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Effects of LXR on renin-induced pressor increses in rats

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EFFECTS OF LXRα ON RENIN-INDUCED PRESSOR INCREASES IN RATS

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Heather DiMaio Knych
August 2000
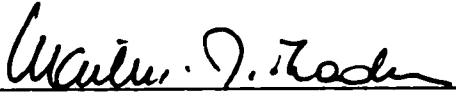
APPROVED FOR THE DEPARTMENT OF BIOLOGY

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Dr. D.C. Holley

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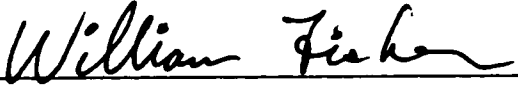
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APPROVED FOR THE UNIVERSITY

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Abstract

EFFECTS OF LXRα ON RENIN-INDUCED PRESSOR INCREASES IN RATS

by Heather DiMaio Knych

This study characterizes rat vasopressor responses to intravenous administration of human and porcine angiotensinogen for the purpose of developing a model with which to evaluate functional inhibitors of renin. A dose dependent increase in mean arterial pressure (MAP) was seen following administration of 0.001, 0.01, 0.1 and 1.0 mg/kg body weight of angiotensinogen. T0901317, an LXRα agonist with good oral bioavailability had a significant effect on plasma lipids but no effect on plasma renin activity or the angiotensinogen induced vasopressor response. In addition, this compound had no effect on MAP in either Spontaneously Hypertensive or renal hypertensive rats. These data indicate that LXRα agonism does not lead to inhibition of the renin angiotensin system (RAS) mediated vasopressor effects and that this class of agents do not appear to be useful for the development of antihypertensive agents based on their effects on renin release or other components of the RAS.

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Lastly, but certainly not least, thank you to my family and especially my husband John for always listening and simply being there for me. Your continued support, in spite of everything, is something I can never thank you enough for.

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I.   Introduction

In past years significant advances have been made in hypertensive therapy, however, there has been limited success in developing renin inhibitors. The renin inhibitors that have been developed have numerous drawbacks, including poor solubility, low affinity for renin and minimal oral bioavailability. Renin inhibitors, however, still have one potential advantage over current anti-hypertensive therapies, that is, specificity. The enzyme renin has one known substrate or target, angiotensinogen. This makes renin manipulation a good candidate for antihypertensive drug intervention.

Regulation of a physiologically important molecule through up-regulation or down -regulation of the gene encoding it is growing in popularity as a means of clinical intervention and therapy. However, in order to regulate at the level of transcription, we must elucidate the gene and possible controlling transcription factors. It has recently been suggested that a member of the nuclear receptor superfamily, LXR alpha, may play a role in the regulation of the renin gene (Victor Dzau, personal communication). If this were found to be the case, then regulation of LXR alpha would offer a new therapeutic modality for controlling hypertension. By producing a small molecule that can block or stimulate LXR alpha, we could potentially control the transcription of the gene responsible for the release of renin.

A.   LXR alpha Literature Review

1.0 The Nuclear Receptors Superfamily

Since the late 1980s, an intensive effort has been dedicated to the study of a group of novel gene products belonging to the nuclear receptor superfamily. Nuclear receptors are a family of transcriptional regulators that play important roles in a wide variety of metabolic and developmental processes. The ligands or hormones for these receptors are naturally occurring. The alterations in the transcription of genes caused by these nuclear receptors is due to an interaction between the DNA binding domain, located on the nuclear receptor, and the corresponding response element on the target gene (Mangelsdorf et al, 1995). Also present on the nuclear receptor is a ligand binding domain (LBD) for its specific ligand. The ligand binding domain contains a dimerization interface, a copressor interaction domain and a transcriptional activation function (Mangelsdorf, 1995). Binding of ligand changes the transcriptional status of the LBD from repressed to active.

A) NH2 — [A/B | C | D | | E | F] — COOH

Transactivation Domain   DNA binding Domain   Hinge   Ligand Binding Domain

B) 5' – AGGTCA-3'   DR   IR   ER

C) RXR + nuclear receptor
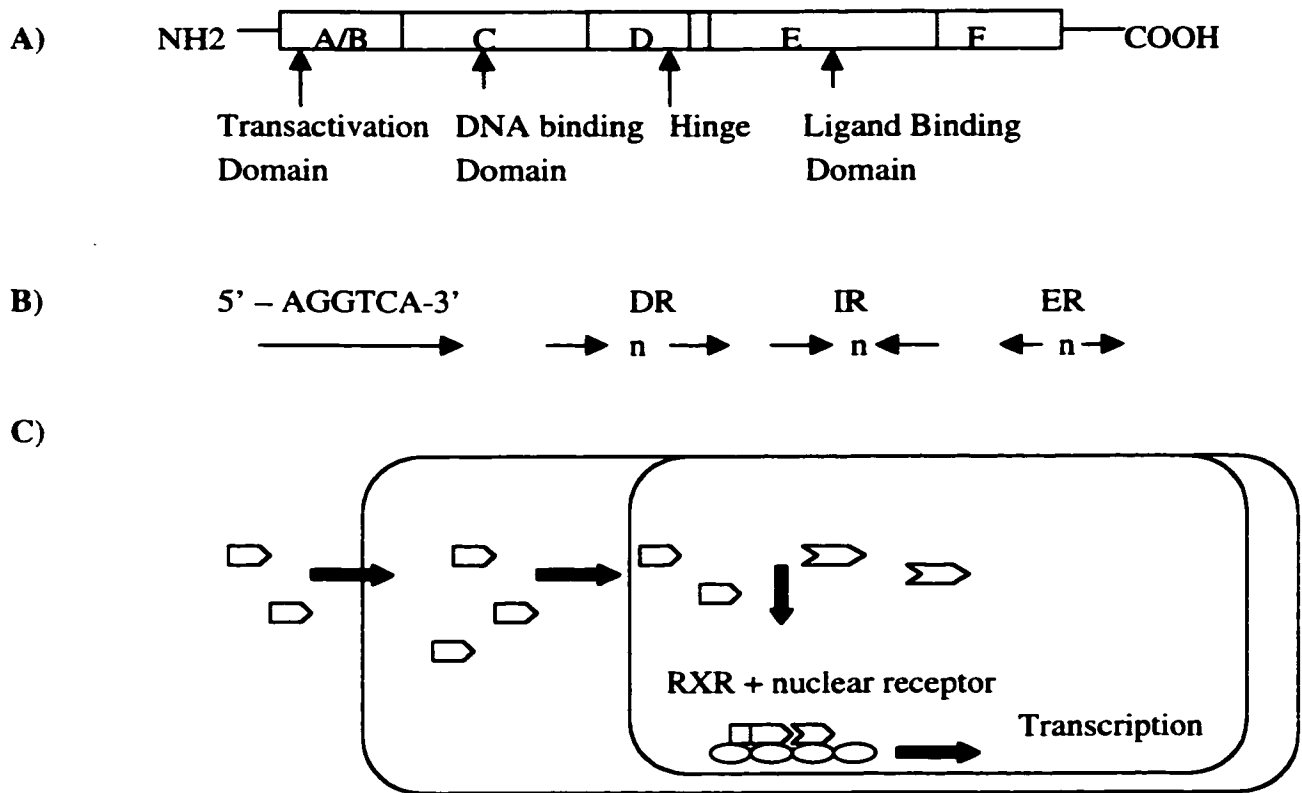
Transcription

Figure 1. Nuclear Hormone Receptors are Ligand Dependent Transcription Activators. (A.) Schematic representation of a nuclear receptor. Functional domains of nuclear receptors, indicating N-terminal A/B domain that often contains a ligand-independent transactivation function, the highly conserved C domain that mediates DNA binding, the hinge region D, the conserved ligand binding domain, E, and a highly variable C-terminal sequence F. The ligand binding domain also mediates dimerization, ligand-independent repression, and ligand dependent transactivation functions (Westin et al, 2000).

(B.)   A typical nuclear response element is composed of DRs (direct repeats), IRs

(inverted repeats) and ERs (everted repeats) of the core sequence AGGTCA. Specificity

for different hormone responses is given by the number of nucleotides (n).

(C.) Simplified diagram of the Mechanism of action of a typical nuclear receptor

heterodimer.

The nuclear receptor ligands, as well as the functions of many of these ligands

have been extensively studied within the last ten years. The nuclear receptors are a

diverse group of gene products, possessing a number of different ligands and signaling

pathways. This thesis focuses on one nuclear receptor in particular, the Liver X Receptor

alpha (LXR alpha). LXR alpha expression is predominately found in metabolic organs,

such as the liver, kidneys, and the intestine (Willy et al, 1995). In addition, LXR alpha is

found to a lesser extent in the spleen and adrenals.

1.1 Heterodimerization with the Retinoid X Receptor

Many members of the nuclear receptor superfamily must form a heterodimeric

complex with the Retinoid X Receptor (RXR) for full functionality. LXR alpha falls into

this grouping. Fusing the ligand binding domain of LXR alpha, derived from the human

liver, with the DNA binding domain of GAL4 created a LXR alpha hybrid protein. This

hybrid was only able to drive expression of luciferase in CV-1 cells when it was able to

form a heterodimer with a VP16-RXR alpha hybrid (Willy et al, 1995). The necessity

for RXR was further demonstrated by the use of gel retardation assays. When LXR alpha was added together with RXR, a strong complex was formed (Willy et al, 1995; Lehman et al, 1997). This led to the conclusion that high affinity DNA binding required the formation of LXR-RXR heterodimers. Further gel retardation assays showed a much slower migration and a weaker complex formation with the DR4-LXRE (LXR Recognition Element) when LXR alpha was used in the absence of RXR .

Each nuclear receptor binds to a unique hormone response element (HRE) within the promoter region of a gene. For those nuclear receptors that bind to DNA as a heterodimer with RXR, the recognition element is a nearly perfect tandem repeat of the nuclear receptor half site recognition sequence AGGTCA. These direct repeats of the core sequence are usually spaced with 1, 2, 3, 4 or 5 nucleotides (DR1, DR2, DR3, DR4 or DR5). It is slight differences in the half site recognition sequence along with different numbers of spacer nucleotides, which determine response element specificity (Mangelsdorf et al, 1995).

As was mentioned earlier, formation of a heterodimer with RXR is necessary for LXR alpha to activate transcription. To determine the polarity of the RXR-LXR alpha heterodimer on the LXRE, site directed mutagenesis was first used to create a mutant LXR alpha receptor. This mutant was then used in electrophoretic mobility shift assays and transient transfections to determine the polarity of the RXR-LXR alpha binding using two mutant LXRE sequences. None of these receptors could bind the LXRE sequences alone but the RXR-LXR alpha heterodimer was able to bind the LXRE when the mutant LXR alpha half site was in the 3' position. Therefore, the RXR-LXR alpha heterodimer

binds to the DNA with the LXR occupying the 3' half site and the RXR occupying the 5' half site (Willy et al, 1997).

RXR — LXR

5' ⊂◯◯◯◯◯◯◯◯◯⊃ 3'

Figure 2. Simplified diagram of the orientation of the RXR-LXR heterodimer when binding to the LXRE (LXR response element). RXR occupies the 5' site while LXR occupies the 3' site.

1.2 LXR alpha Ligands

Recently, it has been discovered that the oxysterols are ligands for the LXR alpha receptor (Janowski et al, 1996; Lehman et al, 1997; Peet et al, 1998; Janowski et al, 1999). These include, 22 (R) –hydroxycholesterol, 24(S)-hydroxycholesterol and 24(S), 25 epoxycholesterol (Janowski, et al, 1996; Lehman, et al, 1997). LXR alpha forms a heterodimer with the retinoid X receptor (RXR). The LXR/RXR heterodimer then binds a response element within a gene. A LXR response element has been found within the animal as well as human Cyp7a gene (Lehman et al, 1997). This suggested that LXR alpha might regulate cholesterol homeostasis. An increase in dietary cholesterol leads to an increase in the catabolic elimination of cholesterol as bile acids. Cyp7a is known to be the rate limiting enzyme in the bile acid synthesis pathway (Lehman et al, 1997). Essentially, when there is an increase in cholesterol more oxysterols bind to LXR alpha leading to an upregulation of components of cholesterol metabolism.

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The role of LXR alpha in cholesterol regulation is further supported by the use of LXR alpha knockout mice. These mice lack the LXR alpha gene and also have a rapid accumulation of cholesterol esters in the liver when fed a diet high in cholesterol. This further suggests a link between LXR alpha and cholesterol elimination. These mice are unable to upregulate the Cyp7a gene thus having an altered bile acid metabolism (Peet et al, 1998).

B.   Literature Review of Hypertension

2.0 Hypertension

Hypertension is a condition in which a person has higher blood pressure than that which is considered normal. In humans a mean arterial pressure greater than 110 mmHg under resting conditions is considered hypertensive. In extreme cases, mean arterial pressure is greater than 140 mm Hg and this is considered severe (Ganong, 1997). Hypertension is usually due to an increase in peripheral resistance resulting from vasoconstriction or narrowing of peripheral blood vessels. Hypertension is the most common cause of cardiovascular disease. Chronic hypertension can lead to serious problems including damage to blood vessels in the kidney, heart and brain. This in turn can lead to renal failure, coronary artery disease, cardiac failure and/or stroke.

2.1   Essential Hypertension

Essential hypertension is defined as elevated blood pressure with an unknown etiology. This type of hypertension accounts for 85-90% of all hypertension. Essential hypertension starts out with intermittent exaggerated pressor responses to stimuli, such as heat or cold, that wouldn't affect normal individuals. Eventually, the blood pressure response becomes sustained. The disease can then progress even further resulting in narrowing of blood vessels and spasm of the arterioles leading to hypertrophy of their musculature.

2.2   Renal Hypertension

Renal hypertension is a form of hypertension that results from constriction or obstruction of the renal arterial blood supply. This leads to an increase in renin secretion and ultimately an increase in blood pressure. In addition, the inability of the kidney to secrete Na^+ due to constriction of the renal artery contributes to the hypertensive response. This thesis is primarily concerned with these potential renal mechanisms of hypertension.

3.0   The Renin-Angiotensin System (RAS)

The Renin-Angiotensin system is an essential factor in the maintenance of fluid volume and blood pressure. Both renin and its inactive form, prorenin, are stored in the juxtaglomerular cells in the distal tubules of the nephron. They are released in response to a drop in glomerular afferent arteriolar blood pressure, sympathetic nerve stimulation, or a reduced rate of sodium ion delivery to the distal tubule. Renin only remains in the bloodstream for a short period of time and during this time it acts on its one and only substrate, angiotensinogen. Angiotensinogen is produced by the liver and circulates in the bloodstream. When it comes into contact with renin it is cleaved, releasing a 10 amino acid sequence known as Angiotensin I. This enzymatic cleavage of angiotensinogen by renin is the rate limiting step of the RAS, making it an ideal target for drug action. Angiotensin I, itself, has very mild vasoconstrictive properties. It is further cleaved to produce an eight amino acid sequence known as angiotensin II. This enzymatic cleavage occurs as angiotensin I travels through the lungs and other tissues

where it comes in contact with angiotensin converting enzyme (ACE). Figure 3 shows a schematic of the biochemical reactions of the RAS.

