect that the chronic administration of the  $Y_1$  receptor antagonist would lead to a lower arterial blood pressure in SHR by lowering direct NPY vasoconstriction and causing a leftward shift in the renal-pressure natriuresis relationship.

It has long been known that chronic treatment of SHR with  $\alpha_1$ -adrenoceptor blockers does not alter the course of hypertension in SHR [64]. Because  $\alpha_1$ -adrenoceptors are primarily responsible for sympathetically-mediated direct vasoconstriction in the kidney, these data would appear to rule out the involvement of renal sympathetic nerves in the pathophysiology of genetic hypertension. However, chronic denervation of the SHR kidney both delays the development of hypertension and attenuates the maximum increase in blood pressure[8-11] in SHR, findings consistent with a role for the renal sympathetic nerves in hypertension, yet inconsistent with the results of studies with  $\alpha_1$ -adrenoceptor blockers. Moreover, the profound effects of renal denervation on the natural history of hypertension in SHR are seemingly at odds with the important role of the renin-angiotensin system in genetic hypertension. Our hypothesis would unify these facts by postulating that the problem is not over-activation of  $\alpha_1$ -adrenoceptors or over-activation of the renin-angiotensin system, but rather normal levels of stimulation of  $Y_1$  receptors, and perhaps to a lesser extent  $Y_2$  receptors, leading to potentiation of the renal vasoconstrictor responses to normal levels of Ang II in SHR, but not WKY, kidneys. These ideas were further explored in chapter four and chapter five and discussed more later in this

chapter. From chapter three, it would appear that  $Y_1$  receptors would take the leading role in this scenario because the efficacy of  $Y_2$  receptors with regard to potentiating Ang II-induced renal vasoconstriction seems quite low.

Why does  $Y_1$  and  $Y_2$  receptor activation potentiate Ang II-induced renal vasoconstriction in SHR, but not WKY, kidneys? One possibility is that  $Y_1$  and  $Y_2$  receptor levels are elevated in the kidneys of SHR. However, we are unable to detect an increase in either  $Y_1$  or  $Y_2$  receptor mRNA or protein in either whole kidneys or preglomerular microvessels freshly isolated from whole kidneys. Thus, it appears that the mechanism for the enhanced  $Y_1$  and  $Y_2$  receptor activation does not involve over-expression of receptors, but rather enhanced coincidence signaling between the Ang II and  $Y_1$  and  $Y_2$  receptor signal transduction pathways in SHR kidneys. In this regard, previous work from our laboratory indicates that the  $G_i$  pathway mediates potentiation of Ang II-induced renal vasoconstriction by  $\alpha_2$ -adrenoceptors in SHR[12]. The present study shows that pertussis toxin, which ADP ribosylates and inactivates  $G_i$ , blocks the potentiation of Ang II-induced renal vasoconstriction by  $Y_1$  and  $Y_2$  receptors in the SHR. Thus, like  $\alpha_2$ -adrenoceptors,  $Y_1$  and  $Y_2$  receptors appear to enhance renovascular responses to Ang II in the SHR by activating the  $G_i$  pathway. Several other studies implicate over-expression of  $G_i$  proteins in some tissues in SHR[65-67]. However, our studies suggest normal levels of  $G_i$  proteins in SHR preglomerular microvessels[70], and indicate a role for the  $G_i$  protein/phospholipase C(PLC)/protein kinase C/c-src/phosphatidylinositol 3-kinase pathway in the interaction between Ang II and the  $G_i$  signal transduction pathway in SHR[71]. Although we do not know why coincidence signaling between the Ang II signal transduction pathway and the  $G_i$  signal transduction pathway is enhanced in SHR kidneys, it appears to be due to a

downstream event in the aforementioned signal transduction pathway. Our leading theory is the action each signaling pathway has on PLC. In this respect, Ang II binds the AT<sub>1</sub>R which is coupled to the G<sub>q</sub> pathway. Once the G<sub>q</sub> pathway is activated, the heterotrimeric G-protein releases its  $\alpha$  subunit ( $\alpha_q$ ), and  $\alpha_q$  then binds and activates PLC; however, PLC augments the intrinsic GTPase activity of  $\alpha_q$  (that is to say that PLC is a GTPase-activating protein (GAP) and therefore “GAPs”  $\alpha_q$ ). Therefore PLC increases the rate of GTP hydrolyses by  $\alpha_q$  resulting in the release of the subunit and deactivation of PLC. Activation of the G<sub>i</sub> pathway results in the release of its  $\beta\gamma$  subunit ( $\beta\gamma$ ).  $\beta\gamma$  binds PLC and inhibits PLC’s GAP activity so it has much less ability to inactivate  $\alpha_q$ . Therefore, if PLC is activated by  $\alpha_q$  and  $\beta\gamma$  binds PLC, PLC can no longer release  $\alpha_q$  resulting in increased signaling/response.[72] The demonstrated mechanism mentioned here would suggest that WKY and SHR should have increased responses when both these signaling pathways are activated, yet our results reveal that only SHR demonstrates an increased response. There has been the suggestion of a scaffolding protein that protects PLC from  $\beta\gamma$ . We are currently investigating this protein as the potential target where SHR and WKY differ resulting in only SHR having an increased response. However, additional studies are required to identify more precisely the involved mechanisms.

