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Antihypertensive Mechanisms of Metformin

by

J. C. Morales

A Dissertation submitted to the Graduate Faculty in Biology

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy, The City University of New York

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Abstract

Antihypertensive Mechanisms of Metformin

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Metformin, an antihyperglycemic agent, has been reported to have hypotensive properties in humans and experimental animals. However, the specific mechanisms by which metformin lowers blood pressure are not completely understood. Acute metformin injections decrease both blood pressure and renal sympathetic nerve activity in Spontaneously Hypertensive Rats (SHRs). In addition, acute metformin has been shown to decrease vascular smooth muscle reactivity.

Although the present body of literature on metformin provides evidence that acute metformin lowers blood pressure levels through sympathetic withdrawal and lowered vascular reactivity, it is unclear that these findings can be extrapolated to explain the observed hypotension reported during chronic treatment. In addition, no previous study has examined the possibility that metformin may simultaneously reduce sympathetic neural flow and vascular reactivity.

This study addresses whether long-term metformin treatment decreases blood pressure by decreasing sympathetic nerve activity and/or vascular smooth muscle reactivity.

The first section of this study was devoted to determine whether a 3-week oral treatment of metformin reduced plasma catecholamines and blood pressure in SHRs. To assess the influence of long-term treatment of metformin on the sympathetic nerve activity, the levels of plasma catecholamines, specifically norepinephrine (NE), epinephrine (Epi) and dopamine (DA) were measured. NE plasma concentrations were lowered in metformin rats. However, Epi concentrations in plasma were increased by metformin treatment. DA concentrations in plasma were not affected by metformin.

The second section of this study complemented the former section. This method assessed sympathetic nerve activity involvement by quantifying the fall in blood pressure produced by ganglionic blockade. Venous catheters and radiotelemetry devices were implanted and the animals were allowed to recover and habituate. Metformin slightly but not significantly reduced the fall in BP to Hex indicating a reduction in sympathetic nerve activity.

The third section of this study examined vascular contractility to pressor agents in rats that have been chronically treated with metformin. To improve the possibility of detecting alterations in regional reactivity, rats were instrumented for recording both arterial blood pressure and vascular resistance in two major vascular beds. An α_1-adrenergic agonist, phenylephrine, was used to assess pure α-adrenergic responsiveness rather than the mixed α- and β-adrenergic stimulation produced by NE. Responses to angiotensin II were recorded to determine whether altered reactivity, to metformin, is specific to a single pressor

system. In this section, it was found that metformin increased the vascular reactivity to both phenylephrine and angiotensin II.

In summary, metformin lowered NE spill-over release but increased Epi spill-over release. In addition, metformin tended cause an attenuated fall in blood pressure in response to Hexamethomium bromide. Surprisingly, metformin caused an increase in blood pressure reactivity to both phenylephrine and angiotensin II. Phenylephrine and angiotensin II caused expected changes in regional blood flow, however, no differences were observed between the metformin and control groups. In conclusion, metformin caused a chronic decrease in BP that was caused by a decrease in sympathetic nerve activity, but was not secondary to a decrease in vascular reactivity. In fact, we observed an increase in vascular reactivity in metformin treated rats.

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Grandma, Mom, Dad, brothers (Claudio, Gabriel and Mario) there is very little that I can say that can comprise my feelings and admiration to you as a whole. How lucky and proud I am to be a part of you. I find it very difficult to see myself at this plateau without your help, teachings and sacrifices.

To my wife, I say thanks and more thanks, for letting me play in the sandbox a little longer. I do appreciate all that you do which I don't acknowledge out-loud. This is very much your doing... Congratulations and respects.

After all is said and done, I want to send a message to my kids, Christopher, Rebekah and you. Do what you feel is in you and NOT what the majority does at the time. Research and digest the information before acting on anything. Be patient with your life. Be compassionate with your decisions. Whether right or wrong, it always works out. If you draw a plan, be flexible with it. And most of all, NEVER stop. You can do anything. I hope this work gives you a clue to your potential.