Asp – Arg – Val – Tyr – Ile – His – Pro – Phe – His – Leu – Val – Ile – His – Asn – R

Angiotensinogen

Renin

Asp – Arg – Val – Tyr – Ile – His – Pro – Phe – His – Leu

Angiotensin I

Converting Enzyme

Asp – Arg – Val – Tyr – Ile – His – Pro – Phe
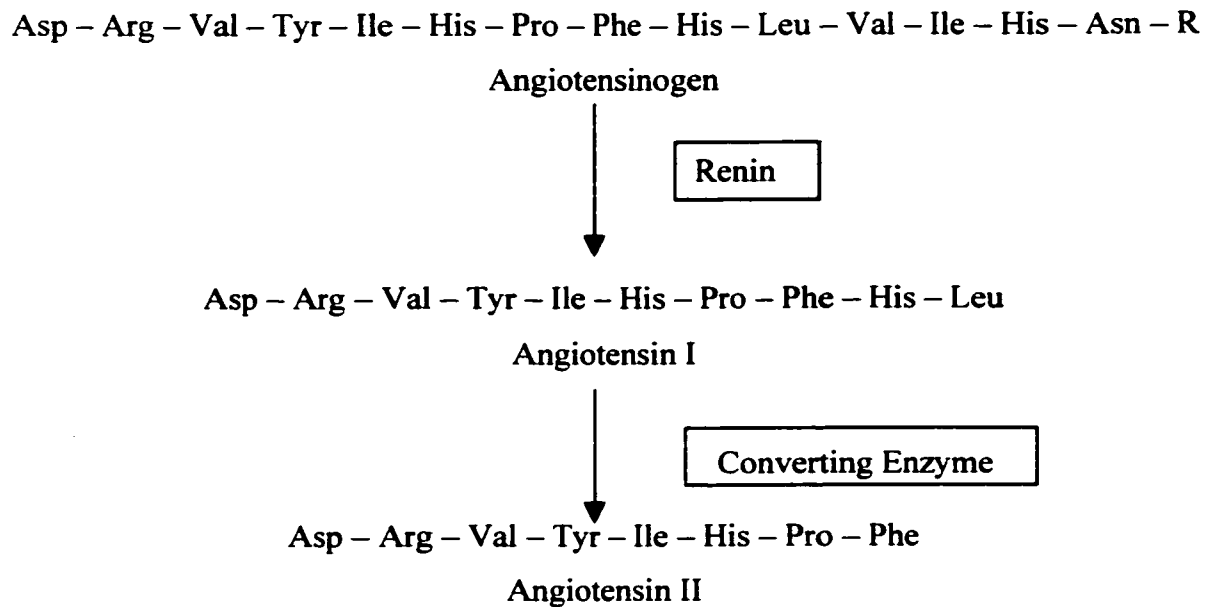
Angiotensin II

Figure 3. Chemistry of the renin-angiotensin system. The amino terminal of human angiotensin is shown. R denotes the remainder of the protein molecule.

Angiotensin II has a number of effects, one of which is vasoconstriction of smooth muscle in the blood vessel walls. This leads to an increase in blood pressure. Angiotensin II also acts upon cells in the brain to produce sympathetic nervous signals to stimulate drinking and sodium appetite. In addition, Angiotensin II increases the production and release of aldosterone from the renal cortex. Essentially, aldosterone increases the re-uptake of sodium. It modifies the reabsorption of sodium in the

collecting duct. Sodium reabsorption is increased and water passively follows causing the isotonic sodium ion solution to be reabsorbed. This modifies the concentrations of Na^+ and K^+ in the body fluids thus affecting the volume of these fluids. This process saves Na^+ and K^+ and maintains blood pressure by maintaining body fluid volume. The net effect of the renin-angiotensin-aldosterone system is to prevent sodium depletion and hypotension.

While renin is important for maintenance of blood pressure, chronic high levels can lead to renal hypertension. Chronic increased activity of renin leads to an increase in angiotensin II and a subsequent chronic increase in peripheral resistance. Retention of large amounts of sodium by the kidneys, due to high angiotensin II levels, also contributes to the development of hypertension. Hypertension can lead to more serious health problems including an increased incidence of heart failure, strokes, and renal failure. In addition, there is a disease state in which renin secreting tumors can lead to hypertension (Kuroda, 2000).

3.1   Renin

Renin is synthesized by, stored in and released from the juxtaglomerular cells of the juxtaglomerular apparatus in the kidney. It is first synthesized in an inactive form known as prorenin, which contains 406 amino acids. Renin is formed when the 23 amino acid leader sequence and the pro sequence are removed from the N terminal end of the prorenin molecule. It is a 340 amino acid glycoprotein with a molecular weight of 37,326 in humans. The renin molecule consists of two domains with the active site in

between. It has 2 aspartyl acid residues located at positions 104 and 292 that are juxtaposed in the cleft and essential for activity. Prorenin and renin are released into the circulation at a rate of 10 prorenin for every 1 renin. This makes the rate of renin secretion one of the primary determinants of the renin angiotensin system. The half- life of renin in the human circulation is 80 minutes.

The secretion of renin is controlled by a number of factors. One factor that controls renin release is renal vascular receptors, which are located within the afferent arteriole. The juxtaglomerular cells of this afferent arteriole function as a renal baroreceptor that responds to stretching of the arterial wall. Decreased input to these stretch receptors (less stretch), resulting from a fall in blood pressure leads to an increase in renin release. A second factor controlling the release of renin is receptors which are sensitive to changes in the rate of Na^+ and Cl^- delivery to the distal tubule. Decreases in Na^+ and Cl^- stimulate the release of renin via a $Na^+/K^+/2Cl^-$ cotransporter. In addition, increased renal nerve activity also contributes to the release of renin. Circulating catecholamines in the body stimulate both beta adrenergic and alpha receptors. This leads to an increase in sympathetic nervous system activity, thereby increasing renin secretion, in particular via beta adrenergic receptors. Lastly, there is a negative feedback loop between angiotensin II and renin whereby angiotensin II acts directly on receptors in juxtaglomerular cells to inhibit further renin release. The rate-limiting step in the renin-angiotensin system is the renin-angiotensinogen reaction. The main source of this renin is found in the kidneys. In this particular study we will attempt to remove the main source of renin from the body by performing a bilateral nephrectomy. The net effect

would be the same as if we had administered a renin inhibitor, circulating angiotensinogen will not be cleaved, there will be diminished generation of angiotensin I and angiotensin II and a subsequent decrease in blood pressure.

In one study where a bilateral nephrectomy was performed, plasma renin levels fell to less than 2% of control values 24-48 hours post nephrectomy (Campbell et al, 1993). In addition, there was a four to eightfold increase in angiotensinogen levels. Angiotensin I and angiotensin II levels were also significantly decreased by 48 hours postnephrectomy (Campbell et al, 1993).

3.2 Angiotensinogen

As was discussed earlier, angiotensinogen is cleaved to angiotensin I by renin. Angiotensinogen is synthesized in the liver and is then released into the circulation where it encounters renin. Rat and human angiotensinogen are both glycoproteins with molecular weights ranging from 52-60 KD. In humans angiotensinogen has four possible glycosylation sites for renin to bind. All four sites are used for generation of angiotensin I. The renin-angiotensinogen reaction is the rate limiting step in the renin-angiotensin system. The concentration of angiotensinogen in the circulation is close to its K_m with respect to renin, making it an important factor in the generation of angiotensin II.

Many researchers have addressed the role of angiotensinogen in hypertension. In the rabbit kidney and perfused rat kidney model, injection of angiotensinogen leads to renal vasoconstriction. This in turn leads to an increased release of renin and subsequent hypertension. In addition, when angiotensinogen antibodies were injected into the rat,

the subsequent angiotensinogen neutralization led to a drop in blood pressure (Menard et al, 1983). In another example, homozygous mutant angiotensinogen deficient mice had undetectable plasma angiotensinogen levels and systolic blood pressures 35% lower than wild type (Morgan et al, 1996).

We theorized that administration of angiotensinogen, administered as either an intravenous bolus or an infusion, could elicit an immediate hypertensive response. An intensive literature search failed to turn up any information on this topic. We feel that by introducing exogenous angiotensinogen into the body there would be a subsequent increase in circulating angiotensinogen available for renin to cleave. This in turn would lead to an increase in the production of angiotensin I and angiotensin II and ultimately an increase in blood pressure.

The renin-angiotensinogen reaction is the rate limiting step in the renin-angiotensin system. By blocking renin, initiation of biochemical reactions in the renin-angiotensin system is prevented. Inhibitors of renin bind to the active site of renin blocking its ability to hydrolytically cleave angiotensinogen. Renin inhibitors decrease plasma renin activity, systemic vascular resistance and systemic blood pressure.

3.3 Angiotensin II

As discussed previously, angiotensin II has very potent vasoconstrictive properties. Angiotensin II has a direct effect on arteriolar smooth muscle leading to constriction. In addition, it can increase blood pressure by acting on the brain and autonomic nervous system. In the brain, angiotensin II resets the baroreceptor reflex

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control of heart rate leading to an increase in blood pressure. In the autonomic nervous system, angiotensin II stimulates ganglia and increases the release of epinephrine and norepinephrine from the adrenal medulla and acts on adrenergic nerve terminals, facilitating sympathetic transmission (Ganong, 1997).

Angiotensin II causes renal vasoconstriction leading to an increase in proximal tubular sodium reabsorption and inhibition of renin release. In addition, angiotensin II directly stimulates the synthesis of aldosterone by the adrenal cortex. Angiotensin II also stimulates drinking and an increase in vasopressin and ACTH secretion by its actions on the central nervous system. Angiotensin II is unable to cross the blood brain barrier and, therefore, exerts its effects by acting on the circumventricular organs. It is the area postrema that is responsible for the pressor effect and the subfornical organ and the organum vasculosum of the lamina terminalis which lead to an increase in water intake (Ganong, 1997).

Angiotensin II has a half life of 1-2 minutes. It is metabolized by a number of peptidases as it passes through most vascular beds. This generates a number of biologically inactive metabolites. However, one metabolite, angiotensin III, does have mild pressor activities. It is formed when an aminopeptidase removes the Asp residue from the N terminal of the angiotensin II peptide.

3.4 Angiotensin II Receptors

There are two different classes of angiotensin II receptors. These receptors have been characterized based on their affinity for peptide and nonpeptide antagonists. AT_1

receptors have a high affinity for non-peptide antagonists and a low affinity for peptide antagonists. AT_2 receptors have a high affinity for peptide antagonists and a low affinity for non-peptide antagonists. Both receptors bind angiotensin II with equal affinity. The AT_1 receptors are serpentine receptors coupled by a G protein to phospholipase C. When angiotensin II binds to the receptor, there is a phospholipase C mediated generation of inositol triphosphate and diacylglycerol, an increase in cytosolic free Ca^{2+} levels and a subsequent smooth muscle contraction. There are two subtypes of AT_1 receptors. AT_{1A} receptors are located in the blood vessel walls, the brain and other organs. AT_{1B} is found in the anterior pituitary and adrenal cortex. Angiotensin II down-regulates the AT_{1A} vascular receptors while it up-regulates adrenocortical receptors.

AT_2 are serpentine receptors with seven transmembrane domains. The second messengers by whom these receptors interact are not known, but they do not appear to act via a G protein, like the AT_1 receptors. These receptors are plentiful in fetal and neonatal life and are found in the brain and other organs in the adult. The functions of the AT_2 receptor are not well known.

4.0   Anti-Hypertensive Therapy

4.1 Renin Inhibitors

Angiotensin Converting Enzyme (ACE) inhibition has been a very effective way of treating hypertension. However, this form of therapy lacks selectivity for the renin-angiotensin system because it also affects bradykinin inactivation, leutinizing hormone

releasing hormones, and others. Renin, on the other hand, has one known target, angiotensinogen, making renin inhibition therapy very specific.

The first renin inhibitors to be developed were antibodies against renin. These antibodies were found to be effective in eliciting a hypotensive response and lowering plasma renin activity. However, renin antibodies proved to be orally inactive because they, as proteins, are not easily absorbed from the gastrointestinal tract. In addition, these antibodies have the potential to induce an antigenic response with repeated administration.

The second class of renin inhibitors were synthetic derivatives of the prosegment of the renin precursor. Renin is synthesized as an inactive precursor, prorenin. Peptides derived from the pro segment of this precursor have been found to inhibit renin (Luther et al, 1989). However, these inhibitors have a very low potency, making them less useful as renin inhibitors.

Another class of renin inhibitors that originally showed promise were the pepstatin analogs. Pepstatin was isolated from the actinomyces cultures and was found to be a potent inhibitor of pepsin, and to a lesser extent, renin. Pepstatin and its analogs have been shown to inhibit renin *in vitro* and to exhibit hypotensive effects *in vivo*, however, they do have their drawbacks. Pepstatin analogs have poor solubility, low intrinsic activity, and a short duration of action.

The fourth class of renin inhibitors are the angiotensinogen analogs. This class consists of modified angiotensinogen fragments, which compete for renin activity. They form an inactive product several amino acids shorter than angiotensinogen I (Frishman et

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al, 1984). Due to their peptide nature and associated limitations, they have not been developed as antihypertensive drugs.

4.1.1 Pepstatin

Pepstatin is a pentapeptide acid protease inhibitor produced in culture by actinomyces. Pepstatin inhibits many aspartyl proteases and has a high affinity for pepsin and a lower affinity for renin. As its name implies, pepstatin contains two statin residues. It is the central residue that determines the binding affinity. Pepstatin has an IC50 of 1.4 x 10^{-6} M against human renin and 8.3 x 10^{-6} M against rat renin (Oldham et al, 1984). Pepstatin has efficacy *in vivo* as well. A drop in blood pressure of 40.1 ± 3.8% has been demonstrated following a 30 minute infusion of 70 ug/kg/min of pepstatin in normotensive rats. In the same study, an infusion of 150 ug/kg/min of pepstatin for 30 minutes showed a decrease in blood pressure of 50.2 ± 4.6%. It has also been demonstrated that pepstatin, administered at 150 ug/kg/min, is able to inhibit the activity of exogenous renin (Oldham et al, 1984). In another study performed in hypertensive rats, an average blood pressure drop of 48.3 ± 8.2 mm Hg was seen following pepstatin injection. In addition, pepstatin was also found to significantly decrease plasma renin activity levels in these rats. Angiotensin I levels dropped from 501 ± 125 pmol to 289 ± 74 pmol following intravenous injection of pepstatin (Oldham et al, 1984).

4.1.2   Renin Inhibitory Peptide (RIP)

His-Pro-Phe-His-Leu-Leu-Val-Tyr, also known as Renin Inhibitory Peptide (RIP), is an analog of a segment of the renin substrate. It is a competitive inhibitor of renin with an inhibitory constant (K_i) of 42 μM. This compound retains the central statin but the amino acids surrounding this statin are replaced by amino acids having a high affinity for the corresponding renin substrate (Hoover et al, 1995). Renin Inhibitory Peptide has proven very effective *in vivo*. When infused into normotensive monkeys, no change in blood pressure was observed. However, when exogenous renin, angiotensin I and angiotensin II were injected, an infusion of 0.2 mg/kg/min of RIP led to a significant inhibition of the pressor response to renin but no inhibition of angiotensin I or II was observed (Haber, 1983). From this investigators were able to conclude that Renin Inhibitory Peptide is renin specific. Renin Inhibitory Peptide also has one major advantage over pepstatin, it is much more soluble.

4.2   Angiotensin Converting Enzyme Inhibitors

The most commonly prescribed anti-hypertensive agents are angiotensin converting enzyme (ACE) inhibitors. ACE is a dipeptidyl-carboxypeptidase. Inhibitors of this enzyme cleave off histidylleucine from angiotensin I to form angiotensin II. Most of the angiotensin converting enzyme is localized within endothelial cells. ACE is especially prevalent in the lungs. As blood passes through the lungs and other organs, the circulating angiotensin I is cleaved to angiotensin II.

Although many anti-hypertensive therapeutics do target angiotensin converting enzyme, it has dual functions in the body making it a less than ideal target. As mentioned earlier, ACE is also responsible for the inactivation of bradykinin. Therefore, by inhibiting this enzyme, you are not only inhibiting hypertension but inactivation of bradykinin as well. Bradykinins are potent vasodilators but also activate inflammatory processes.