To the best of our knowledge, chapter three represents the first investigation of the interaction between Ang II and Y<sub>1</sub> and Y<sub>2</sub> receptors on renovascular resistance in either normotensive or hypertensive animals. However, Mohy El-Din and Malik have examined the effects of high concentrations of NPY (17 nmoles/L), a potent endogenous agonist that activates non-selectively Y<sub>1</sub>, Y<sub>2</sub> and Y<sub>5</sub> receptors[25,34], on Ang II-induced renal vasoconstriction in isolated, perfused kidneys obtained from normotensive Sprague-Dawley rats [73]. Their studies

showed that NPY at the concentration employed transiently increased basal renal perfusion pressure by approximately 58% and potentiated the changes in renal perfusion pressure induced by bolus injections of Ang II by approximately 32%. Thus, the work by Mohy El-Din and Malik indicates that high concentrations of a “broad-spectrum” PP-fold peptide receptor agonist enhance Ang II-induced renal vasoconstriction in normotensive animals. In the present study, we did not examine the interaction between Ang II and concentrations of LPNPY greater than 10 nmoles/L because at higher concentrations LPNPY caused a marked and sustained increase in basal renal perfusion pressure that would have confounded interpretation of the interaction between LPNPY and Ang II.

The focus of chapter three was on the interaction between  $Y_1$  and  $Y_2$  receptors and Ang II. It is well known that in many vascular preparations[74,75], including the isolated, perfused rat kidney[76], neuropeptide Y enhances norepinephrine-induced vasoconstriction. Whether this interaction is greater in SHR, compared with WKY, kidneys is an important and open question that should be addressed in future studies. Along these lines, a very recent study by Vonend et al[77] indicates that  $Y_1$ -receptor activation enhances purinergic, nonadrenergic renal vasoconstriction in isolated, perfused kidneys from young and adult WKY and young stroke-prone SHR, but not in kidneys from adult stroke-prone SHR. Thus, the greater enhancement of vasoconstrictor responses in SHR, compared with WKY, kidneys by  $Y_1$  receptors may occur with only some (for example Ang II) vasoconstrictors. Indeed, our previous studies demonstrate that activating the  $G_i$  pathway with the  $\alpha_2$ -adrenoceptor agonist UK14,304 potentiates renovascular responses to Ang II, but not to the  $\alpha_1$ -adrenoceptor agonist methoxamine in the SHR kidney[59].

## **7.2 Naturally Occurring PP-Fold Receptor Agonists, Especially Y<sub>1</sub> Agonists, Enhance Ang II Induced Renal Vasoconstriction And Synergize With α<sub>2</sub>-Adrenoreceptor Agonists**

Y<sub>1</sub> receptors augment renovascular responses to Ang II in SHR kidneys. The renal microcirculation should be exposed to NPY (released from renal sympathetic nerves) and PYY (secreted by intestinal cells into the systemic circulation). NPY and PYY are Y<sub>1</sub>-receptor agonists. These facts provide a compelling rationale for the hypothesis that the naturally-occurring Y<sub>1</sub>-receptor agonists NPY and PYY potentially could play a role with regard to enhancing Ang II-induced renal vasoconstriction in genetic hypertension. Accordingly, a major goal of chapter four was to determine and compare the effects of NPY and PYY on renovascular responses to Ang II in isolated, perfused kidneys obtained from SHR. Importantly, this chapter confirmed that both NPY and PYY have the ability to potentiate renovascular responses to Ang II in SHR kidneys. Moreover, this potentiation appears to be mediated mostly by the Y<sub>1</sub> receptor because the effects of both NPY and PYY are abrogated by the highly selective Y<sub>1</sub>-receptor antagonist BIBP3226.