Finally I dedicate this work to three people, for their expectations and continual support. I wish that you could have been around to enjoy this day...To "Mi Vieja", Claudio and Carol, you truly got me...

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Introduction

Metformin is a drug used to lower blood glucose concentrations in patients with non-insulin-dependent diabetes mellitus (NIDDM). Recent studies have shown that in addition to lowering blood glucose, metformin also lowers blood pressure in humans and experimental animals. However, the mechanisms of how metformin lowers blood pressure are not understood. These studies will provide new knowledge about how long-term metformin lowers blood pressure in insulin-resistant patients with elevated blood pressure and in other types of hypertension. It is anticipated that this new information will help us to understand why metformin lowers blood pressure in certain human subjects (Chan *et al.*, 1993;Giugliano *et al.*, 1993b;Landin *et al.*, 1991) but not in others (Calle-Pascual *et al.*, 1995;Campbell *et al.*, 1987). Because current studies suggest that controlling not only hyperglycemia but also LDL cholesterol and blood pressure are important to protect diabetics from atherosclerosis (Hsueh and Law., 1998), the use of metformin to potentially lower insulin resistance and blood pressure could be of great advantage to NIDDM patients suffering from hypertension. The clinical relevance of these studies is illustrated by the fact that diabetic individuals with arterial hypertension exhibit markedly increased cardiovascular morbidity and mortality. In fact, it has been estimated that 35-75% of diabetic complications can be attributed to arterial hypertension (Bild and Teutsch., 1987;Epstein and Sowers., 1992). Therefore, a determination of the hypotensive actions of metformin will be of potential relevance to human disease states associated with insulin resistance, dislipidemia, and atherosclerosis.

History of Metformin: The Papyrus Ebers of 1550 BC are the earliest culture to record treatment for diabetes mellitus involving the use of plants. Their treatment was a high-fiber diet of wheat grains and ochre (Copenhagen *et al.*, 1937). Since the availability of insulin, folklore medicines for diabetes have almost disappeared in developed societies (although they continue to be the cornerstone of therapy in underdeveloped regions). Renewed interest to alternative medicines and natural therapies has re-ignited a wave of research interest in traditional practices. Metformin is one such example of this interest.

Metformin is found in the French lilac. The traditional use of *Galega officinalis* (goat's rue or French lilac) in medieval Europe can be explained by high contents of the hypoglycemic substance guanidine (Hermann and Melander., 1992). Although guanide was evaluated as toxic for clinical use, the alkyl diguanides (synthalin A and synthalin B) were introduced as oral antidiabetic agents in the 1920s. Like their predecessor, these agents were discontinued due to their toxic effects on animals. This occurred at the same time that insulin was becoming widely available through volume production made possible by advances in molecular techniques.

Diguanides prompted the development of large numbers of biguanides from which only three became used for diabetic treatment: Phenformin, Buformin and Metformin. In 1977, the FDA banned the used of Phenformin due to reported cases of lactic acidosis. Since insulin, at the time, had no reported side effects, interest in Biguanide for diabetic treatment diminished. Insulin later was found to cause complication with immune responses. These reports caused a search for a new drug that would have a hypoglycemic effect. The logical place to begin the search was with the traditional plant treatments. The scientific community had little toxicological information available concerning traditional antidiabetic plants. The biguanide literature found itself centered-stage, again. Metformin was the biguanide of choice over phenformin because it is excreted unchanged by the kidneys (Beckmann., 1969). Re-evaluation of lactic acidosis generated by metformin was initiated. Lactic acidosis, a rare but serious adverse effect in metformin-treated patients, was estimated to occur in < 0.01 to 0.08 case per 1000 patient (Bailey and Day., 1989) when compared to phenformin which occurred 20 times more often (Krentz *et al.*, 1994). These new studies supported that metformin is not a major cause of lactic acidosis. In practice, where metformin is prescribed, lactic acidosis in not regarded as a major problem.