Lisinopril (Zeneca), is an example of an angiotensin converting enzyme inhibitor. It is a lysine derivative of enalaprilat and is converted to an active agent by hydrolysis in the liver. In addition to lowering blood pressure ACE inhibitors, such as lisinopril, are also useful in the treatment of diabetic neuropathy, diminishing proteinuria, and in the stabilization of renal function. Angiotensin converting enzyme inhibitors are also taken to treat congestive heart failure and following myocardial infarction (Goodman et al, 1980).

Lisinopril is slowly absorbed, reaching peak levels in the blood at seven hours. The half-life of lisinopril is twelve hours and it is typically administered to humans once daily at doses of 10-80 mg (Katzung, 1998).

4.3   Angiotensin II Receptor Blockers

4.3.1   Saralasin

Saralasin is a competitive inhibitor of angiotensin II. Like angiotensin II it binds to both AT_1 and AT_2 receptors with equal affinity. Saralasin is an analogue of angiotensin II where the asparagine in position 1 has been replaced with sarcosine. In

addition, alanine replaces phenylalanine in position 8 (1-Sar-8-ala-angiotensin II). It

blocks the pressor response and aldosterone releasing effects of angiotensin II. Saralasin

also has weak agonist activity (Katzung, 1998).

5.0   Renin Gene Regulation

It has recently been suggested that the LXR alpha gene may also regulate the gene

that controls the production and release of renin (Victor Dzau, personal communications).

If this were found to be the case then this would offer a new therapeutic route of

controlling hypertension. By producing a small molecule that can block or stimulate

LXR alpha we could potentially control the transcription of the gene involved in

production and/or release of renin

6.0   Current Rat Models of Hypertension

Two of the most commonly used rat models for hypertension are the

Spontaneously Hypertensive Rat (SHR) and the "Two Kidney" Goldblatt hypertension

model. SHR hypertension is a genetic anomally and is characterized by an increase in

peripheral vascular resistance. This form of hypertension is not associated with an

increase in plasma renin activity. However, inhibitors of the renin-angiotensin system

such as ACE inhibitors are very effective antihypertensive agents in this model

(Birkenhager et al, 1984).

In the "Two-Kidney" Goldblatt Hypertension model, the artery to one kidney is

occluded, leaving the renal vessels to the other kidney open. The decreased renal arterial

pressure in the constricted kidney causes renin release and increased angiotensin I formation. The increased renin secretion by this kidney causes the other kidney to retain salt and water, leading to hypertension (Cangiano et al, 1979).

7.0   Aim of this thesis

The main goal of this thesis was to test the hypothesis that LXR alpha plays a role in the release and/or production of renin. The potent LXR alpha agonist, T0901317 was used this to test this hypothesis. Animal models were designed to functionally evaluate the effects of inhibitors of renin and other RAS components. These were subsequently used to test the hypothesis that LXR alpha agonists could influence renin activity.

II. General Methods

1.0 Animals

Male Sprague Dawley Rats (Harlan, San Diego, CA) between 8 and 12 weeks or Spontaneously Hypertensive rats (Harlan, San Diego, CA) between 25 and 28 weeks were used for all studies. Rats were acclimated in the Tularik animal facility(South San Francisco, CA) for 6-7 days before initiation of treatment. During acclimation, animals were examined for abnormalities indicative of health problems and body weights were recorded for all animals prior to treatment. Environmental controls for the animal room were set to maintain $21 \pm 2°$ C, relative humidity of $50 \pm 20\%$ and a 12-hour light/dark (fluorescent) cycle, set to come on at 06:00 and turn off at 18:00. Animals were group housed, 3-6 animals per cage in an autoclaved microisolator or Thoren cage (Thoren, Hazelton, PA). Certified rodent diet (PMI Picolab 5058 Chow, Purina, Richmond, IN) was provided *ad libitum*. Domestic autoclaved, hyperchlorinated (0.5 mL Cl /Liter of water) water was provided *ad libitum*. All protocols were reviewed and approved by the Tularik and San Jose State University Institutional Animal Care and Use Committees and are in compliance with the Guide to Care and Use of Animals (NRC, 1996).

2.0 Drugs and Formulations

Ketamine/Xylazine Anesthesia (Henry Schein, Port Wahsington, NY): A 50/50 (v/v) solution of Ketamine/ Xylazine (100 mg/kg b.w./20mg/kg b.w.) was prepared,

given an expiration date of one month and stored at 4°C. Anesthesia was administered ip.

Angiotensinogen (Sigma, St. Louis, MO): Both Porcine and human synthetic angiotensinogen were prepared according to the following procedures. Four different dosing solutions were prepared at 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml and 0.001 mg/ml in glass scintillation vials. A 1 mg/ml stock solution of angiotensinogen was prepared by weighing out the angiotensinogen and dissolving it in 5% dextrose. The 1 mg/ml solution was diluted to make a 0.1 mg/ml solution. This was then diluted to a 0.01mg/mL solution, which was in turn diluted to make a 0.001mg/mL solution. The original stock solution of 1 mg/ml was given an expiration date of 1 week and was stored at 0° C. This solution was used for all experiments performed within that one week period. All other dosing solution were disposed of at the end of each day.

Pepstatin (Sigma, St. Louis, MO): Pepstatin was dissolved in 100% dimethylsulfoxide, 55.5 mg/ml. Dilution into aqueous solution was accomplished by injecting 75ul of this pepstatin solution into 2.425 ml phosphate buffered saline (PBS), pH 7.0, while the latter was vigorously agitated on a Roto-mixer. A suspension was obtained which was further diluted with PBS to obtain a final dose of 150 ug/kg/min b.w. (Oldham et al, 1984). The infusion rate was 150 ul/kg/min b.w..

Renin Inhibitory Peptide (Sigma, St. Louis, MO): Renin Inhibitory Peptide is a renin inhibitor with a high affinity for the renin substrate, angiotensinogen. RIP (His-Pro-Phe-His-Leu-Leu-Val-Tyr) was dissolved in 2% dimethylsulfoxide. PBS was then

be added to bring the final concentration to 1 mg/ml. The infusion rate was 200 ug/kg/ml b.w.

Lisinopril (Sigma, St. Louis, MO): Lisinopril was dissolved in autoclaved water at a concentration of 1 and 10 mg/mL.

Saralasin (Sigma, St. Louis, MO): Saralasin was and dissolved in 5% dextrose at a concentration of 1 mg/mL.

Enalapril (Sigma, St. Louis, MO): Enalapril was dissolved in autoclaved water at a concentration of 10 mg/mL.

T0901317 (Tularik Inc., So. San Francisco): From previous studies with T0901317 we determined that the compound was poorly soluble and, therefore, for intravenous administration it was necessary to dissolve the compound in a mixture of Ethanol(EtOH)/Dimethylacetamide(DMAC)/PolyethyleneGlycol(PEG)/Water (10/10/30/50) (Sigma, St. Louis, MO). A 1mg/kg b.w. dose was administered intravenously. A 1 mg/mL solution of T0901317 was made by first milling it in a morter with 10% EtOH and then adding 10% DMAC, 30% PEG and 50% water to bring the solution to its final volume.

T0901317 was dosed orally at both 5 mg/kg b.w. and 10 mg/kg b.w. The dosing solutions were made up at 2.5 mg/ml and 10 mg/ml (5 mg/kg b.w. and 10 mg/kg b.w. respectively). The compound was weighed out, dissolved in 1% by volume Tween 80 (Sigma, St. Louis, MO), and ground in a morter. 1% Methylcellulose (Sigma, St. Louis, MO) was then added to bring the suspension up to its final volume.

3.0 Surgical Procedures

3.1 Carotid Artery Cannulation

To measure blood pressure, all animals had a cannula inserted into the carotid artery. Animals were anesthetized with a 50/50 v/v solution of Ketamine/Xylazine (100 mg/kg b.w. and 20 mg/kg b.w. respectively) administered intraperitoneal, prior to any surgical procedure being performed.

After preparation and assessment for surgical anesthetic plane, a ventral midline neck incision was made. The carotid artery was isolated and clamped. 4-0 silk suture was used to place two ties around the vessel, one above and one below the incision site. The caudal tie was pulled tight while the rostral tie remained loose. A small incision was made between the two ties. A cannula (PE50 tubing, Becton Dickinson, Sparks, MD), filled with a 0.1 IU heparin/saline solution was inserted into the artery via the incision, tied into place and checked for patency.

For Experiment B (T0901317 pharmacokinetic study), animals underwent carotid artery surgery following the same surgical procedure described above. However, these animals had surgery one day prior to the study being performed. In addition, these animals had the cannulas filled with 1000IU/1 mL of heparin and plugged with a wire plug overnight. Immediately prior to dosing, the cannulas of all animals were flushed with a heparin/saline solution and checked for patency.

3.2   Jugular Vein Cannulation

All animals had a jugular vein cannula inserted for intravenous administration of compounds. The jugular vein was identified, isolated and a cannula (PE50, Becton Dickinson, Sparks, MD) inserted following a similar procedure described above for the carotid artery cannulation. Infusion of renin inhibitors was done via the jugular vein and it was therefore necessary in these studies to cannulate the femoral vein as well as the jugular vein to administer the angiotensinogen. For this procedure, a small cut was made in the inner thigh skin area and the femoral vein identified, isolated and cannulated according to the procedure similar to that described above for carotid artery cannulation.

For Experiment B, (T0901317 pharmacokinetic study), the same procedures were followed for jugular vein cannulation as are described above. As with the carotid artery cannulation, the procedure was performed one day prior to the study being performed. Cannulas were externalized through a skin incision in the back of the neck, were filled with 1000IU/1 ml heparin and were plugged with a wire pin overnight.

3.3   Femoral Vein Cannulation

A small incision was made in the inner thigh area and the femoral vein identified and isolated. 4-0 silk suture was used to place two ties around the vessel, one above and one below the future vessel incision site. The top tie was pulled tight while the bottom tie remained loose. A small incision was made between the two ties. A heparinized cannula, filled with heparin/saline, (PE50, Becton Dickinson,Sparks, MD) was inserted into the vein via the incision, tied into place and checked for patency.

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3.4   Bilateral Nephrectomy

For Experiment A it was necessary to perform a bilateral nephrectomy. The procedure was performed as follows. A dorsoventral incision was made into the abdominal cavity on both the right and left sides of the body. Each kidney was isolated from surrounding connective tissue and exteriorized. The renal artery was clamped using a pair of hemostats. 4-0 silk (Henry Schein, Port Washington, NY) was then placed around the renal artery as far from the kidney as possible towards the midline. The silk was then tied securely and the kidney removed. Both the muscular and the skin layers were sutured closed using 4-0 Ethilon suture (Ethicon Inc., Somerville, NJ).

3.5   Renal ligation surgery

On Day 1 of treatment, all animals underwent renal ligation surgery. A 1.5 inch dorsoventral incision was made into the body cavity on the left side of the animal. The left renal artery was isolated and ligated completely with 4-0 silk suture (Cagniano et al, 1979): The renal vein was left intact. The muscular layer and skin layers were sutured closed with 4-0 ethilon suture. As mentioned previously, animals also had a cannula inserted into the carotid artery on Day 1.

4.0   Blood Pressure Measurements

Blood pressure and heart rate were measured from the carotid artery by running an extension of PE50 tubing from the cannula to pressure transducers (Harvard

Apparatus, Holliston, Mass). Mean arterial pressure was recorded using the Gould Po-ne-mah Physiology Platform system running on a PC. The system was calibrated using a modified sphygmomanometer.

5.0   Blood Collection Via the Tail Artery

Animals were anesthetized with isoflurane anesthesia using a nose cone. All animals were placed on their backs and the tail artery identified. A 21 G needle and syringe, with the plunger removed, was then inserted into the artery at a 45 degree angle and blood was allowed to flow back into the syringe. The needle was then removed and the blood transferred into a tube.

6.0   Statistical Analysis

All measurements presented are means $\pm$ standard deviation. Statistical significance between groups was determined using repeated measures ANOVA as calculated by the SAS system, version 6.11 (SAS Institute, Cary, NC) running on a Silicon Graphics Computer under the UNIX operating system. When comparing differences between groups, $p < 0.05$ was considered statistically significant.

7.0 Experiment A

The purpose of this study was to characterize the vasopressor responses to intravenous administration of human (synthetic) and porcine (extract) angiotensinogen in

an attempt to develop a model with which to evaluate functional inhibitors of the renin-angiotensin system, in particular functional inhibitors of renin.

In this experiment, all groups of animals underwent arterial and venous cannulation (procedure described above) approximately 15 – 20 minutes prior to mean arterial pressure measurements. The first group of animals were dosed with either porcine or human synthetic angiotensinogen at a dose of 0.001, 0.01, 0.1, and 1.0 mg/kg b.w. Blood pressure measurements were allowed to return to normal between doses. All remaining groups of animals received all doses of angiotensinogen stated above with blood pressure being allowed to return to normal between doses. Another group of animals was given either the renin inhibitor, pepstatin, or renin-inhibitory peptide as a constant infusion via the jugular vein cannula starting 30 minutes prior to and continuing throughout administration of porcine angiotensinongen. The doses of inhibitor were 150 ug/kg/min b.w. and 200 ug/kg/min b.w. for pepstatin and renin inhibitory peptide respectively. In this case, the doses of angiotensinogen were administered via the femoral vein cannula. A third group of animals received all doses of porcine angiotensinogen immediately following or 16 hours post nephrectomy (procedure described above). Another group of rats were dosed with either 1 mg/kg b.w. or 10 mg/kg b.w. lisinopril intravenously, via a tail vein, approximately 10 minutes prior to administration of the porcine angiotensinogen. The last group of animals were given 5 mg/kg b.w. saralasin subcutaneously approximately 30 minutes prior to porcine angiotensinogen administration.

8.0 Experiment B

The pharmacokinetics of the LXR agonist, T0901317 following oral and intravenous administration were characterized in order to evaluate whether this compound could be used to assess pharmacological responses after oral administration. Animals were dosed either by oral gavage (5 or 10 mg/kg b.w.) or intravenously (1 mg/kg b.w.) via a jugular vein cannula.

Blood was collected from the carotid artery cannula at 0, 5, 15, 60, 120, 240, 360 and 1440 minutes following administration of T0901317. Following each sample, all cannulas were flushed with a 1000 IU/1 mL heparin/saline solution. Due to the large volume of blood taken over the course of the study, animals were supplemented with approximately 0.5 ml of whole blood from a donor rat via the jugular vein cannula as needed (aproximately 4 times over the course of this study).

Animals receiving orally administered T0901317 underwent one of two procedures. Those dosed with 5 mg/kg b.w. T0901317 had blood taken from the tail artery at 0, 1, 6 and 24 hours postdose with the animals under isoflurane anesthsia. Animals given the 10 mg/kg dose of T0901317 were cannulated one day prior to dosing. They underwent procedures identical to those for the animals receiving the compound intravenously. All cannulas were then flushed with a heparin/saline solution and checked for patency immediately prior to dosing. Blood samples were then collected at 0, 30, 60, 120, 240, 360 and 1440 minutes postdose. Due to the large volume of blood taken from

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the cannulated rats, over the course of the study, animals were supplemented with approximately 0.5 ml of whole blood as needed., from a donor rat, via the jugular vein cannula (approximately 4 times over the course of this study).

Approximately 0.5 mL of blood was collected at each timepoint, into microtainer tubes containing EDTA. Following collection, the tubes were placed on ice until centrifugation. Samples were centrifuged at 12,000x g for 4 minutes. Following centrifugation, plasma was separated, placed in labeled tubes and stored at -80° C until analyzed.