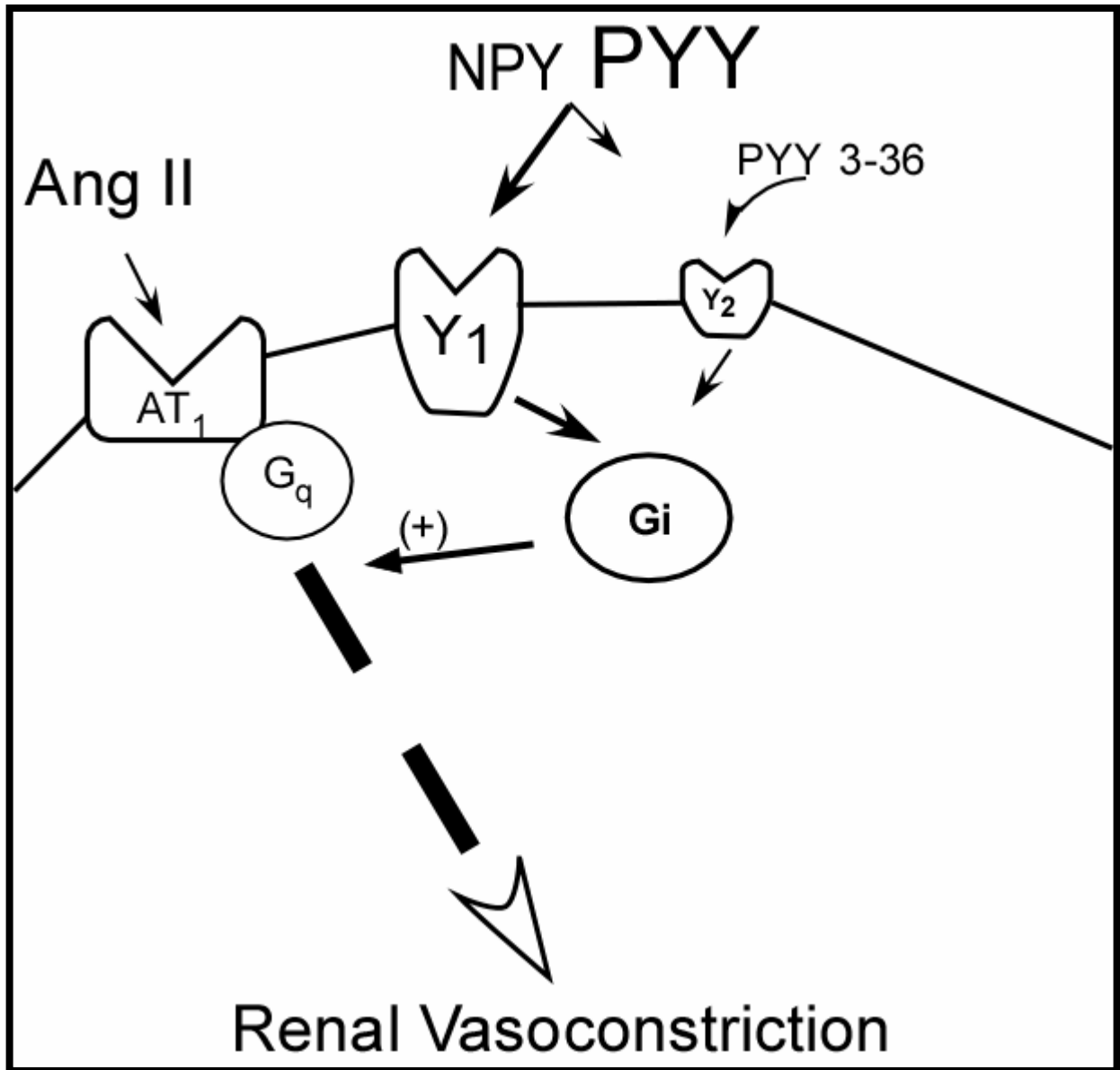
The present study illustrates the capacity of NPY and PYY to potentiate renovascular responses in genetic hypertension. In this regard, as represented in figure 7.4, our results demonstrate:

- 1) The threshold concentration of naturally-occurring PP-fold Y<sub>1</sub>-receptor agonists for enhancing the responses to Ang II in SHR is equal to or less than 1 nmole/L.



- 2) The potential of Y<sub>2</sub>-receptor agonists to alter responses to Ang II is minuscule.

In respect to the primary finding in this chapter, as discussed in the chapter four summary, this concentration (1 nmole/L) is an upper limit for the *in vivo* threshold concentration because small peptides commonly bind tightly to various surfaces. Because sympathetic nerve terminals release NPY directly onto vascular smooth muscle, it seems likely that such concentrations (1 nmole/L) should be achieved locally with elevated renal sympathetic tone; however, direct evidence for this concept is not available. With regard to PYY, in rats systemic levels of immunoreactive PYY can reach 0.1 nmoles/L to 0.25 nmoles/L.[61] Although this concentration is only 10 to 20% of the nominal *in vitro* threshold concentration required for potentiation after a few minutes of exposure, it is conceivable that over longer periods of time, these lower concentrations could accumulate causing a potentiated response to Ang II in kidneys from genetically-susceptible animals and human beings.