Diabetes Mellitus—a brief review: A chronic disease of absolute or relative insulin deficiency or resistance, diabetes mellitus is characterized by disturbances in carbohydrate, protein, and fat metabolism. A leading cause of death by disease in the United States, this syndrome contributes to about 50% of myocardial infarctions and about 75% of strokes, as well as to renal failure and peripheral vascular disease (Morsiani, 1989). It is also the leading cause of new blindness. This condition occurs in two forms: insulin-dependent diabetes mellitus (IDDM), and the more NIDDM. IDDM, also referred to as type I or childhood-onset diabetes, usually occurs before the age of 30; the patient is usually thin and requires exogenous insulin and dietary management to achieve control. Conversely, NIDDM, also referred to as type II adult-onset diabetes, usually occurs in obese adults after age 40 and is most often treated with diet and exercise with some combination of hypoglycemic drugs, although treatment rarely may include insulin therapy (Ireland *et al.*, 1980). Although the ultimate causes of IDDM and NIDDM are still under extensive investigation, it is apparent that there is overlap among signaling components, such as the hormones, neuropeptides, and receptors involved in these two disorders, and it is believed that these signaling components play a prominent role in their association.

Insulin Production and Secretion: Under normal conditions, the production of insulin is controlled at many levels, including translation, protein folding and protein trafficking. The insulin peptide undergoes a series of post-translational modifications from preproinsulin, its initial transcriptional state to proinsulin, finally to the active transcriptional state, the insulin molecule. The proinsulin molecule serves as an aid that leads to the correct protein structural alignment of the dipeptide chains, A & B, that make up the insulin molecule by alignment of the disulfide bonds. The proinsulin molecule is further cleaved into insulin and a second peptide called the C-peptide. Both of these peptides are packed and released together. The C-peptide is thought to maintain the affinity and integrity of the insulin receptor (Ido *et al.*, 1997).

The pancreas is an organ of great interest due to its role in homeostasis. The pancreas releases insulin from β-cells in a circadian manner (Peschke and

Peschke., 1998). In general, the pancreatic β-cells respond to an increase in glucose level by releasing insulin in a triphasic pattern. The first phase is interpreted as the sympathetic priming of the pancreatic cells. The second phase is the release of insulin from vesicles, which are readily available. After a meal, the increase in blood glucose stimulates the pancreas to release insulin. The third phase is the release of newly synthesized insulin. The patterns of secretory release are usually a reflection of individual eating patterns. Briefly, it is generally accepted that glucose is metabolized within the β-cell and this metabolism is the signal that causes insulin release. The metabolism of glucose means that the glucose transporters (Glut) sequester glucose into the cell. Once glucose is sequestered by Glut, glucose is deposited at specific sites, such as the cytosol for glycolysis. This leads to the production of metabolites originating from glucose which make it possible for the cell to manufacture molecules, such as ATP, which make it possible for the cell to meet the energy requirements set by homestasis. Briefly, Glucose is used by both prokaryotic and eukaryotic cells for energy and macromolecule synthesis and modification. Glucose is involved in the synthesis of glycoaminoglycans, mucins, and glycosylation of proteins (Seifter and Englard., 1990). Through the anaplerotic reactions of the citric acid cycle and the pentose phosphate pathway, glucose can be transformed into amino acids and the ribose moiety of nucleotides. Virtually all animal cells use glucose for energy and posses elements of glycolytic pathway. The chemical potential released during the breakdown of glucose is converted and temporarily stored in the high energy phosphate bonds of ATP for subsequent hydrolysis.