Plasma samples for each timepoint of each dose group were pooled for analysis. Pooling of the samples does eliminate the potential to assess the variability between samples, however, it provides the average plasma concentration analytically rather than mathematically and greatly reduces analytical and machine time. This procedure is routinely done in pharmacokinetic studies. Drug concentrations were determined using a Sciex API365 LC/MS/MS (Sciex, Ontario, Canada) operated with electrospray ionization and in the MRM mode.

The actual calculations were conducted using a custom designed EXCEL spreadsheet using the following formulas: Clearance = Dose/AUC, Vdss = Dose* $AUMC/AUC^2$, $t_{1/2} = 0.693/ K_e$(Girabaldi and Perrier, 1982).

9.0 Experiment C

The purpose of this experiment was to evaluate the effects of T0901317, an LXR alpha agonist, on the following parameters: the angiotensinogen-mediated vasopressor

response in anesthetized normal Sprague Dawley rats, the arterial blood pressure in renal hypertensive and spontaneously hypertensive rats and plasma lipid profile in normal Sprague Dawley rats. In the first study, we used a dose of 10 or 30 mg/kg b.w. (determined from Experiment B) dosed orally either 15, 120, 360, or 1440 minutes prior to angiotensinogen administration. For this study we used only one dose of 0.1 mg/kg b.w. porcine angiotensinogen. As with experiment A, animals had the carotid artery and jugular vein cannulated approximately 15 minutes prior to angiotensinogen administration. In a second study, we administered 10 mg/kg b.w. T0901317 or vehicle chronically once a day for 5 days . On day 5 animals were dosed approximately 2 hours prior to angiotensinogen administration. Once again, we used only one dose of 0.1 mg/kg b.w. porcine angiotensinogen. Cannulation took place 15 minutes before blood pressure measurements were recorded. In addition, blood was taken prior to angiotensinogen administration for analysis of plasma renin activity and plasma lipids. Blood samples (0.5 − 1.0 mL), for clinical chemistry and plasma renin activity analysis, were taken via the carotid artery cannula. Blood was collected into pre-chilled tube containing EDTA. The tubes were kept on ice until centrifuged in a refrigerated centrifuge. Following centrifugation, plasma was pipetted using pre-chilled pipet tips into pre-chilled eppendorf tubes. All samples were stored at -80°C until analyzed.

All plasma samples were analyzed for plasma cholesterol, triglycerides, high density lipoprotein (HDL), aminotransferase (ALT), and alkaline phosphatase using a Hitachi 704 auto analyzer (Boehringer Mannheim, Indianapolis, IN) (Westgard et al,

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1981). Plasma renin activity was measured using a radioimmunoassay for angiotensin I by AntiLytics Incorporated (Gaithersburg, MD).

A third study was done in which renal hypertensive rats (procedure described above) were given 10 mg/kg b.w. T0901317, vehicle, or 10 mg/kg b.w. enalapril (positive control) once a day for 5 days. Animals underwent arterial and venous cannulation on Day 1 (along with renal ligation surgery) and blood pressure measurements were taken on Day 5. A fourth study was performed with spontaneously hypertenive rats. These animals were given 10 mg/kg b.w. T0901317, vehicle or 10 mg/kg b.w. lisinopril (positive control) once a day for 5 days. Animals had the carotid artery cannulated on Day 4 and blood pressure measurements taken on Day 5.

III. Results

Experiment A:

1.0 Effects of Porcine Angiotensinogen on Mean Arterial Pressure

An increase in mean arterial pressure was detected upon administration of all doses of porcine angiotensinogen. The log dose response curve is shown in Figure 4. When the dextrose vehicle was administered an average increase of 3.5 ± 4.4 mm Hg was seen. When we administered 0.001 mg/kg b.w. an average increase in blood pressure of 26.1 ± 6.33 mm Hg was observed. In addition, there was a substantial change in the average blood pressure following administration of 0.01, 0.1 and 1.0 mg/kg b.w. ($57.6 \pm$ 14.9, 69.8 ± 19.7 and 56.6 ± 15.1 mm Hg respectively). There was a significant difference between the vehicle control group and all the other dose groups as determined by ANOVA ($p < 0.05$). Additionally, there was no significant difference between the 0.01, 0.1 and 1.0 mg/kg b.w. dose groups. The ED_{50} for porcine angiotensinogen was calculated by extrapolation using the dose response insert graph shown in Figure 4. This value was equal to approximately 0.0023 mg/kg. While the dose response curve is not necessarily representative of a typical dose response (see discussion section for further clarification), the results of this experiment clearly show that porcine angiotensinogen does elicit a substantial hypertensive response.

2.0 Effects of Human Synthetic Angiotensinogen on Mean Arterial Pressure

Similar results were seen with administration of human synthetic angiotensinogen as were seen with porcine angiotensinogen. The dose response curve can be seen in Figure 5. Control animals had an average increase of 4.3 ± 2.5 mm Hg. There was only a slight increase in MAP when 0.001 mg/kg b.w. angiotensinogen was administered (4.7 ± 1.2 mm Hg). The remaining three doses yielded a statistically significant increase in MAP ($p<0.05$). At 0.01 mg/kg b.w. there was an average increase of 22.7 ± 7.6 mm Hg and at 1.0 m/kg b.w. angiotensinogen, an average increase of 41.6 ± 7.2 mm Hg. There was no significant difference between the 0.1 and the 1 mg/kg b.w. doses, while the difference between these two doses and 0.01 mg/kg was significant ($p<0.05$). The ED_{50}, once again determined by extrapolation from the insert curve shown in Figure 5, was approximately 0.009 mg/kg. This ED_{50} is much higher than that seen for porcine angiotensinogen (see discussion section for further explanation). While the human synthetic angiotensinogen did not elicit as dramatic of an increase in blood pressure, administration of 0.01, 0.1 and 1.0 mg/kg do clearly elicit a statistically significant hypertenisve response.
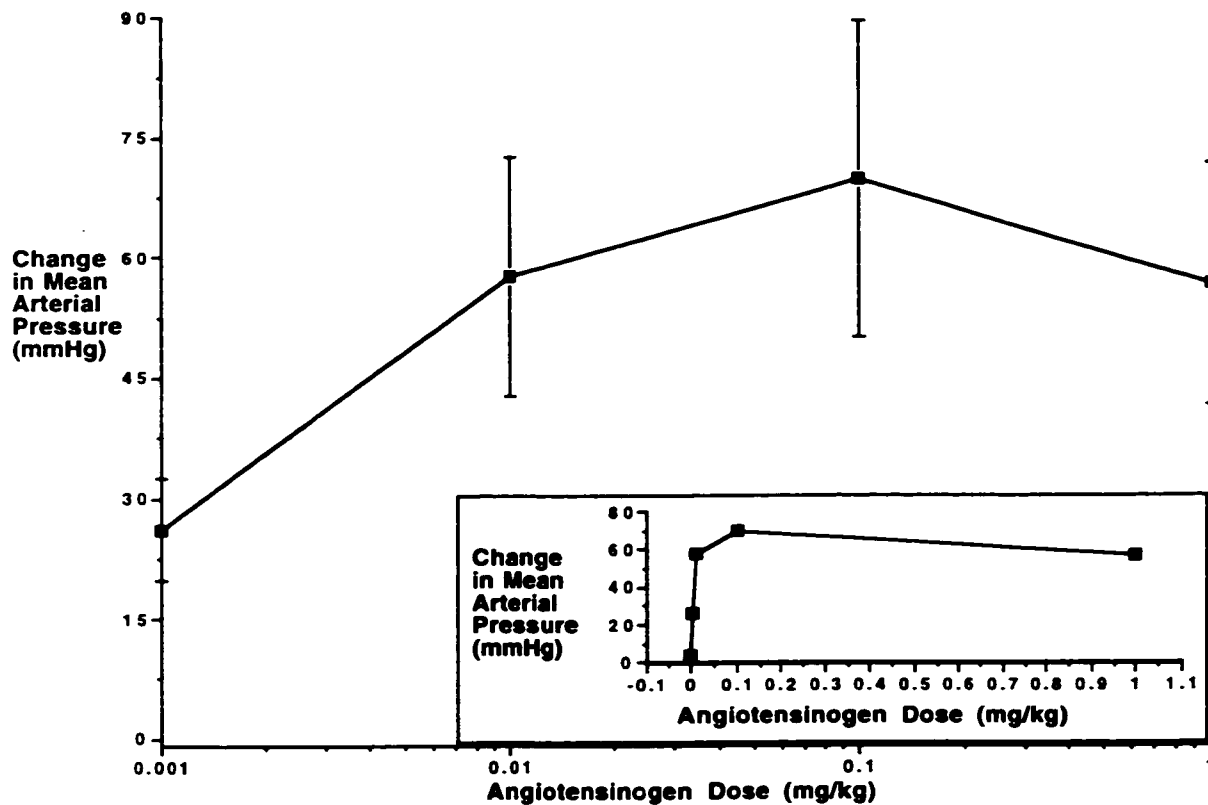
Figure 4. The effects of of 0.001, 0.01, 0.1 and 1.0 mg/kg b.w. porcine angiotensinogen

(i.v.) on mean arterial blood pressure in anesthetized rats. Symbols represents the mean

arterial pressure ± standard deviation, n = 4. Insert graph shows the best fit curve

through the data points and was used to calculate ED_{50} value for porcine angiotensinogen.
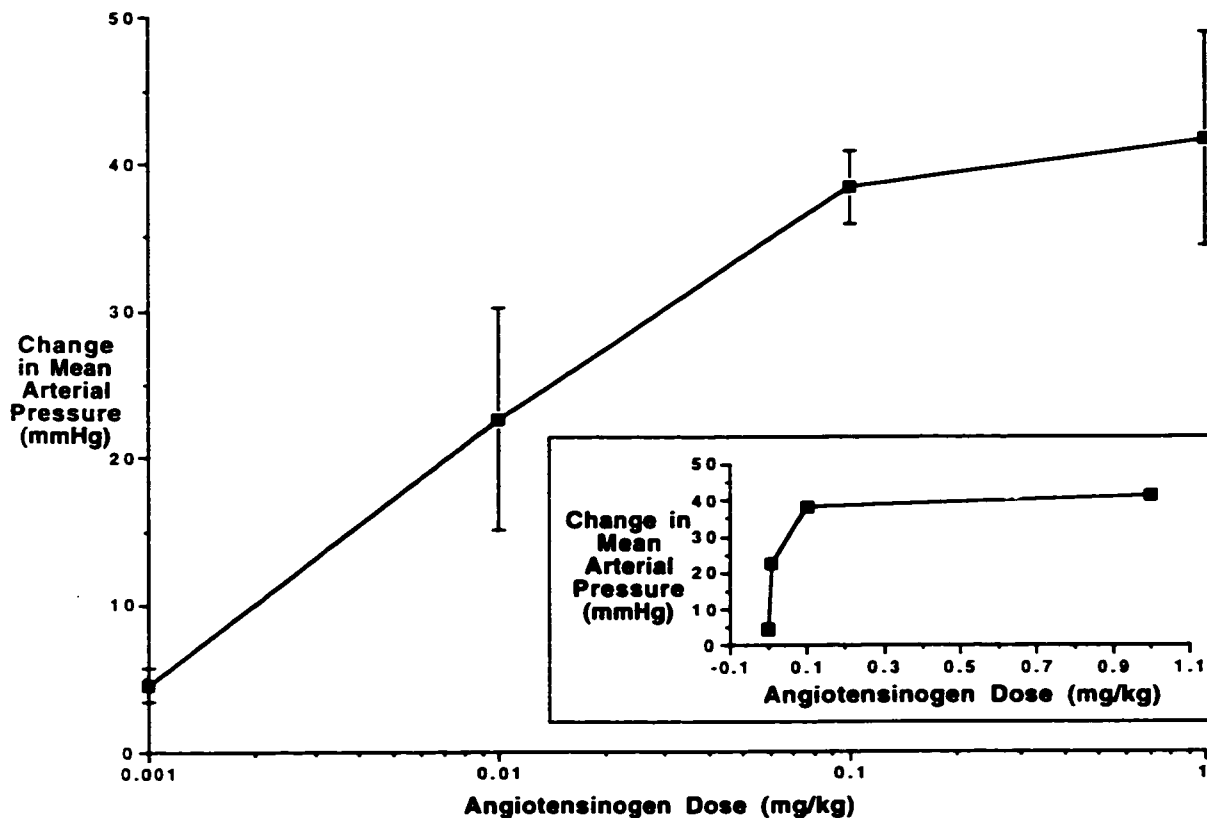
Figure 5. The effects of 0.001, 0.01, 0.1 and 1.0 mg/kg b.w. human synthetic angiotensinogen (i.v.) on mean arterial blood pressure in anesthetized rats. Symbols represents the mean arterial pressure $\pm$ standard deviation, n = 4. The insert graph shows the best fit curve through the data points and was used to calculate the ED_{50} value for human synthetic angiotensinogen.

3.0 Effect of Pepstatin

As is shown in Figure 6, significant increases in MAP were seen when angiotensinogen was administered in the presence of pepstatin (150 ug/kg b.w./min). At 0 mg/kg b.w. angiotensinogen, blood pressure in the control rats increased an average of

3.5 ± 4.4 mm Hg and in the pepstatin treated rats it increased an average of 9.3 ± 4.2 mm

Hg. When control rats were administered 0.001 mg/kg b.w. angiotensinogen, MAP

increased an average of 26.1 ± 6.3 mm Hg as compared to an increase of 27.3 ± 14.0 mm

Hg for the pepstatin treated group. When the animals were given 0.01 mg/kg b.w.

angiotensinogen, MAP in control rats increased an average of 57.6 ± 14.9 mm Hg.

However, pepstatin treated rats showed an average increase in MAP of 44.6 ± 14.0 mm

Hg. When control rats were given 0.1 and 1.0 mg/kg b.w. angiotensinogen, they showed

an average increase of 69.8 ± 19.7 mm Hg and 56.5 ± 5.1 mm Hg respectively.

Similarly, pepstatin treated rats showed an average increase of 53.0 ± 25.0 mm Hg and

38.3 ± 11.5 mm Hg when dosed with 0.1 and 1.0 mg/kg angiotensinogen. Statistically,

there was no significant difference in blood pressure increase between the control group

and the group infused with pepstatin. The results generated here clearly show that

pepstatin is not an effective renin inhibitor (see discussion section).