**FIGURE 7.4**

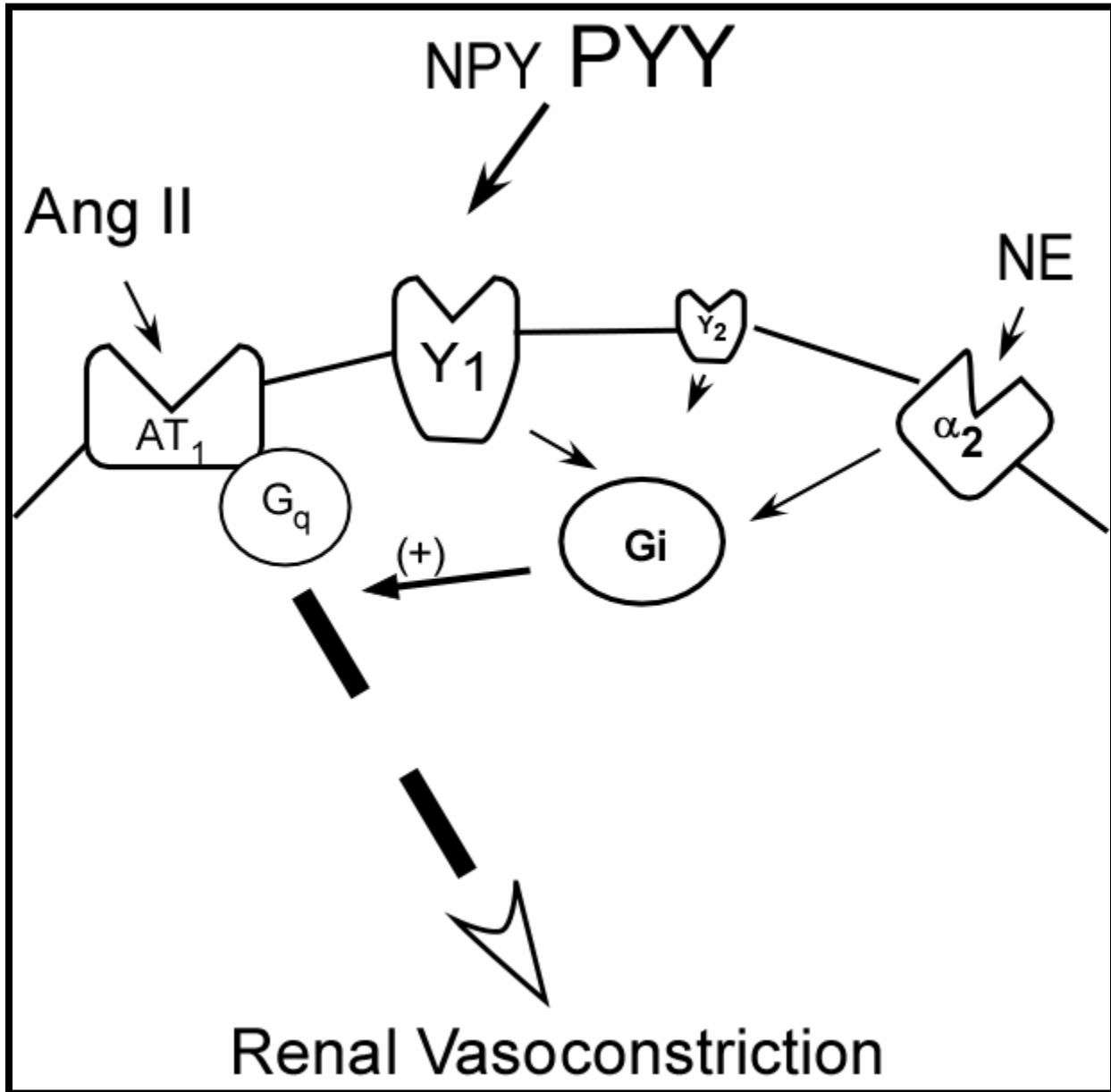
This is a diagram of the results that compare the naturally occurring PP-fold peptides' ability to augment Ang II signaling in SHR. PYY demonstrates the most ability to augment Ang II-induced renal vasoconstriction while PYY<sub>3-36</sub> seems to slightly affect Ang II signaling. This data suggests that the Y<sub>1</sub>-G<sub>i</sub> pathway is more responsible for effecting change in renal responses to Ang II as compared to the Y<sub>2</sub>-G<sub>i</sub> pathway.

Related to the discussion of whether endogenous levels of NPY and PYY are high enough to enhance renal vascular responses to Ang II is the concept that renal vascular smooth muscle cells are bathed by circulating catecholamines and catecholamines released from renal sympathetic nerves and are simultaneously under the influence of circulating PP-fold peptides and PP-fold peptides released from renal sympathetic nerves. Catecholamines can activate  $\alpha_2$ -adrenoceptors, and like  $Y_1$  receptors,  $\alpha_2$ -adrenoceptors are also coupled to  $G_i$  proteins. Because  $\alpha_2$ -adrenoceptors and  $Y_1$  receptors couple to  $G_i$  proteins, it is possible that co-activation of  $\alpha_2$ -adrenoceptors synergizes with PP-fold peptides with regard to enhancing renovascular responses to Ang II in kidneys from genetically hypertensive rats. Indeed chapter four shows, as also demonstrated in figure 7.5, that:

- 3) Low-level stimulation of  $\alpha_2$ -adrenoceptors synergizes with NPY and PYY with regard to potentiating renovascular responses to Ang II in isolated, perfused kidneys from SHR.