Anaerobic glycolysis of glucose to lactate can yield two net ATP molecules per glucose from direct phosphorylation of ADP. Aerobic glycolysis of glucose to pyruvate and its further breakdown through the Krebs cycle can generate as much as 38 ATP energy equivalent per glucose molecule by electron transport and oxidative phosphorylation. Glycogen, normally found in significant quantities in muscle and liver, can be considered a temporary form of stored glucose. When carbohydrate digestion exceeds the energy expenditure, in the presence of insulin most of the excess glucose is synthesized into glycogen in the liver and muscle. A small fraction of excess glucose can be used for *de novo* fatty acid synthesis in the liver and adipocytes. Subsequent lipolysis releases the stored chemical potential. The metabolism of glucose also results in the closure of ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane, leading to depolarization of the cell, with a consequent influx of extracellular Ca^{2+} through voltage sensitive channels. These events lead to an increase in intracellular Ca^{2+} causing the cells to release vesicles containing insulin.

When insulin reaches the target cell, it binds to a specific receptor. These insulin receptors are αβ-heterodimers that binds insulin at the extracellular α-subunit. This immediately activates the intrinsic tyrosine kinase activity in the β-subunit resulting in autophosphorylation of tyrosine residues in 3 regions of the receptor: the juxtamembrane region, the regulatory region, and the carboxyl terminal region. The process of autophosphorylation of the insulin receptor is crucial. Any mutations that inhibit this process lead to severe insulin resistance (Odawara *et al.*, 1989). The phosphorylated tyrosine activates several cytosolic docking proteins, including the insulin receptor substrate (IRS); IRS-1, IRS-2, IRS-3, IRS-4 and Gab1. Activated insulin receptor substrate molecules serve as multisite docking proteins that bind specific Src homology 2 (SH2)- containing proteins. IRS molecules serve as "traffic controllers" which traffic signals to the appropriate signal transduction cascade to result in protein synthesis, glycogenesis, proliferation, and the activation of the facilitative glucose transporters.

These signaling pathways are responsible for glucose transport across the plasma membrane, which is vital to the entire cell entity for survival. Glucose is required by cells for oxidative and nonoxidative ATP production and for anabolic reactions. The ubiquitous use of glucose as the common currency of metabolism is unquestionably a result of the extreme abundance in nature of glucose in the form of cellulose and starch, synthesized from the glucose produced by the dark reactions of photosynthesis.

The facilitative glucose transporters are described as passive glucose carriers. A passive carrier is an energy-independent system that can only transport its substrate down a concentration gradient. The constant catabolism of glucose for cellular respiration creates lower intracellular glucose levels than in the extracellular spaces. This carrier is efficient when the cell is exposed to a constant level of the substrate, in this case glucose. The main function of the glucose transporters is to mediate the exchange of glucose between the blood and the cytoplasm of the cell. These glucose transporters form pathways between three sources of glucose: the blood, the extracellular fluids, and the cellular cytoplasm. Cells control the intake of glucose by expressing and regulating several facilitative glucose transporter isoforms, Glut1 through Glut5 and Glut7, which have distinct kinetic properties and distinct locations. For example, Glut1 is expressed in many fetal and adult tissues; it is abundant in red blood cells, endothelia and immortalized cell lines. Glut1 functions to maintain basal intracellular glucose and increase glucose supply for growth and for cells that are dividing. Glut1 is also involved in transporting glucose across the blood brain barrier and other tissue barriers (Czech., 1995). Glut2 is found in hepatocytes, pancreatic β-cells, intestine, and kidneys (Kahn., 1992). Glut3 is found in many tissues in humans, and controls the basal transport of glucose in many human cells. In other species, Glut3 regulates the uptake of glucose from cerebral fluid into the brain parenchymal cells (Kahn., 1992). Glut4 is found in skeletal muscle, heart, and adipose cells. It is involved in rapid increases of glucose transport in response to elevated blood insulin. It also plays a role in glucose disposal (Czech., 1995). Glut5 is found in the intestine, specifically in the jejunum, adipose cells, muscle, brain, and kidneys. Glut5 is thought to transport fructose because it has a higher K_M (~17mM) for fructose (Kahn., 1992). Glut6 is a pseudogene that is not expressed at the protein level (Kayano *et al.*, 1990). Finally, Glut7 is found mainly in hepatocytes; its function is to mediate the flux of glucose across the membrane of the endoplasmic reticulum.