4.0   Renin Inhibitory Peptide (RIP)

Figure 6 also shows the dose response curve for animals treated with Renin

Inhibitory Peptide (His-Pro-Phe-His-Leu-Leu-Val-Tyr). Infusion of RIP at a rate of 200

ug/kg b.w./min significantly inhibited the hypertensive response seen upon

administration of angiotensinogen, when compared to the control group (p<0.05). When

given the vehicle control, these animals had an increase in MAP of 3.5 ± 4.4 mm Hg and

the treated rats had an increase of 8.0 ± 4.6 mm Hg. Administration of 0.001 mg/kg b.w.

angiotensinogen led to an increase of 26.1 ± 6.3 mm Hg in control rats and 13.3 ± 11.6

mm Hg in RIP treated animals. At 0.001, 0.01, and 1.0 mg/kg b.w. angiotensinogen, there was an increase in blood pressure of 57.6 ± 14.9 mm Hg, 69.8 ± 19.7 mm Hg and 56.5 ± 15.1 mm Hg in the control rats. When the treated rats were given the same doses, there was an increase in blood pressure of 47.3 ± 11.8 mm Hg, 35.0 ± 15.1 and 9.3 ± 12.7 mm Hg (0.01, 0.1 and 1.0 mg/kg b.w. respectively). This represents a statistically significant decrease in MAP for the RIP treated rats ($p < 0.05$). In addition, there was a notable difference in blood pressure increases at both the 0.1 and 1.0 mg/kg b.w. doses, between the pepstatin group and the RIP group ($p < 0.05$). Even though the shape of the RIP curve does not follow the typical dose response pattern (see discussion), it can be ascertained from this study that RIP is an effective antihypertenisve agent. The inhibition of the hypertensive response by RIP, which is known to inhibit renin, suggests that the angiotensinogen induced hypertenisve response may be mediated by renin.
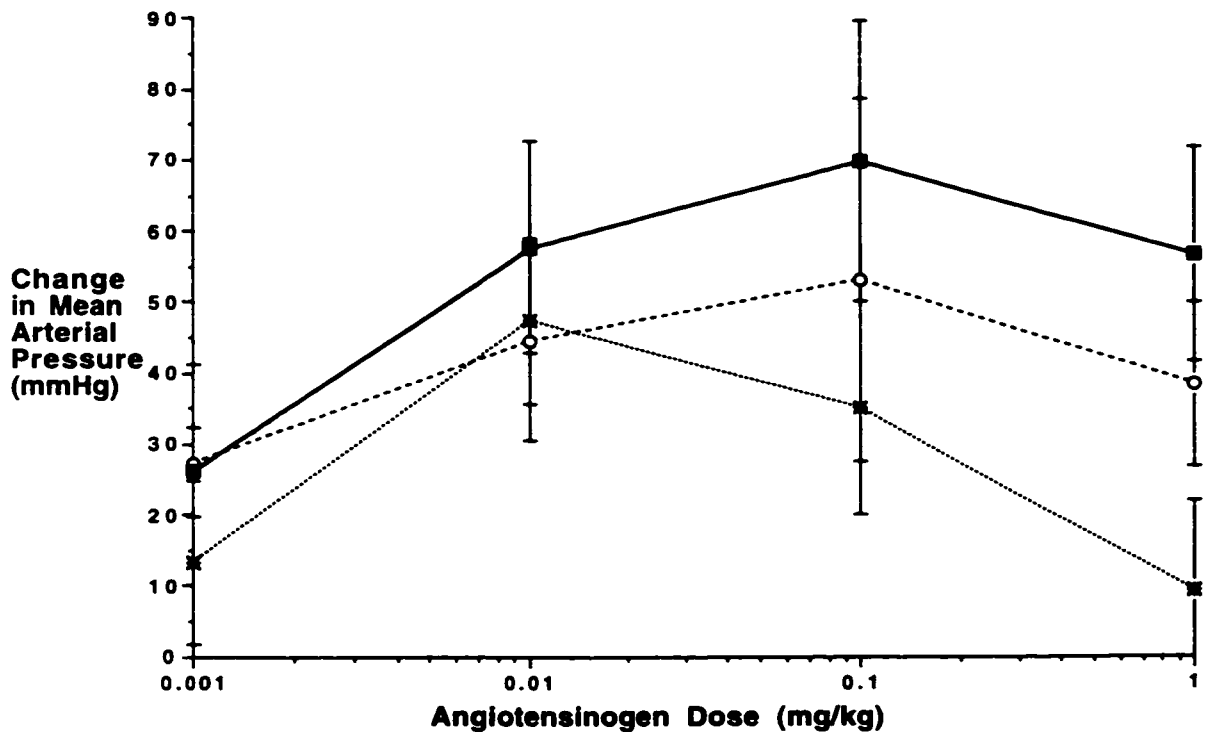
Figure 6. Change in mean arterial pressure following administration of 0.001, 0.01. 0.1

and 1.0 mg/kg porcine angiotensinogen during pepstatin (150 µg/kg b.w./min) and renin

inhibitor peptide (200 µg/kg b.w./min) infusion. The symbols represent the mean arterial

blood pressure ± standard deviation, n = 3 - 4. ■ = control, = O pepstatin and ✳ = RIP.

5.0 Effects of nephrectomy immediately prior to angiotensinogen adminstration on Mean Arterial Pressure

As is demonstrated in Figure 7, there was a small difference in average blood

pressure change between the control group and the rats nephrectomized immediately

prior to dosing. There was a significant difference between both groups at all doses

(p<0.05). The vehicle control had an average increase of 3.5 ± 4.4 mm Hg in the control group and 10.3 ± 3.9 mm Hg in the nephrectomized group. In addition, when 0.001 mg/kg b.w. angiotensinogen was administered there was an average increase in blood pressure of 26.1 ± 6.3 mm Hg in the control group and only 17.2 ± 6.7 mm Hg in the nephrectomized animals. At 0.01 mg/kg b.w. of angiotensinogen, there was an average rise in MAP of 57.6 ± 14.9 mm Hg in control animals and 40.5 ± 10.7 mm Hg in the nephrectomized group. MAP of 69.8 ± 19.7 mm Hg and 56.6 ± 15.1 mm Hg in control animals and 56.8 ± 9.2 mm Hg and 42.3 ± 11.5 mm Hg in the nephrectomized animals. The significantly decreased hypertensive response seen in the nephrectomized animals as compared to the control animals can be attributed to the removal of the main source of renin, the kidneys.

6.0   Effect of nephrectomy 16 hours prior to angiotensinogen administration on Mean Arterial Pressure

There is an even greater difference in MAP when 7. In the control group, and the 0.001 mg/kg b.w. of angiotensinogen, there was an average increase of 3.5 ± 4.4 mm Hg and 26.1 ± 6.3 mm Hg, respectively. In the nephrectomized group this was much lower. In the control group, the average increase was 1.75 ± 2.6 mm Hg and at 0.001 mg/kg b.w. it was 12.8 ±5.7 mm Hg. At 0.01 mg/kg b.w. the average increase in MAP was 57.7 ± 14.9 mm Hg for control animals and 27.8 ± 10.3 mm Hg for nephrectomized animals. When we gave a dose of 0.1 mg/kg b.w., MAP rose 69.8 ± 19.7 mm Hg in control animals and only 25.5 ± 8.6 mm Hg in the nephrectomized animals. Lastly, at a dose of

1 mg/kg b.w. angiotensinogen, blood pressure increased an average of 56.5 ± 15.1 mm

Hg in the control group and only 11.5 ± 3.6 mm Hg in the nephrectomized animals.

Overall, there was a statistically significant difference between the control group and the

animals given angiotensinogen 16 hours post nephrectomy (p<0.05) and between animals

given angiotensinogen immediately following nephrectomy and those receiving

angiotensinogen 16 hours post nephrectomy.
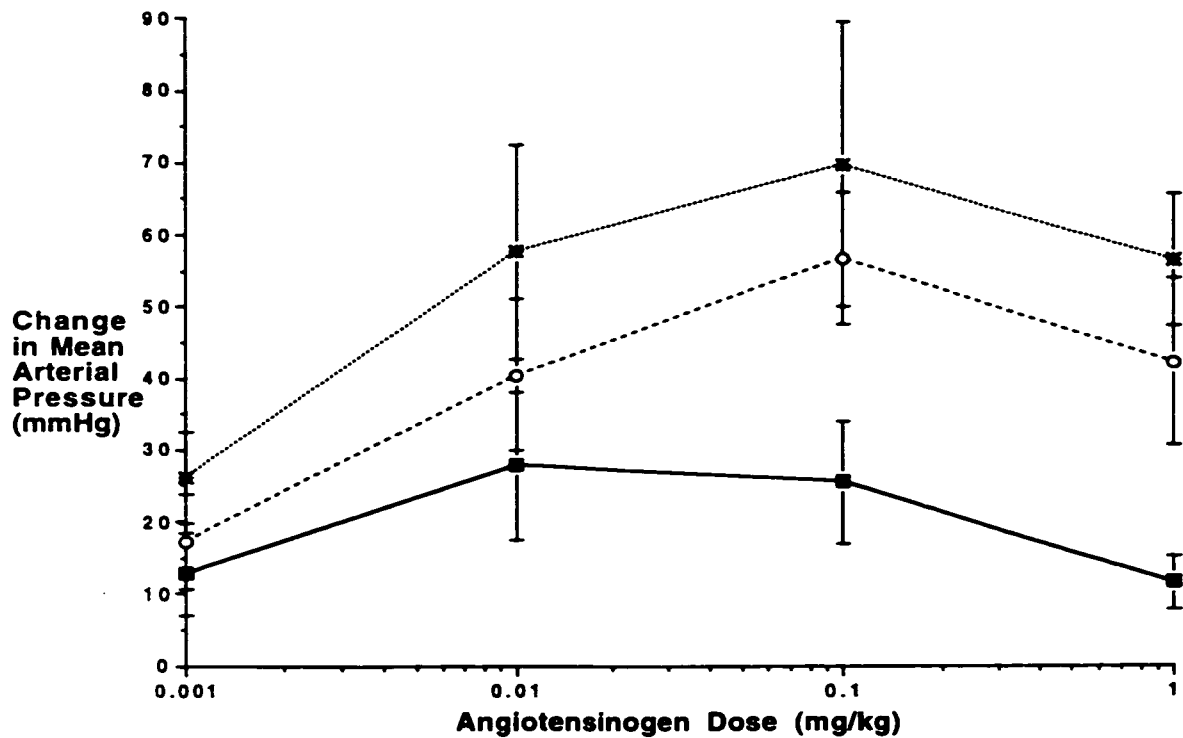
Figure 7. Change in mean arterial pressure following administration of 0.001, 0.01, 0.1

and 1.0 mg/kg b.w. porcine angiotensinogen (i.v.) to anesthetized rats following

nephrectomy. Symbols represent the mean arterial pressure ± standard deviation, n = 3 -

4. ✱ = control, ■ = Nephrectomy 16 hours predose and O = Nephrectomy 15 min.

predose.

7.0   Effect of 1 mg/kg Lisinopril (i.v.) on Mean Arterial Pressure

At a dose of 0.001 mg/kg b.w. angiotensinogen, there was an average increase in blood pressure of 26.1 ± 6.3 mm Hg in the control animals and only 7.8 ± 6.9 mm Hg in the animals dosed with 1 mg/kg b.w. lisinopril (i.v.) 15 minutes prior to angiotensinogen administration. There was inhibition of blood pressure increases at 0.01 mg/kg b.w. angiotensinogen as well, with an increase of 57.7 ± 14.9 mm Hg in control animals and 20.5 ± 3.3 in lisinopril treated animals. When given 0.1 mg/kg b.w. angiotensinogen, blood pressure increased an average of 69.8 ± 19.7 mm Hg in control animals and only 40.2 ± 12.8 mm Hg in lisinopril treated animals. Lastly, MAP increased an average of 56.6 ± 15.1 mm Hg in control animals and 32.5 ± 8.1 in lisinopril treated animals following treatment with 1 mg/kg angiotensinogen. There was a significant decrease in MAP in the treated animals as opposed to the control group ($p < 0.05$). Therefore, it can be concluded that lisinopril, dosed at 1 mg/kg iv is an effective form of anti-hypertensive therapy.

8.0   Effect of 10 mg/kg Lisinopril on Mean Arterial Pressure

We saw similar results in the 10 mg/kg b.w. lisinopril group as with the 1 mg/kg b.w. lisinopril group.   When animals were administered 0.001 mg/kg b.w. angiotensinogen, MAP increased an average of 26.1 ± 6.3 mm Hg in control rats and only 6.0 ± 3.1 mm Hg in treated rats. At 0.01 mg/kg b.w. angiotensinogen blood pressure

increased an average of 57.7 $\pm$ 14.9 mm Hg in control animals and 20.2 $\pm$ 7.1 mm Hg in the lisinopril treated animals. Lisinopril treated animals given 0.1 mg/kg b.w. angiotensinogen had an average MAP increase of 38.5 $\pm$ 6.7 mm Hg. 1 mg/kg b.w. angiotensinogen administration caused the control animal's blood pressure to rise an average of 56.6 $\pm$ 15.1 mm Hg and the lisinopril treated animals 35.8 $\pm$ 15.8 mm Hg. There was a statistically significant difference between the control group and the 10 mg/kg b.w. lisinopril treated group ($p < 0.05$) and no difference between the 1 mg/kg b.w. and 10 mg/kg b.w. lisinopril groups.
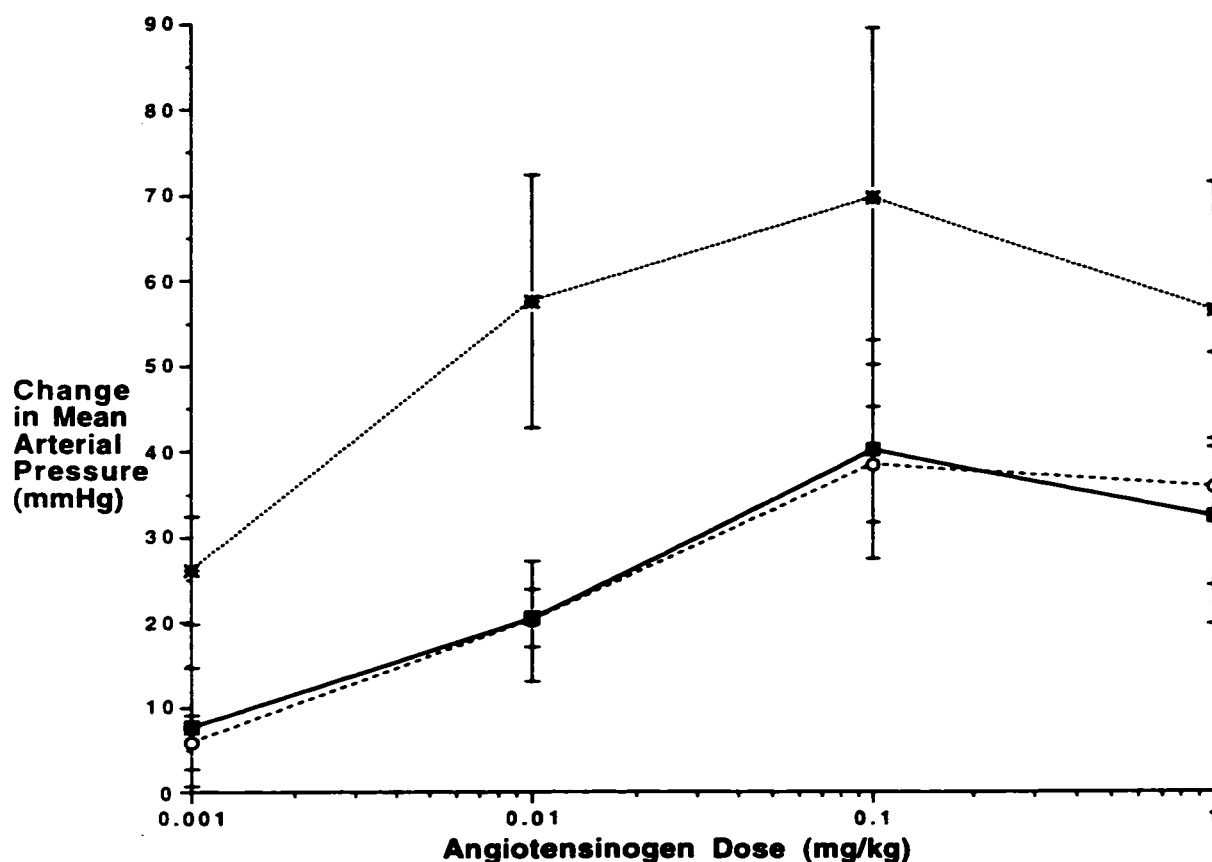
Figure 8. Change in mean arterial pressure following administration of 0.001, 0.01, 0.1 and 1.0 mg/kg b.w. porcine angiotensinogen (i.v.) to anesthetized rats 15 minutes after administration of 1 and 10 mg/kg b.w. lisinopril. Symbols represent mean arterial pressure ± standard deviation., n = 3 – 4. ■ = 1 mg/kg b.w. lisinopril, ✳ = control and O = 10 mg/kg b.w. lisinopril.