This study also demonstrates that:

- 4) Selective  $Y_1$ -receptor activation with LPNPY and selective  $\alpha_2$ -adrenoceptor stimulation with UK14,304 interact to determine the renovascular response to Ang II in kidneys from genetically hypertensive animals and that this interaction occurs *in vivo* and is more robust in SHR compared with WKY rats.



**FIGURE 7.5**

This is a diagram of the results that reveal a synergy between the naturally occurring PP-fold peptides and catecholamines, such as NE. Low level activation of the Y<sub>1</sub>-G<sub>i</sub> pathway can combine with low level activation of the α<sub>2</sub>-adrenoreceptor-G<sub>i</sub> pathway to potentiate Ang II induced renal vasoconstriction in SHR.

The findings discussed above suggest that whether endogenous levels of NPY and/or PYY are high enough to enhance renovascular responses to Ang II may depend critically on the prevailing levels of sympathoadrenal tone. A broader interpretation of our findings is that any  $G_i$ -activating factor has the potential to synergize with any other  $G_i$ -activating factors with regard to augmenting renovascular responses to Ang II. In this respect, activation of multiple  $G_i$ -activating factors would lead to increases in free  $\alpha_i$  subunits. These subunits would then have the ability to bind to PLC, trapping bound  $\alpha_q$  subunits from being released, and increase the Ang II-induced activation of PLC which would lead to increased renal vascular responses. If this is the case, whether NPY and/or PYY enhance renovascular responses to Ang II may depend on the prevailing levels of all three factors (NPY, PYY and catecholamines) as well as the prevailing levels of other  $G_i$  activators such as adenosine via the  $A_1$  receptor [78]. Our fundamental hypothesis, therefore, is that the net effect of Ang II on renovascular resistance in kidneys from genetically-susceptible subjects will depend on the total mix of  $G_i$  activators. We hypothesize that NPY, PYY and catecholamines represent three important players in this regard, but doubt whether this is an exhaustive list and other endogenous  $G_i$  activators most likely participate and will be discovered.

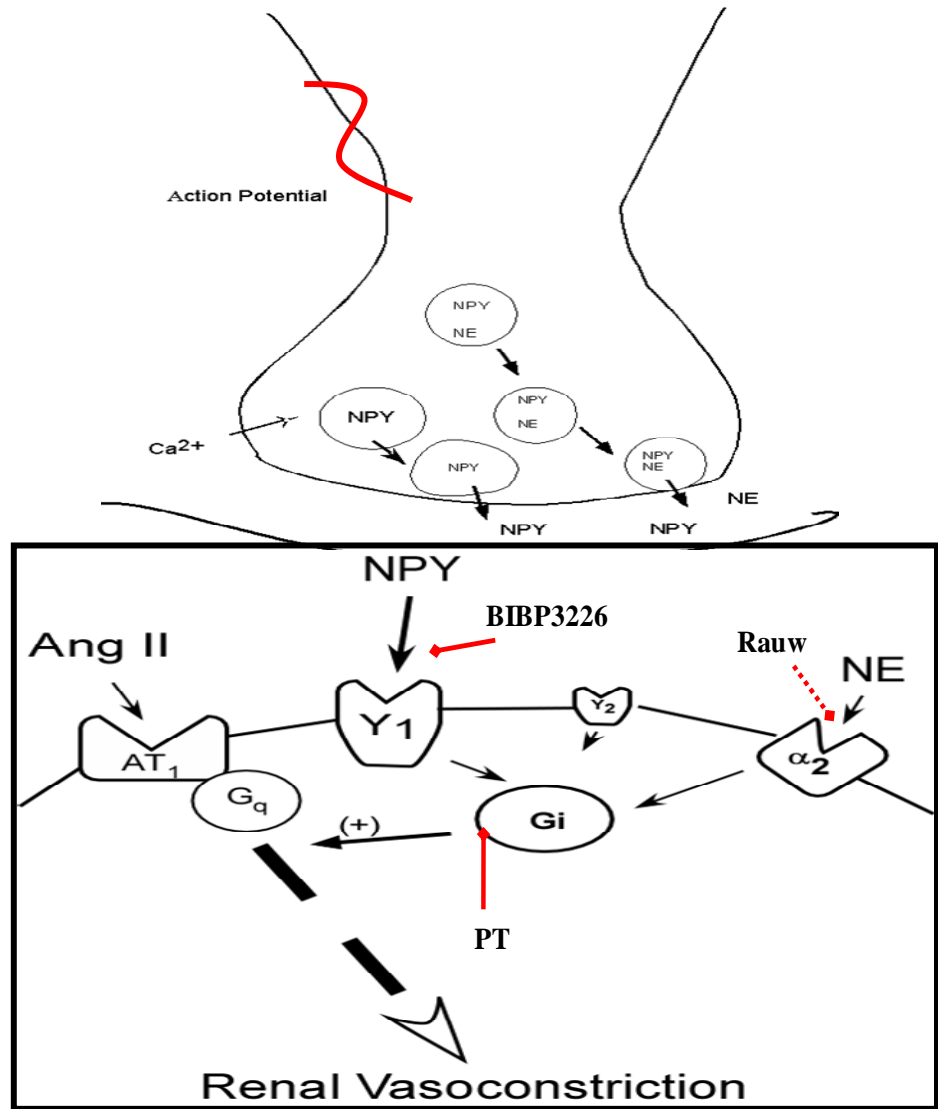
Inhibitors of dipeptidyl peptidase IV are being developed for treatment of type 2 diabetes [51-56,79], and would be expected to prevent the proteolytic cleavage of PYY to PYY<sub>3-36</sub>. Another implication of this study is that in some patients, inhibitors of dipeptidyl peptidase IV could adversely affect renal function and arterial blood pressure by raising PYY levels. Indeed, in further studies we have confirmed that administration of a dipeptidyl peptidase IV inhibitor acutely increases arterial blood pressure in SHR (see chapter 6).