Although stimulation of glucose transport appears to require the activity of the insulin-activated receptor kinase, glucose transporters are not phosphorylated by insulin, suggesting that other substrates of the kinase, like

IRS, help to regulate glucose transport. The internalization process of glucose by these transporters is still not clear. It is thought that the invagination of the plasma membrane, which is the main glucose transport step, is caused not by one population of glucose transporters but by at least two, Glut1 and Glut4.

Pathophysiology of IDDM: The etiology of IDDM is not known but interesting hypothesis have been proposed in the last few years. The presence of certain childhood diseases (Draznin *et al.*,) originating from viruses such as Coxsackie virus B4, retrovirus type 3, Venezuelan-encephalitis virus and rubella virus are thought to lead to or be precursors of IDDM. These traumatic insults generated by the viral infection may cause the decrease and finally the depletion of β-pancreatic cells, which are responsible for the production and secretion of insulin. It has also been proposed that IDDM is an autoimmune disease (Yokota *et al.*, 1998) and that viral infection is what "instigates" the macrophages to attack the β-cells, thus lowering the number of β-pancreatic cells available to secrete insulin, leading to hypoinsulenima and hyperglycemia. The hyperglycemic effect is caused by a decrease in insulin that disrupts of the glucose influx into the cell, resulting in an increased concentration of glucose in the extracellular fluid and the blood. In response to an overall decrease in insulin production, the remaining β-pancreatic cells still producing insulin can and do increase insulin production and release. Although temporarily helpful, this increase in production and release leads to an early type of necrosis of the β-pancreatic cell, ultimately decreasing the amount of insulin secretion. As a final mechanism of IDDM, viral infections can also cause mutations that alter the sequence of the insulin molecule. The insulin sequence, when mutated, can generate an insulin molecule that can either be completely inactive or able to bind to the target receptor in an abnormal manner with respect to the native insulin molecule.

In normal physiological states, the systemic concentrations of insulin flux from low levels during fasting to high levels during feeding states. These fluxes of insulin production and secretion suggest that the β-pancreatic cells are not under constant stimulation. Glucose stimulation of β-pancreatic cells does not generate a continuous stimulus to release insulin into the systemic system. In fact, it is now accepted that pulsative release of insulin and not a steady-state release is more efficiently used by the body during consumption of glucose (Peschke and Peschke., 1998). Therefore, the viral infections, autoimmune responses, and mutations discussed previously can adversely affect the release pattern of synthesized insulin.

The resulting lack of insulin in the body has been linked to many pathological states that range from starvation, dehydration, neuropathy, nephropathy, micro- and macrovascular complications, the lack of gene activation and deactivation or regulation, and modifications at the morphological level that lead to functional complications including alterations in the basement membrane of blood vessels.

<u>Pathophysiology of NIDDM</u>: As in IDDM, the etiology of NIDDM is also not well understood. NIDDM can be characterized by impaired β-cell function, insulin resistance or a combination of the two. It has been shown that the insulin secretory patterns are usually negatively affected in NIDDM. For example,

subjects with NIDDM usually secrete more insulin under basal conditions (Polonsky *et al.*, 1988). Moreover, the amplitude of the secretory pulse is reported to be small and the rate of the pulse is abnormally increased when compared to normal subjects. This suggests that the cycles of insulin release are shorter and more irregular than those reported in normal subjects (O'Rahilly *et al.*, 1988). Together these differences in the pattern of insulin release may also contribute to the pathology of NIDDM. In addition, NIDDM is often characterized by insulin resistance. Insulin resistance is evidenced by the fact that cell's insulin receptors are not able to maintain the signal transduction pathway that removes or allows glucose transportation into the cell. Insulin resistance can be due to receptor desensitization or any abnormality in the insulin receptor signal transduction cascade. Withers et al., (Withers *et al.*, 1998) showed that the disruption of the IRS-2 gene impairs insulin signaling and pancreatic β-cell functioning. IRS-2 knockout mice suffered from high levels of blood glucose, and insulin, indicating an increase in insulin resistance. This mutation may therefore represent a model of NIDDM diabetes. The mechanisms leading to insulin resistance in NIDDM are complex but may in part also be due to the chronically elevated insulin levels often observed in NIDDM.