9.0   Effect of 5 mg/kg b.w. Saralasin (s.c.) on Mean Arterial Pressure

Saralasin inhibited MAP increases due to angiotensinogen administration at some, but not all doses evaluated. The vehicle control group showed an increase of 2.3 ± 2.1 mm Hg while the treated animals had an average increase 2.6 ± 0.4 mm Hg.

Administration of 0.001 mg/kg b.w. angiotensinogen led to an average increase 13.6 ±

13.0 mm Hg for the control animals and 11.8 ± 13.6 mm Hg for the treated animals. 0.01

mg/kg b.w. angiotensinogen gave an increase of 48.3 ± 5.5 mm Hg in the control group

and only 16.9 ±10.2 mm Hg in the treated group. This was statistically significant

(p< 0.05) Angiotensinogen dosed at 0.1 mg/kg b.w. resulted in an average increase in

blood pressure of 38.0 ± 12.0 mm Hg in control animals and 26.3 ± 6.2 mm Hg in treated

animals. Lastly, a dose of 1.0 mg/kg b.w. angiotensinogen yielded an increase of 17.6 ±

11.9 mm Hg in control rats and 31.0 ± 8.0 mm Hg in treated rats. It is very obvious that

the shape of the saralasin dose response curve is very different from that of the RIP and

pepstatin (see discussion section for explanation).
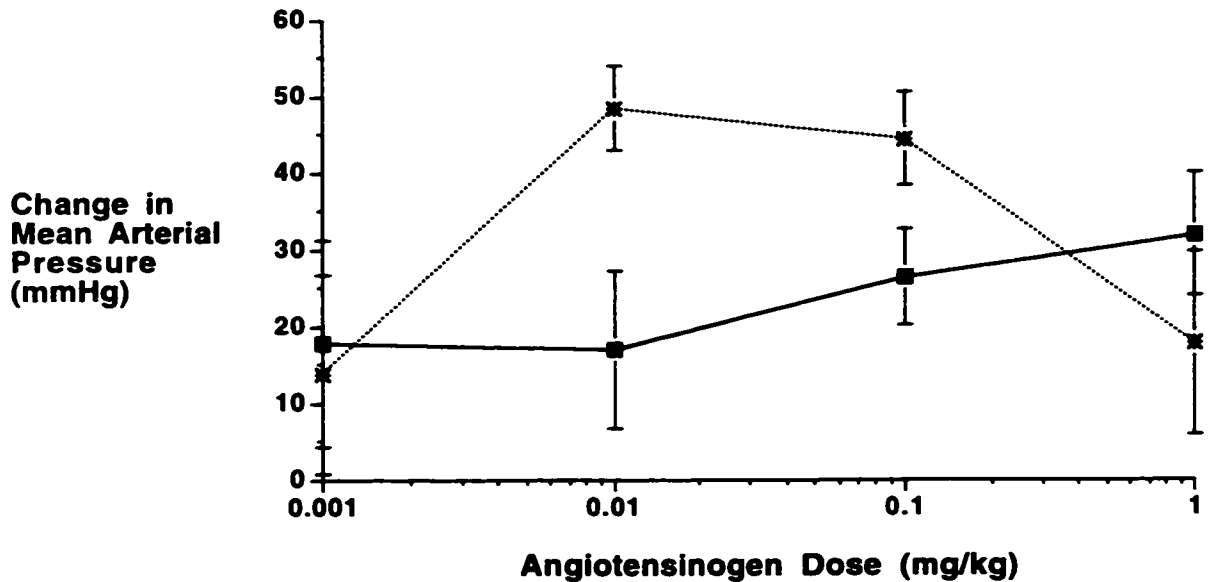
Figure 9. Change in mean arterial pressure following administration of 0.001, 0.01, 0.1 and 1.0 mg/kg b.w. porcine angiotensinogen (i.v.) to anesthetized rats 30 minutes following subcutaneous administration of 5 mg/kg b.w. saralasin. Symbols represent mean arterial pressure ± standard deviation, n = 3. ■ = saralasin treated rats and ✻ = control.

Experiment B:

1.0   Plasma Concentrations and Pharmacokinetics of T0901317 following Intravenous Administration

No adverse clinical symptoms were noted following administration. The plasma concentrations of T0901317 after intravenous administration of 1 mg/kg b.w. are shown

in Figure 10. Mean peak plasma concentrations (n=3) was 1375 ng/mL at 5 minutes after dosing (first sample point after dosing). The area under the plasma concentration vs. time curve (AUC) from 0 to 24 hours after dosing was 3645.29 hr x ng/mL. The overall plasma elimination half-life was calculated as 4.6 hours. The volume of distribution at steady state (Vdss) was 1.49 L/kg.
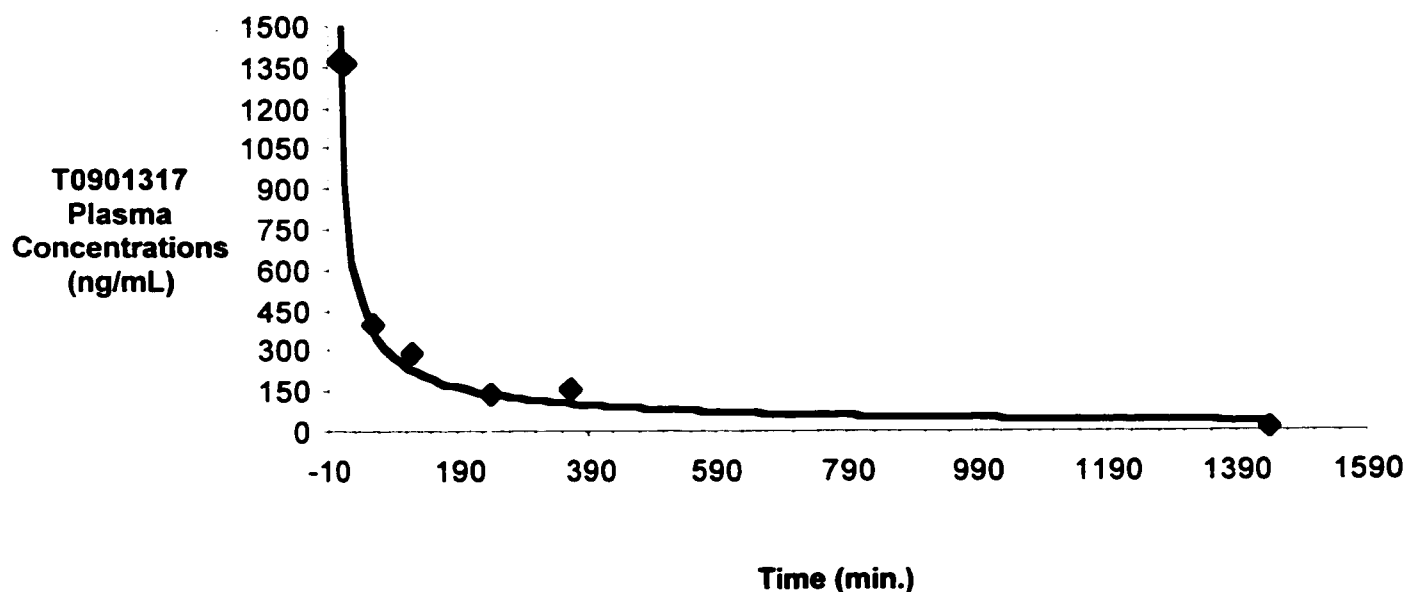
Figure 10. Plasma concentrations following intravenous administration of 1 mg/kg b.w. T0901317 in Sprague Dawley rats. T0901317 was dissolved in 10% EtOH/10% DMAC/30% PEG/50% water. Symbols represents mean plasma concentration values, n = 3.

2.0 Plasma Concentrations and Pharmacokinetics of T0901317 Following Oral administration

The plasma concentrations following 5 or 10 mg/kg b.w. T0901317 in rats are shown in Figure 8. The peak plasma concentration after 5 mg/kg b.w. was 450 ng/mL at 1 hour following dosing (first sample point after dosing). Plasma levels decreased at later timepoints. At 24 hours, a plasma concentration of 100 ng/mL was still detected. After 10 mg/kg b.w., peak levels were approximately twice as high as following the 5 mg/kg b.w. dose. However, plasma concentrations did not appreciably decline between 1 and 24 hours after dosing. AUC_{0-24h} after 5 and 10 mg/kg were 5941.92 hr x ng/mL and 22523.41 hr x ng/mL respectively. The oral bioavailability, calculated using the 1 mg/kg b.w. i.v. and 5 mg/kg b.w. p.o. doses, was 61.3%.
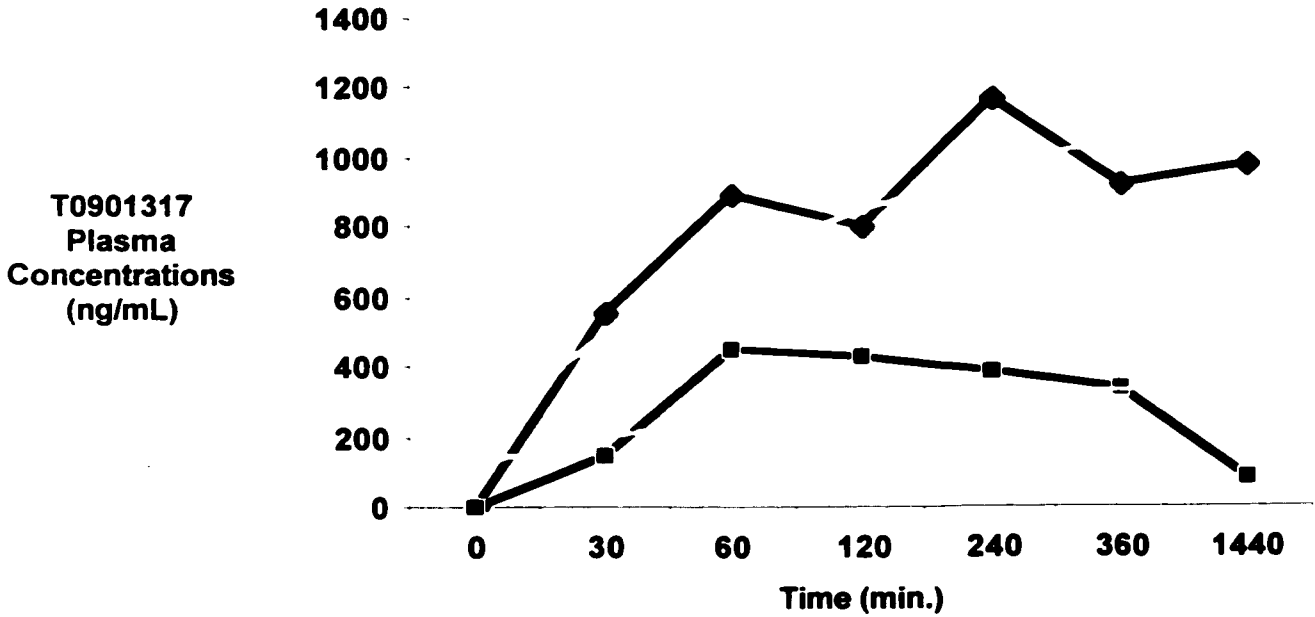
Figure 11. Plasma concentrations following oral administration of 5 and 10 mg/kg b.w. T0901317 in Sprague Dawley rats. T0901317 was suspended in a solution of 1% by volume Tween 80 and 1% Methylcellulose. The squares represent the mean plasma concentration values of the animals dosed with 5 mg/kg b.w. T0901317 and the diamonds represent the mean plasma concentration values of the animals dosed with 10 mg/kg b.w. T0901317. N = 3/dose group.

Experiment C

1.0 Effects of acute administration of T0901317 on angiotensinogen-induced vasopressor effects

A bolus intravenous administration of 0.1 mg/kg b.w. porcine angiotensinogen to anestetized rats resulted in an increase of MAP of 50 to 90 mm Hg (Figures 1 and 2). There was no significant difference in the pressor responses between control animals and animals which were dosed with T0901317, 10 or 30 mg/kg b.w., at various time points after drug administration (Figures 9 and 10).
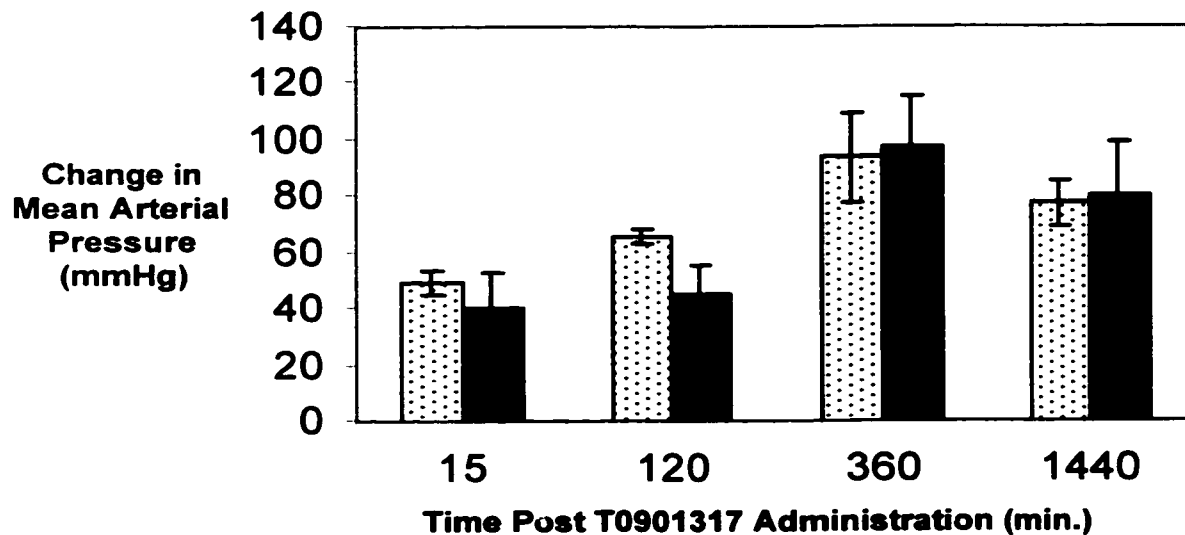
Figure 12. Effects of acute oral administration of 10 mg/kg b.w. T0901317 on mean arterial pressure in Sprague Dawley rats following intravenous administration of 0.1 mg/kg b.w. porcine angiotensinogen. Dotted bars represent the average change in blood pressure ± standard deviation of animals given 0 mg/kg b.w. 1% Methylcellulose (n = 4/ per timepoint). Solid bars represent the average change in MAP ± standard deviation of animals given T0901317 (n = 4/timepoint).

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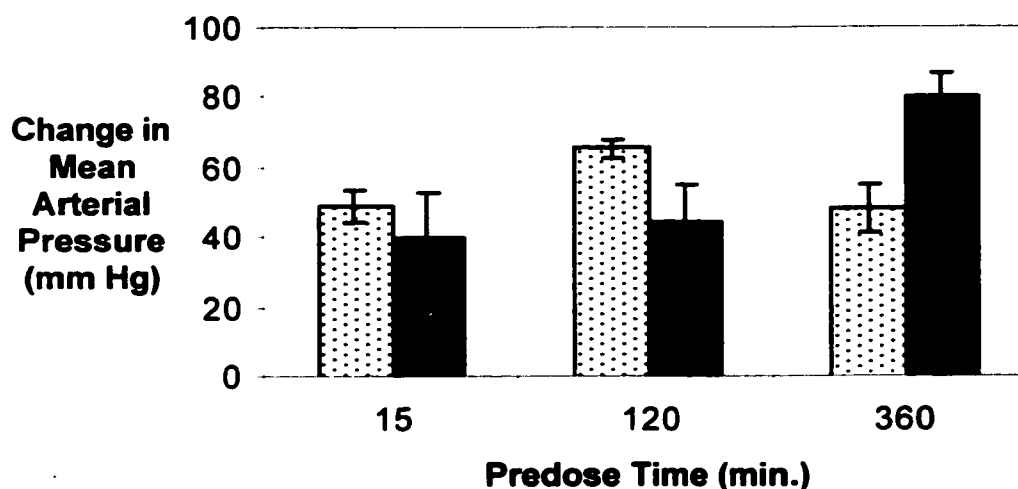
Figure 13. Effects of acute oral administration of 30 mg/kg T0901317 on MAP in Sprague Dawley rats following intravenous administration of 0.1 mg/kg b.w. porcine angiotensinogen. Dotted bars represent the average change in blood pressure $\pm$ standard deviation of animals given 0 mg/kg b.w. 1% Methylcellulose (n = 4/ per timepoint). Solid bars represent the average change in blood pressure $\pm$ standard deviation of animals given T0901317 (n = 4/timepoint).