### **7.3 Endogenously Released PP-Fold Receptor Agonists, Via Nerve Stimulation, Enhance Ang II-Induced Renal Vasoconstriction In SHR**

Numerous studies show that the renin-angiotensin system is critical for the development and maintenance of high blood pressure in SHR, and transplantation studies demonstrate that the SHR kidney is essential to the pathophysiology of hypertension in the SHR. Other investigations demonstrate that the renal sympathetic nervous system contributes to the pathophysiology of hypertension in SHR as well. However, a coherent hypothesis that explains the co-involvement of the renin-angiotensin system, the sympathetic nervous system and the kidneys in SHR hypertension is lacking.

The results of chapter five demonstrate that renal sympathetic nerves enhance renovascular responses to Ang II in SHR, but not WKY, kidneys, and this finding provides a critical link that connects the three pathophysiological systems (i.e., renal system, renin-angiotensin system and renal sympathetic nervous system). This unifying hypothesis proposes that hypertension in SHR is due in part to a genetic abnormality in the renal microcirculation that allows the renal sympathetic nerves to potentiate Ang II-induced renal vasoconstriction, thus causing long-term changes in renal function.

In chapter five, our results, also represented in figure 7.6, confirm:



**FIGURE 7.6**

This is a diagram of the results that demonstrate renal nerve stimulation as a potentiator of renal responses in SHR. Stimulation of the sympathetic nerves would lead to the release of NPY and NE that synergize to have a potentiating effect on renal response induced by Ang II. Rauwolscine, an  $\alpha_2$ -adrenoreceptor blocker, slightly blocks RNS potentiation of renal responses. BIBP3226 and PT completely abrogate RNS potentiation of renal responses suggesting that the Y<sub>1</sub>-G<sub>i</sub> pathway plays the predominate role in RNS potentiation.

- 1) A physiological level (5 Hz) of stimulation of the renal periarterial nerves, results in a near doubling of the renovascular response to a physiological level (100 pmoles/L) of Ang II in kidneys from SHR.
- 2) In kidneys from WKY rats, periarterial nerve stimulation does not significantly alter vasoconstrictor responses to Ang II.
- 3) BIBP3226 and pertussis toxin completely abrogate renovascular responses to renal nerve stimulation while rauwolscine has little effect on increased renovascular responses to nerve stimulation.

Thus, we conclude that in kidneys from genetically hypertensive rats, but not kidneys from normotensive rats, physiological levels of renal sympathetic nerve stimulation enhance renovascular responses to physiological levels of Ang II.

The results of this chapter further show that blockade of  $G_i$  with pertussis toxin abolishes the ability of renal sympathetic nerve stimulation to enhance renovascular responses to Ang II in SHR kidneys. This finding indicates that renal sympathetic nerve stimulation augments renovascular responses to Ang II in SHR kidneys via a  $G_i$  signal transduction pathway. Importantly, previous research as well as research from earlier chapters three and four, indicate that the  $G_i$  signal transduction mechanism mediates in part the enhanced renovascular response to Ang II in SHR. For example, pertussis toxin, an inhibitor of  $G_i$ , abolishes the increased renovascular response to Ang II in SHR *in vivo*[18]. The fact that pertussis toxin blocks both the enhanced renovascular responses to Ang II in SHR kidneys *in vivo* and the ability of renal sympathetic nerves to augment Ang II-induced renal vasoconstriction *in vitro* is critical support for the hypothesis that enhanced renovascular responses to Ang II in SHR are mediated in part via activation of renal sympathetic nerves. Published studies by us[19] and others[69]



demonstrate that pertussis toxin is antihypertensive in SHR, and the results of the present study are consistent with that conclusion. The antihypertensive action of pertussis toxin in SHR is also consistent with our aforementioned unifying hypothesis regarding the pathophysiology of hypertension in SHR.