Since glucose can not get into the cell, the molecule is "trapped" in the peripheral circulation, leading to hyperglycemia in NIDDM subjects. Because of the high concentration of glucose in the systemic system, the osmotic equilibrium is shifted, resulting in water leaving the cells, leading to dehydration and other problems such as the abnormal glycosylation of proteins.

Hypertension in general: Hypertension is defined as blood pressure greater than 140/95 mmHg. Primary hypertension, or essential hypertension, is the term used when the exact cause of elevated blood pressure has not been determined. Currently, hereditary history of hypertension, obesity and chronic stress are factors considered to promote primary hypertension. Secondary hypertension is caused by abnormal hormonal production outside the cardiovascular system. Any condition resulting in excessive production of antidiuretic hormone (also referred to as ADH), renin, aldosterone or Epi will probably lead to secondary hypertension. Many forms of kidney disease may also lead to secondary hypertension caused by fluid retention or excessive renin production. Whether primary or secondary, hypertension causes an increase in the work load of the heart, usually causing an enlargement of the left ventricle. The increase of muscle mass requires a greater oxygen demand. When the coronary circulation cannot keep pace, symptoms of coronary ischemia develop.

Treatment for hypertension consists of a combination of lifestyle changes and physiological therapies will improve peripheral circulation, prevent increases in blood volume and total body weight, and reduce plasma cholesterol levels. These strategies may be sufficient to control hypertension if it has been detected before significant cardiovascular damage has occurred. The majority of treatments make use of antihypertensive drugs, such as calcium channel blockers, β-blockers, diuretics, and vasodilators, used alone or in combination.

Hypertension in IDDM and NIDDM

Hypertension is observed in about 50% of the population suffering from IDDM and NIDDM (Harris., 1998), which is in contrast to the 30% incidence in the general population.

Hypertension in IDDM subjects is attributed mainly to the renal failure and nephropathy. As a result, sodium retention is implicated in the hypertension of IDDM, since the sodium content in the body is greater in diabetics than in normal subjects. An increase in water retention has also been reported, which is thought to be due to the hyperglycemic effect.

Glucose intolerance, insulin resistance, hyperinsulinemia, dyslipidemia, altered nitric oxide synthase activity, and obesity are thought to be contributing factors to hypertension in NIDDM. The most consistently linked factor with NIDDM and hypertension has been obesity. Obesity itself is an independent risk factor for hypertension although the blood pressure elevating mechanisms are unknown.

Nitric oxide synthase (NOS) activity is reduced in platelets from IDDM and NIDDM patients (Rabini *et al.*, 1998). This decrease in NOS activity leads to a decrease in nitric oxide production at the area of the vessel surface intimate with the blood. Decreased nitric oxide leads to an increase in vessel constriction and an increase in blood pressure along with an acceleration in the pathogenesis of micro- and macroangiopathy.

To complicate the story, hypertension is usually not a single event, comprising a single biochemical change, but a chain of events. For example, the hyperglycemia observed in both NIDDM and IDDM can cause nonenzymatic glycosylation on long-lived vessel wall proteins such as type IV collagen. The increased cross-linking of collagen, which is generated by glycosylation, is thought to be glucose dependent. Cross-linking of collagen affects the morphology of smooth muscle cells, mesangial cells, and endothelial cells that can later lead to vascular occlusion (Raabe *et al.*, 1998;Trovati and Anfossi., 1998). Another structure affected by nonenzymatic glycosylation is the basement membrane. The basement membrane is generally composed of type IV and type V collagen, heparin sulfate, dematan sulfate and chondroitin sulfate proteoglycans and laminin, and entacin. The function of the basement membrane, if interrupted, can lead to a number of changes in the physiology of the vessel resulting in increased thickness of the basement membrane and elevated microvascular pressure (Temelkova-Kurktschiev *et al.*, 1998;Wagenknecht *et al.*, 1998). Extrapolating this morphological event from one microvessel to a large number of microvessels can generate a synergistic effect resulting in an increase in blood pressure.