2.0 Effect of multiple doses of T0901317 on angiotensinogen-induced vasopressor effects and selected plasma chemistry parameters

Five consecutive daily oral doses of T0901317 (10 mg/kg b.w.) had no significant effect of the magnitude of the vasopressor response to 0.1 mg/kg b.w. porcine angiotensinogen in anestetized rats (Figure 11). In marked contrast, significant changes were observed in selected clinical chemistry parameters (Table 1). In particular, plasma

total cholesterol and plasma HDL cholesterol were significantly reduced in animals

treated with T0901317, while plasma triglycerides increased fivefold. Plasma glucose,

LDH and alkaline phophatase were not significantly different between the groups. Plasma

AST, ALT and albumin were mildly elevated in animals treated with T0901317. Plasma

renin activities were not significantly different between vehicle and T0901317 treated

groups (Figure 12).

```
                    80 ─────────────────────────────────────
                    70 -
                                                      ┬
Change in           60 -                              │
  Mean              50 -              ┬          ┌─────┐
Arterial            40 -              │          │█████│
Pressure            30 -        ┌.....┤          │█████│
(mmHg)              20 -        │.....│          │█████│
                    10 -        │.....┴          │█████│
                     0 ─────────┴.....┴──────────┴█████┴───
                             Control      T0901317
```
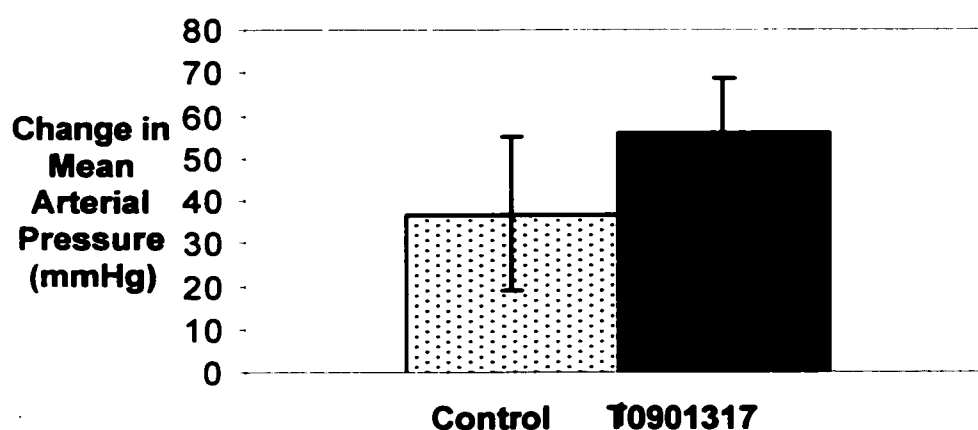
Figure 14. Effects of chronic (qd x 5) oral administration of 10 mg/kg b.w. T0901317 on

mean arterial pressure in Sprague Dawley rats following intravenous administration of

0.1 mg/kg b.w.porcine angiotensinogen. Animals were dosed with angiotensinogen

approximately 2 hours following drug administration of Day 5. Dotted bars represent the

average change in blood pressure ± standard deviation of animals given 0 mg/kg b.w. 1%

Methycellulose (n =4). Solid bars respresent the average change in blood pressure ±

standard deviation of animals given T0901317 (n = 4).
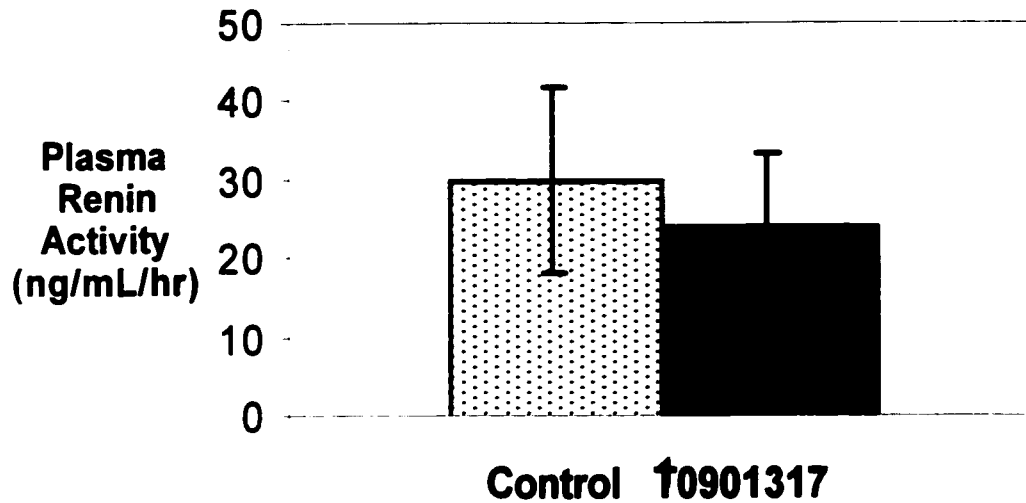
Figure 15. Effects of chronic (qd x 5) oral administration of 10 mg/kg b.w. T0901317 on

plasma renin activity in Sprague Dawley Rats following intravenous administration of 0.1

mg/kg b.w. porcine angiotensinogen. Blood samples were taken approximately 2 hours

following drug administration on Day 5. Dotted bars represent the average plasma renin

activity $\pm$ standard deviation of animals given 0 mg/kg b.w. 1% Methylcellulose (n = 4).

Solid bars represent the plasma renin activity $\pm$ standard deviation of animals given

T0901317 (n = 5).

	Vehicle Control	T0901317	P value
Total Cholesterol (mg/dL)	83.7 ± 35.5	54.6 ± 23.6 *	0.04
Triglycerides (mg/dL)	65.7 ± 21.0	355.8 ± 225.4 *	0.001
HDL Cholesterol (mg/dL)	54.7 ± 13.2	24.2 ± 7.8 *	0.00009
Glucose (mg/dL)	244 ± 87.9	229.5 ± 87.5	0.36
Albumin (g/dL)	4.55 ± 0.1	4.86 ± 0.21*	0.03
LDH (U/L)	187.8 ± 152.1	172.2 ± 74.3	0.845
AST (U/L)	113.8 ± 24.1	189.8 ± 36.1*	0.009
ALT (U/L)	52.5 ± 13.7	76.4 ± 7.2*	0.01
Alkaline Phosphatase (U/L)	93.3 ± 31.9	131.0 ± 26.7	0.09

Table 1. Effects of 5 day administration of 10 mg/kg b.w. T0901317 on selected clinical chemistry parameters. All values represent averages ± standard deviation. * denotes a statistically significant difference ($p < 0.05$). For total cholesterol, triglycerides, HDL cholesterol and glucose n = 9 per dose group. For all other tests n = 4 for the vehicle control and n = 5 for the T0901317 treated group.

3.0   Effect of T0901317 on arterial pressure in hypertensive rats

Mean arterial pressure in rats, 5 days following complete ligation of the left renal artery and treated with vehicle was146.7 $\pm$ 20.0 mm Hg. In renal artery ligated animals treated with enalapril (10 mg/kg/day b.w.), mean arterial pressure was significantly lower at 116 $\pm$ 0 mm Hg.   In renal artery ligated rats treated with T0901317 (10 mg/kg/day b.w.), mean arterial pressure was not statistically different from vehicle treated rats with an average mean arterial pressure of 165.3 $\pm$ 19.1 mm Hg. (Figure 13).

Five-day oral treatment with lisinopril (10 mg/kg b.w.) in spontaneously hypertensive rats resulted in a significantly lower arterial pressure as compared to SHR treated with vehicle or with 10 mg/kg/day b.w. T0901317. There was no statisticallly significant difference between the T0901317 and vehicle-treated groups. Data are shown in Figure 14.
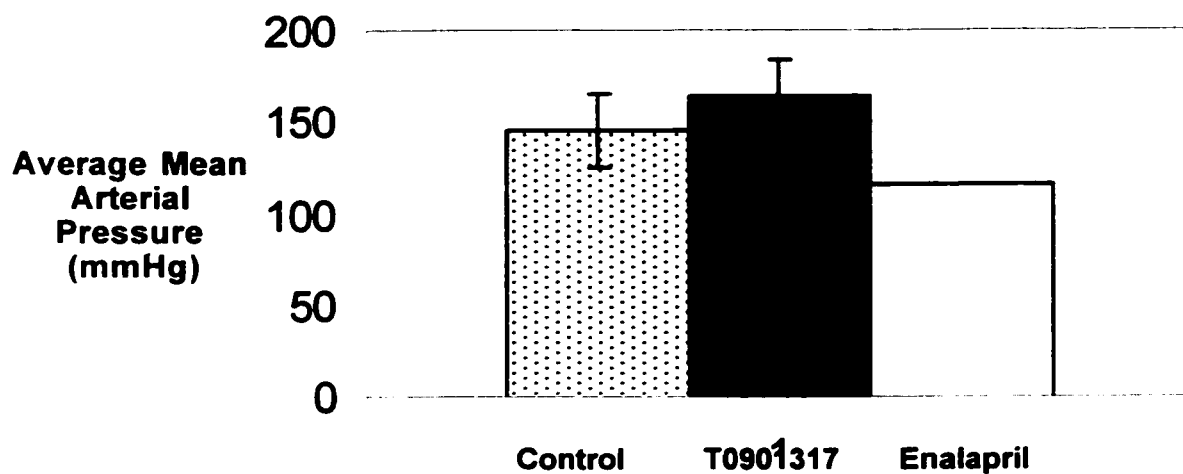
Figure 16. Effects of chronic (qd x 5) oral administration of 10 mg/kg b.w. T0901317 on mean arterial pressure in renal hypertensive rats. Enalapril was used as a positive control. MAP measurements were taken approximately 15 minutes following administration of drug on day 5. Dotted bar represents the mean arterial pressure ± standard deviation of aniimals given 0 mg/kg 1% Methylcellulose (n= 3). Solid bar represents the mean arterial pressure ± standard deviation of animals given T0901317 (n=3). Open bar represents the mean arterial pressure ± standard deviation of animals given enalapril (n=3).
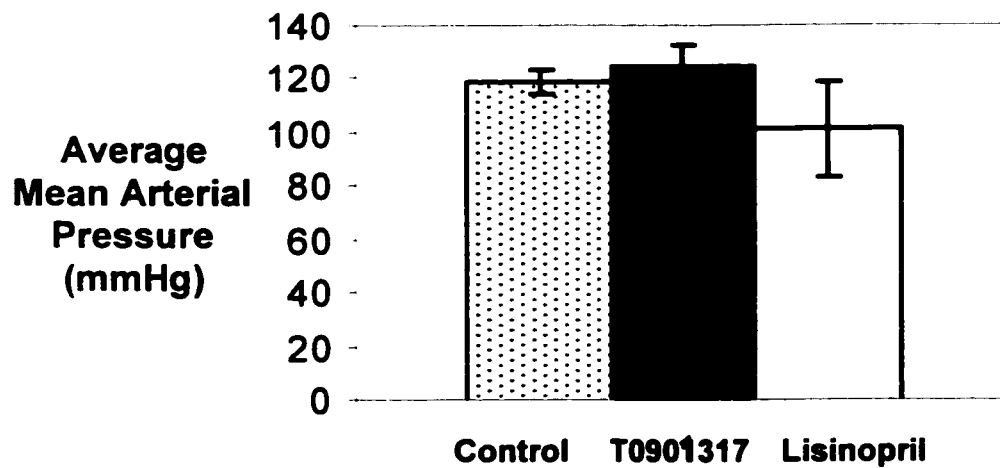
Figure 17. Effects of chronic (qd x 5) oral administration of 10 mg/kg b.w. T0901317 on

mean arterial pressure in conscious Spontaneously Hypertenisve rats. Lisinopril was

used as the positive control. MAP measurements were taken approximately 15 minutes

following administration of drug on day 5. Dotted bar represents the mean arterial

pressure ± standard deviation of animals given 0 mg/kg b.w. 1% Methylcellulose (n = 5).

Solid bar represents the mean arterial pressure ± standard deviation of animals given

T0901317 (n = 4). Open bar represents the mean arterial presure ± standard deviation of

animals given lisinopril.

IV.   Discussion

Experiment A

This study shows that intravenous administration of exogenous angiotensinogen to anesthetized rats causes a pressor response that requires an intact and functional renin-angiotensin system. This is supported by the findings that renin inhibitors such as Renin Inhibitory Peptide, Angiotensin Converting Enzyme inhibitors such as lisinopril and Angiotensin II receptor blockers such as Saralasin significantly inhibit the pressor effects.

Administration of both human (synthetic) and porcine (extract) angiotensinogen cause pressor reponses. It has been repeatedly demonstrated that a rise or fall in angiotensinogen levels in the circulation leads to an increase or decrease in the production of angiotensin I and subsequently angiotensin II. Therefore, the concentration of angiotensinogen is a rate limiting factor in the generation of angiotensin I. In this study, as expected, angiotensinogen administration led to an increase in mean arterial pressure (MAP) presumably as a result of increased generation of angiotensin I and II.

The ED_{50} values for both porcine and human synthetic angiotensinogen were also determined. The ED_{50} for porcine angiotensinogen was approximately 0.002 mg/kg and for human synthetic it was 0.009 mg/kg. From these two values it is apparent that the porcine angiotensinogen is much more potent than the human synthetic, as is indicated by the lower ED_{50} for porcine angiotensinogen. The greater potency of porcine angiotensinogen is also demonstrated by the much greater increase in mean arterial pressure following porcine angiotensinogen administration as opposed to human

synthetic angiotensinogen. In fact, there was a statistically significant difference between the porcine and human synthetic angiotensinogen (see the results section). This may be explained by the species specificity of renin. Generally, primate angiotensinogen is most effectively cleaved by primate renin. However, non-primate angiotensinogens can be cleaved by renin of their own or other species (Morgan et al, 1996; Menard et al,1983). This species specificity can be attributed to the difference in the bond at the renin cleavage site. Primate angiotensinogen has a Leu[10]-Val[11] bond at the cleavage site as well as a charged histidine instead of a tyrosine (seen in non-primate angiotensinogen) at residue 13.

Upon administration of the vehicle and all doses of angiotensinogen we see an increase in mean arterial pressure. Even though you wouldn't normally expect to see an increase in MAP following administration of a Dextrose vehicle, the increase that is seen in this case is simply due to the increased fluid volume which occurs as a result of an intravenous dose of any type of fluid. Even though human synthetic angiotensinogen does not elicit as great of a hypertensive response as porcine angiotensinogen, there is still a statistically significant increase as compared to the control. In addition, just as with the porcine angiotensinogen, human synthetic angiotensinogen causes an increase in mean arterial pressure that is clearly dose dependent, rising as the dose of angiotensinogen increases. The deviation from the normal sigmoidal shape of a typical dose response curve can be attributed to this particular response requiring a series of different biochemical steps. In addition, had we evaluated intermediate doses between the log-unit dose steps, we might have seen the characteristic sigmoidal shape. The bell

shaped dose response curve may also be the result of impurities. The porcine angiotensinogen that we used is an extract, therefore, the chance of it containing impurities is quite high. These impurities may include vasodilatory peptides. At the higher dose of angiotensinogen, mainly the 1.0 mg/kg b.w. dose, these impurities or vasodilating components may be dosed high enough to counteract the vasopressor response to angiotensinogen. Due to limited time and resources, we were unable to test the purity of the angiotensinogen (porcine and human synthetic). However, this can easily be done either by analyzing the purity of the angiotensinogen solution by using an HPLC or Mass Spectrometer or by performing an immuno-depletion experiment. Nevertheless, it can still be concluded that the response is dependent on the presence of active renin.