Our previous studies show that activation of the  $G_i$  pathway by agonists of  $\alpha_2$ -adrenoceptors or  $Y_1$  receptors or by co-activation of both  $\alpha_2$ -adrenoceptors or  $Y_1$  receptors potentiates renovascular response to Ang II in SHR kidneys. Because renal sympathetic nerves release both norepinephrine and NPY, which activate  $\alpha_2$ -adrenoceptors and  $Y_1$  receptors, respectively, we anticipated that the ability of renal sympathetic nerve stimulation to augment renovascular responses to Ang II would be attenuated somewhat by both rauwolscine (an  $\alpha_2$ -adrenoceptor antagonist) and BIBP 3226 (a  $Y_1$  receptor antagonist), but not completely by either. In contrast to our expectations, rauwolscine did not significantly affect the ability of renal sympathetic nerve stimulation to augment renovascular responses to Ang II, whereas BIBP 3226 completely abrogated stimulation-induced enhancement of Ang II responses. A conflicting issue is that administration of rauwolscine would lead to upregulation of NPY and NE release via blockade of presynaptic  $\alpha_2$ -adrenoceptors; however, complete abrogation of RNS increases in Ang II activity via blockade of the  $Y_1$  receptor suggests that NPY plays a more predominate role in these RNS responses. In correlation, norepinephrine mainly undergoes prejunctional and postjunctional uptake and therefore would have limited access to  $\alpha_2$ -adrenoceptors, which are known to be primarily extrajunctional[80], therefore, NE alone may have difficulty activating its receptor. On the other hand, NPY does not undergo uptake and, even in the absence of NE, would be available to diffuse to both junctional and extrajunctional  $Y_1$  receptors. If this hypothesis is correct, this leaves open the possibility that circulating catecholamines, for

example from the adrenal gland, as well as circulating endogenous agonists of  $Y_1$  receptors, for example PYY released from intestines, may also enhance renovascular responses to Ang II in SHR under the appropriate conditions.

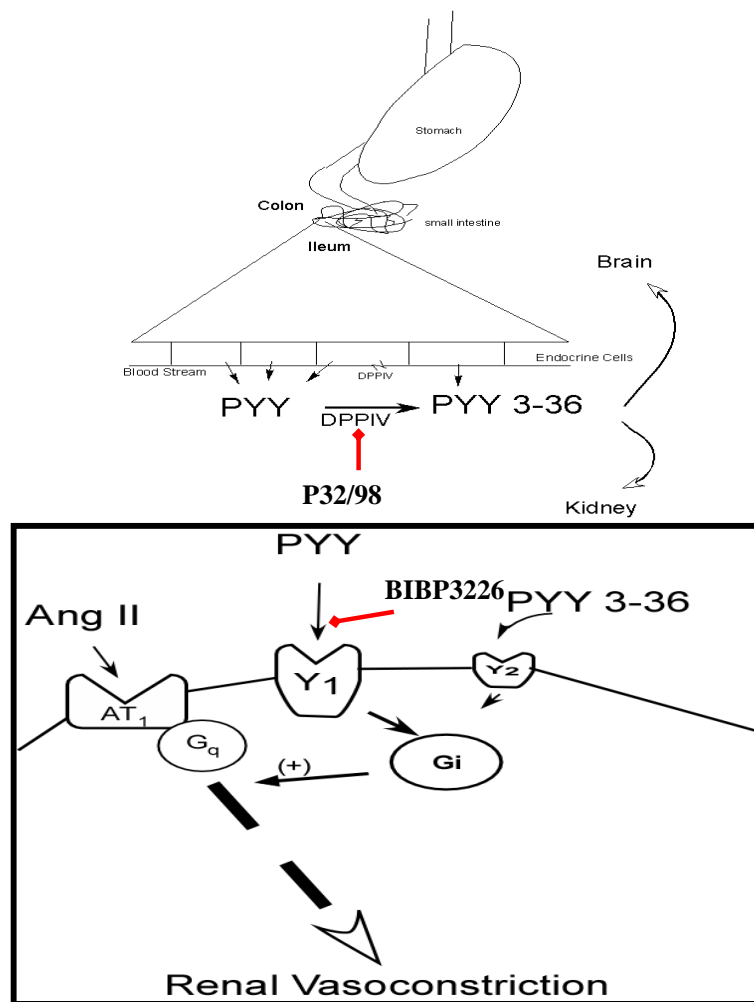
#### **7.4 PYY's Conversion To PYY<sub>3-36</sub> Via Dipeptidyl Peptidase IV May Attenuate The Hypertensive Effect Of PYY**

Chapters three, four, and five of this work have begun to demonstrate a link between the kidney, renin-angiotensin system, and the sympathetic nervous system in the increased blood pressure of the SHR. In this regard, hormones released during sympathetic nerve stimulation, namely NPY and norepinephrine, interact in the kidney with the renin-angiotensin system via a  $G_i$ -dependent mechanism to cause an acute increase in Ang II-induced renovasoconstriction in the genetically susceptible SHR. Yet, NPY is a member in a group of peptides labeled PP-fold peptides. This group also includes PYY which is a gastrointestinally-released  $Y_1$  agonist. Chapter four further demonstrates that PYY has similar if not more ability than NPY to augment Ang II induced renal vasoconstriction, and that in the presence of an  $\alpha_2$ -adrenoreceptor agonist, these two  $G_i$  coupled agonist significantly potentiate Ang II induced renal vasoconstriction in SHR. However, as mentioned in the background, PYY is most often found in the serum in its truncated form (PYY<sub>3-36</sub>) especially following the release of the PP-fold peptides due to a high fat meal.[42-46] The proteolytic cleavage of PYY by DPPIV is the major focus of chapter six.