Finally, the hyperinsulinemia observed in NIDDM may have several cardiovascular actions. These include antiatiuretic effects (Finch *et al.*, 1990;Gans *et al.*, 1991), long-term stimulation of vascular smooth muscle proliferation and hypertrophy (King *et al.*, 1985;Stout *et al.*, 1975), and stimulation of sympathetic nerve activity (Anderson and Mark., 1993). These mechanisms together or independently can lead to the generation of hypertension.

Effects of Metformin on Hypertension in NIDDM

Antihyperglycemic agents, such as metformin, have been used to investigate how hyperinsulinemia may contribute to the development of arterial hypertension in NIDDM. Metformin (also referred to as glucophage) is an agent that is used to lower blood glucose levels in NIDDM patients. Metformin decreases hepatic gluconeogenesis, liponeogenesis, increases insulin sensitivity, and decreases intestinal absorption of glucose (Gilman *et al.*, 1990) although the precise mechanism of action is unknown. Metformin treatment in either IDDM or NIDDM patients increased the number of insulin receptors by up to 20% (Lord *et al.*, 1983;Pagano *et al.*, 1983) and NIDDM patients have been able to exploit the insulin sensitizing properties of this drug.

Since it is possible to generate hypertension from insulin resistance and hyperinsulinemia, then one can reasonably postulate that by reducing insulin resistance with metformin treatments one should lower blood pressure. Landin et al. (1991) tested this hypothesis and found that the blood pressure in humans was decreased after metformin administration. A number of groups have indeed generated data from both animal models and humans in agreement with this observation that metformin lowers blood pressure. Landin et al, (1991) Guigliano et al, (1993a and 1993b), Morgan et al, (1992) Velazquez et al, (1994), and Verma et al, (1994a, 1994b, 1996), have shown that metformin reduces insulin resistance, plasma insulin and arterial pressure.

A most interesting finding, reported by Verma et al., (1994a, 1994b, 1996), was that the blood pressure decrease in metformin treated SHR and fructose-fed rats were reversed by restoration of plasma insulin to pretreatment levels by use of subcutaneous insulin implants. Furthermore, acute administration of metformin also produces short-lasting hypotensive effects in both SHRs (Muntzel *et al.*, 1997;Petersen and DiBona., 1996) and dogs (Sterne., 1969).

Although these findings are intriguing, the relationship between metformin, insulin, and blood pressure has not been consistent across all experiments. In human studies, for example, chronic metformin reduced blood pressure without affecting plasma insulin in two studies (Calle-Pascual *et al.*, 1995;Fanghanel *et al.*, 1996), whereas metformin reduced plasma insulin with no effect on blood pressure in a number of other studies (Dorella *et al.*, 1996;Grant., 1996;Semplicini *et al.*, 1993).

Metformin and Blood Pressure: Nitric Oxide and Smooth Muscle

Despite a great deal of investigation in the potential antihypertensive actions of metformin, little is known regarding the mechanism of the drug's lowerings blood pressure properties over the long –term use. However, some progress has been made in terms of elucidating which cardiovascular control systems are not involved. For example, short-lasting decreases in blood pressure to intravenous metformin were unaffected by pretreatment with nitric oxide synthase inhibitors (Petersen *et al.*, 1997), indicating that neither central nervous system nor endothelial NOS are necessary for metformin-induced depressor responses. Supporting a lack of nitric oxide involvement, blunted arterial contractions by metformin were not affected by removal of the endothelium from vascular strip preparation (Verma *et al.*, 1996).