The hypertensive response reaches its peak at 0.1 mg/kg b.w. and drops off very slightly at 1.0 mg/kg b.w.. We will use 0.1 mg/kg b.w. porcine angiotensinogen for future studies because it does elicit a predictable and consistent response and because it is consistent throughout the studies.

In addition, it would have been beneficial to include a few more data points on the dose response curves. This could have been done by administering more doses of angiotensinogen. Since there was no previous literature on angiotensinogen dose response curves, we chose to start with the doses that were powers of ten apart. It turned out that many times we saw no significant difference in the hypertenisve response between the 0.001, 0.01 and 1.0 mg/kg doses, giving the appearance of only two data points on the dose response curve. Unfortunately, due to limited time and resources, we

waereunable to pursue the effects of more doses of angiotensinogen, however, this would be worthwhile pursuing in future studies.

Intravenous infusion of Renin Inhibitory Peptide (RIP) significantly inhibited the pressor response. This clearly indicates that the response to porcine angiotensinogen is dependent on renin activity. The finding that the higher doses of angiotensinogen were apparently more effectively inhibited than the lower doses may be explained by the experimental design of the study. The construction of the dose-response curve used subsequent injections of increasing doses of angiotensinogen into the same animal. RIP was infused throughout the period of administration of angiotensinogen at 200 ug/kg b.w./min. It is conceivable that the plasma concentration of the inhibitor increases over time, which would lead to higher inhibitory effects at higher doses of angiotensinogen. Depending on the half life of the inhibitor, steady state plasma concentrations may not have been reached for some time.

In addition to RIP, another renin inhibitor, pepstatin was administered, at 150 ug/kg b.w./min. According to previously performed studies (Oldham et al, 1984) if the hypertensive response was due to renin activity, then administration of pepstatin should have inhibited the rise in blood pressure seen upon administration of angiotensinogen. However, we were unable to replicate this in the current studies. Due to the extremely poor solubility of pepstatin and my inability to produce a uniform solution we can not rule out the possibility that the insolubility of the compound compromised the study and that the hypertensive response may be renin induced. We were unable to dissolve pepstatin and therefore, ended up with a suspension with large particles floating in it,

rather than a solution. In addition, while pepstatin does inhibit renin, it is a relatively weak renin inhibitor. This can be explained by the rather poor correspondence of the sidechains of pepstatin and renin indicating a lack of specificity(Hoover et al, 1995). Therefore, there is a lack of specificity as well as a low affinity of pepstatin for renin.

Bilateral nephrectomy, 16 hours prior to angiotensinogen administration effectively inhibited the pressor responses. To the contrary, bilateral nephrectomy immediately prior to angiotensinogen administration showed a much smaller inhibitory effect. As was discussed earlier, by removing the kidneys we removed the major source of renin in the body. Once the renin already in the circulation is removed, there is very little new renin being produced in the body. This means that eventually there will no longer be sufficient renin available to cleave the endogenous or exogenous angiotensinogen. Subsequently we would see a decrease in blood pressure as compared to the control group. Additionally, when the exogenous angiotensinogen was administered to nephrectomized animals we were unable to elicit a hypertensive response of the same magnitude as seen in the control group, once again due to the lack of renin. This is further proof that the angiotensinogen induced hypertensive response is renin induced.

There was also a difference between the two nephrectomized groups. Animals receiving angiotensinogen 12-16 hours post nephrectomy demonstrated lower blood

pressure increases to angiotensinogen than those nephrectomized immediately prior to angiotensinogen administration. This can be attributed to an increased clearance of renin. As mentioned previously, it may take up to 24 hours to remove the renin from the circulation. Therefore, it follows that animals nephrectomized immediately prior to angiotensinogen administration have a higher blood pressure response to angiotensinogen than those nephrectomized 16 hours prior to angiotensinogen administration. That we saw any blood pressure increase at all in nephrectomized animals following angiotensinogen administration suggests that there is a contribution to the hypertensive response by something other than kidney derived renin and the plasma renin angiotensin system. The existence of a local renin-angiotensin system within various tissues has been well documented (Campbell et al, 1993). These systems have been found to exist within various tissues including the vasculature, eyes, pancreas, heart, fat, adrenal cortex, pituitary, pineal and brain. These local renin-angiotensin systems play a minor but evident role in blood pressure regulation. In addition, it has been shown in previous studies that the conversion of angiotensin I to angiotensin II is much more efficient in tissues rather than plasma. This is evidenced by the higher concentrations of angiotensin II as compared to angiotensin I within tissues (Campbell et al, 1993). The cleavage of angiotensinogen to angiotensin I may be accomplished by locally generated renin.

Although the contribution by these tissues renin-angiotensin systems is slight they can still contribute to the observed hypertensive response seen in this study. When we consider that the half-life of renin within the circulation is only eighty minutes, the contribution of the longer lasting tissue generated renin could account for the slight hypertensive response seen following angiotensinogen administration in the animals nephrectomized 16 hours prior to dosing.

Another possible explanation for the slight hypertensive response seen in the nephrectomized animals is the existence of serine proteases other than renin that may also have the ability to cleave angiotensinogen. It is believed that cleavage by these proteases may lead directly to the formation of angiotensin II. This is further supported by a study in which low levels of angiotensin I in the tissues was observed in nephrectomized animals, while angiotensinogen and angiotensin II levels remained high (Campbell et al, 1993). The concurrent high levels of angiotensinogen and angiotensin II, while angiotensin I concentrations remained low, suggest that the angiotensin II is generated by something other than angiotensin I, namely angiotensinogen. In addition, it was reported that the levels of renin were also too low to account entirely for the high levels of angiotensin II, indicating that something other than renin was responsible for the cleavage of angiotensinogen. This pathway would allow for an increase in blood pressure even with low or absent levels of renin.

The significantly decreased hypertensive response in nephrectomized animals following angiotensinogen administration is further evidence that this hypertensive

response seen following the administration of angiotensinogen in nephrectomized animals is elicited by renin. Had even more time elapsed before administration of angiotensinogen following nephrectomy, we would expect to see an even greater drop in the blood pressure response both prior to and throughout angiotensinogen administration. In addition, the shape of the dose response curve is a little peculiar. It does not show the typical continuous incline as dose increases. This could be explained by the extremely low levels of renin. The remaining renin following nephrectomy may be saturated following the administration of the first dose of angiotensinogen. This could be tested by measuring plasma renin activity prior to and possibly throughout the administration of angiotensinogen.

Treatment with lisinopril (1 or 10 mg/kg b.w. i.v.) significantly inhibited the pressor response to angiotensinogen. This demonstrates that the pressor response to angiotensinogen requires conversion of angiotensin I to angiotensin II by ACE. There was no difference between the inhibition by 1 or 10 mg/kg b.w. lisinopril. This indicates that at the dose of 1 mg/kg b.w. lisinopril, ACE inhibition is maximal. The remaining pressor response to angiotensinogen is apparently not dependent on ACE activity. It may be suggested that this remaining response is due to angiotensin II in the preparation of porcine angiotensinogen. This could be evaluated by complete blockade of the angiotensin II receptor by a competitive antagonist. In our study, we used saralasin, which is a partial agonist and thus will not result in complete inhibition of the angiotensin II receptors.

In looking at the dose response for saralasin a significant inhibition of the pressor response at both 0.01 and 0.1 mg/kg b.w. angiotensinogen can be seen. However, at the higher doses, we did not see the same effect. As stated previously this could be due to the impurities within the porcine angiotensinogen extract. In addition, we most likely would have seen pressor inhibition at the highest dose had we used a complete agonist and not just a partial one. For further studies, we will use the 0.1 mg/kg b.w. dose of angiotensinogen because this does shows the most consistent effects.

In summary, the present study demonstrates that intravenous administration of exogenous angiotensinogen results in a vasopressor response that is dependent on renin activity, ACE activity and is finally mediated via angiotensin II receptors. This response may be used to evaluate inhibitors of the Renin Angiotensin System. Because renin inhibition and reduction of plasma renin concentrations by nephrectomy effectively inhibit the response, this model may be used for the evaluation of inhibitors of renin activity and/or release.

Experiment B

This study characterizes the i.v. and p.o. pharmacokinetics of T0901317, an LXRa agonist in rats. The data show that after i.v. administration, the compound is cleared at approximately 2.6 L/kg/hr. The renal blood flow is approximately 100 mL/kg/hr in rats whereas the blood flow to the liver is about 4 L/kg/hr. Therefore, with a compound clearance of 2.6 L/kg/hr we can conclude that the majority of this compound is eliminated and cleared from the body via a hepatic route (i.e. metabolic inactivation),

rather than renal filtration. In addition, the volume of distribution at steady state is 1.5 L/kg. Since the volume of distribution is a measure of the distribution of a compound throughout tissues, we can see that T0901317 distributes out of the intravascular compartment into tissue compartments. This value is substantially higher than the total blood volume in rats (approximately 0.06 L/kg) and higher than total body water volume (approximately 0.7 L/kg). The large volume of distribution, combined with a relatively high clearance results in an overall elimination half-life of about 4.5 hours.

Oral administration of T0901317 at both 5 mg/kg b.w. and 10 mg/kg b.w. demonstrates that the drug remains in the body for a relatively long period of time. The oral half-life of T0901317 was found to be 9.21 hours for 5 mg/kg b.w. and 66.4 hours for 10 mg/kg. It is obvious from these numbers that the different doses produce two very different profiles. It is apparent from the half-life that the 10 mg/kg b.w. dose of T0901317 causes a very prolonged exposure to the compound. This could indicate a number of things. First of all, there could be a very large storage of T0901317, either in the tissues or bound to plasma proteins, and therefore unavailable for removal. Secondly, the drug could go through a number of reabsorption cycles from the gastrointestinal tract (e.g. enterohepatic recirculation). Thirdly, T0901317 could actually inhibit its own metabolism by inhibiting the enzymes necessary for its degradation. Inhibition of these enzymes could occur if the concentration of the drug at the enzymatic site exceeds its K_i. In addition, the compound may form an irreversible complex with the enzyme. Lastly, the prolonged presence of T0901317 following an oral dose of 10mg/kg b.w. may be partially due to prolonged absorption from the gastrointestinal tract. In order to calculate

the oral bioavailability of T0901317 I used the AUC of the 5 mg/kg b.w. dose. The oral bioavailability was found to be 61.3 %. Due to the long half-life and the good oral bioavailability of T0901317 we conclude that further pharmacology studies can be performed using oral administration as the dose route.

Experiment C

When acutely administered in anesthetized rats, T0901317 has no effect on the angiotensinogen induced pressor response. The results of this study indicate that T0901317 has no effect on renin production, renin release or any part of the renin-angiotensin system. Since we saw no inhibition when T0901317 was administered acutely, we next investigated the effects of chronic administration of the drug. However, five day administration of T0901317 had no effect on the angiotensinogen induced pressor response, again indicating that T0901317 has no effect on the renin angiotensin system. In addition, plasma renin levels were not significantly changed in animals treated with T0901317 for five days.

One might argue that the angiotensinogen efficacy model that we used might be too insensitive to detect small differences in renin effects. Thus, we evaluated chronic effects on two models of hypertension that are known to be sensitive to inhibition of the renin-angiotensin system. For the rats that were renally ligated, no sham operated animals were included. The purpose of this experiment was to evaluate any effects of T0901317 on the hypertension caused by renal artery ligation in rats. As such, animals with renal artery ligation and treated with T0901317 were compared with renal artery

ligated rats treated with vehicle. Sham-operated rats would not have added significantly to the evaluation of the results because blood pressure in sham-operated animals is expected to be normal (Cangiano et al, 1979). We administered T0901317 to both rats with renal ligation and spontaneously hypertensive rats. Once again, I saw no effect on blood pressure from administration of T0901317 in either of these two models. This lack of inhibition again strongly indicates a lack of effect of T0901317 on renin production, release or other components of the renin-angiotensin system.

Despite the lack of effect on the renin-angiotensin system, T0901317 did have a clear effect on clinical chemistry parameters. We saw a significant drop in total cholesterol and HDL-cholesterol upon repeated administration of T0901317 ($p<0.05$). As LXR alpha plays a role in cholesterol homeostasis (Mangelsdorf et al, 1995; Willy et al, 1995)), this is what we would expect from an LXR alpha agonist such as T0901317. In addition we saw a significant increase on triyglycerides. Plasma alanine amion transferase , Aspartate amino transferase and albumin were also significantly increased in the T0901317 treated animals. These effects on plasma lipids show that even though T0901317 has no effect on the angiotensinogen induced pressor response, it is still pharmacologically active. In addition, we have previously shown that T0901317 is orally bioavailable. LXR alpha agonists have been shown to upregulate CYP7A, an important enzyme for cholesterol catabolism. CYP7A produces bile acids from cholesterol and these are excreted via the bile into the gastrointestinal tract. The reduction in plasma

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cholesterol in rats treated with T0901317 may be due to this effect via LXR alpha. In addition, it has been shown recently that T0901317 upregulates the gene for Free Fatty Acid Synthase (unpublished observations). The significant rise in plasma triglycerides may be associated with this effect. T0901317 mildly elevated AST and ALT, both indicating hepatocellular damage. Since the elevations were only minor, this compound is not likely to cause major liver toxicity. The effects of liver enzymes may be secondary to the significant elevation of plasma triglycerides as well. In summary, we can conclude from this series of studies that despite the presence of plasma chemistry changes, that T0901317 has no effect on the renin-angiotensin system. Therefore, LXR alpha agonists are not likely to be useful for the development of antihypertensive drugs.

V.   Conclusions

- Intravenous angiotensinogen administration caused a dose dependent increase in blood pressure when administered at 0.001, 0.01, 0.1 and 1.0 mg/kg b.w. The results show that administration of exogenous angiotensinogen administration causes a pressor response that is dependent upon an active Renin Angiotensin System and that inhbitors of renin and/or Angiotensin Converting Enzyme can block the responses. Thus, this model may be useful for the functional assessment of agents that inhibit renin and/or ACE.

- Administration of porcine angiotensinogen shows a much greater hypertensive response than human synthetic angiotensinogen. In addition, the ED_{50} for porcine angiotensinogen (0.002 mg/kg) is much lower than that of human synthetic angiotensinogen (0.009 mg/kg).

- The LXR alpha agonist, T0901317 has an apparent oral bioavailability of 60% following a dose of 5 mg/kg b.w. These data indicate that the oral administration of T0901317 to rats results in significant plasma levels and that the compound may be useful for oral pharmacological studies.

- Acute or chronic administration of T0901317 (10 or 30 mg/kg b.w.) had no significant effects on the vasopressor responses to 0.1 mg/kg b.w. i.v. porcine angiotensinogen.

- Following chronic administration of T0901317, significant changes in plasma lipids were seen, however, no changes in plasma renin activity were observed.

- Chronic administration T0901317 had no effect on the arterial pressure in Spontaneously Hypertensive Rats or renal hypertensive rats.

- The data indicate that LXR alpha agonism does not lead to inhibition of RAS mediated vasopressor effects, and that this class of agents does not appear to be useful for the development of antihypertensive agents based on their potential effects on renin release or other components of the RAS.

VI. References

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