The results from chapter six reveal an important role in protection from acute increases in blood pressure by the cleavage of PYY to PYY<sub>3-36</sub> via DPPIV. In this regard chapter six demonstrates:

- 1) P32/98, a commercially available DPPIV inhibitor, given intravenously causes an acute increase in mean arterial blood pressure in SHR.
- 2) P32/98 has no effect on blood pressure in WKY, normotensive animals.
- 3) BIBP3226, a Y<sub>1</sub> antagonist, completely abrogates increases in blood pressure induced by P32/98 in SHR.

Thus, we conclude, that the conversion of PYY to PYY<sub>3-36</sub> via DPPIV plays a protective



**FIGURE 7.7**

This is a diagram of the results that show the role of dipeptidyl peptidase IV in SHR. Normally, DPP-IV would result in the body having more PYY<sub>3-36</sub> than PYY which would have little to no effect on renal responses, and thus blood pressure. The use of DPP-IV inhibitor P32/98 leads to increases in renal responses, and thus blood pressure, that can be blocked with BIBP3226. This result suggests that P32/98 increased Y<sub>1</sub>-G<sub>i</sub> pathway signaling.

role in the genetically susceptible SHR, but no role in the WKY normotensive animal. We further conclude that the primary signaling pathway by which blockade of PYY to PYY<sub>3-36</sub> elicits increases in blood pressure is through the Y<sub>1</sub> receptor pathway.

The results of previous research, chapters three and four, suggested the possibility that DPPIV may play a role in the renal response to Ang II in SHR. In this regard, our earlier results demonstrated that PYY had a significant effect on Ang II induced renovasoconstriction via the Y<sub>1</sub>-G<sub>i</sub> pathway in SHR, but not WKY kidneys. On the other hand, PYY<sub>3-36</sub> which is known to only act via the Y<sub>2</sub> receptor pathway elicited little to no effect on Ang II induced renovasoconstriction in SHR or WKY, and had no ability to synergize with UK14,304 to elicit an augmentation. To the best of our knowledge, this is the first study where the role of DPPIV inhibition has demonstrated an effect on blood pressure. Altered serum DPPIV activity have been observed in a number of diseases including different malignant processes, liver diseases and osteoporosis, and DPPIV levels have been shown to be influenced by psychiatric disorders. Yet, there has been no correlation between DPPIV levels and blood pressure.[56] One possible explanation is that the parameters surrounding DPPIV levels have only been observed in normal patients. Our results reveal that in normotensive WKY animals blockade of DPPIV had no effect on blood pressure which would confirm no correlation between DPPIV and blood pressure. However, in animals that are genetically prone to hypertension, for example SHR, the level and ability of DPPIV to act may be essential. Concurrent acute increases in blood pressure could eventually have a detrimental effect in the long term.

Importantly, as discussed in the introduction, DPPIV inhibitors have become an important pharmacological controller of blood glucose level.[51-56] Inhibitors of DPPIV result in the upregulation of GLP-1 which leads to increased blood glucose handling. While this could be beneficial in the pathophysiology of type II diabetes, the findings of the current study would suggest that some individuals may not handle this therapy well. In a subset of patients that have similar genetic abnormalities as the SHR, this type of therapy would lead to at least acute increases in blood pressure that over time could become a chronic problem.

## **7.5 Medical Perspectives**

The renal sympathetic nervous system and renin-angiotensin system interact at multiple levels to regulate renal vascular tone, and hence long-term levels of arterial blood pressure. Augmentation of any of these interactions could participate in the pathophysiology of high blood pressure. The present experiments demonstrate that in SHR, but not WKY, kidneys, renal sympathetic nerve stimulation augments the ability of Ang II to constrict the renal circulation. The mechanism of this augmentation mainly involves  $Y_1$  receptors signaling via the  $G_i$  transduction pathway. Previous studies by many investigators implicate the renal system, the renin-angiotensin system and the sympathetic nervous system in the pathophysiology of genetic hypertension. Furthermore, the present study demonstrates that the gastrointestinal system may also play a role in the pathophysiology of genetic hypertension. Similar to renal nerve stimulation, the augmentation observed via gastrointestinal sources mainly involves  $Y_1$  receptors signaling via the  $G_i$  transduction pathway. The implications of the experiments reported here provide a possible explanation for the co-involvement of these systems in genetic hypertension.

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