In other acute experiments, short-lasting reductions in blood pressure to metformin were unaltered by atropine (Sterne., 1969), and were not affected by indomethacin (Petersen and DiBona., 1996), indicating that the drug does not activate cholinergic vasodilatation nor prostaglandin-induced arterial relaxation.

Finally, it has been shown that metformin does not affect platelet-derived growth factor stimulation of vascular smooth muscle incorporation of thymidine (Sharma and Bhalla., 1995). Thus, metformin does not appear to inhibit or stimulate vascular smooth muscle proliferation. Although other studies have reported an inhibitory effect of metformin on thymidine incorporation, the effect was observed only at higher doses (Petty and Pearson., 1992).

Metformin and Blood Pressure: Vascular Contraction

Although metformin has no effect on the vasodepressor systems mentioned above, it does appear to directly alter vascular smooth muscle contractility. For instance, treatment with metformin caused reductions in contractile responses to NE (Peuler *et al.*, 1997;Verma *et al.*, 1996), phenylephrine (Chen *et al.*, 1997), and KCl (Peuler *et al.*, 1997). The mechanism of inhibition is most likely related to calcium regulation since metformin reduced transient increases in intracellular calcium to phenylephrine (Chen *et al.*, 1997), arginine vasopressin (Bhalla *et al.*, 1996;Dominguez *et al.*, 1996), thrombin (Bhalla *et al.*, 1996;Dominguez *et al.*, 1996), and to angiotensin II (Sharma and Bhalla., 1995). These inhibitory actions of metformin were observed in both cultured smooth muscle (Bhalla *et al.*, 1996;Dominguez *et al.*, 1996) and in arterial ring segments (Chen *et al.*, 1997). In series of comprehensive studies by

Chen and colleagues (Chen *et al.*, 1997), metformin caused repolarization of phenylephrine-constricted rat tail artery segments. Theses effects were associated with decreases in intracellular calcium and reductions in isometric force, suggesting that metformin-induced relaxation is caused by repolarization and subsequent reductions in calcium influx (Chen *et al.*, 1997). The repolarization, in turn, may be secondary opening of potassium channels since metformin-induced relaxation of phenylephrine-contracted arteries were attenuated by treatment with tetraethylamonium, a non-specific blocker of potassium channels (Smith *et al.*, 1998).

Taken together, these studies suggest that the hypotensive actions of metformin are mediated, in part, by attenuation of agonist-stimulated elevations in intracellular calcium levels in vascular smooth muscle. Although this hypothesis is intriguing, it is unclear whether acute studies using isolated vascular strips can be extrapolated to the blood pressure-lowering actions of chronic metformin treatment. The proposed experiments will therefore examine long-term metformin effects on blood pressure and whole-animal vascular reactivity in hypertensive rats. It is anticipated that metformin-induced reductions in blood pressure will be associated with attenuated regional pressor responses to vasoconstricting agents.

Metformin and Blood Pressure: Sympathetic Nerve Activity

In keeping with the multiple actions of metformin on carbohydrate metabolism (Bailey and Turner., 1996), it is likely that this drug reduces blood pressure through more than one mechanism. Recent evidence, for example, suggest that metformin lowers sympathetic nerve activity. Of the few experiments with humans examining this possibility, one reported simultaneous reductions in blood pressure and plasma NE levels in hypertensive, obese women (Giugliano *et al.*, 1993a) whereas another, administering the same dose to insulin-resistant hypertensive men, found no change in either parameter (Gudbjornsdottr *et al.*, 1994). In a third study, metformin blunted the rise in NE caused by insulin infusion during glucose clamp (Dorella *et al.*, 1996).

In contrast to the conflicting picture in humans, metformin consistently decreases sympathetic activity in experimental animals, at least in acute studies. For example, Petersen and colleagues found that acute intravenous administration of metformin produced dose-dependent reversible decreases in efferent renal sympathetic nerve activity and blood pressure in SHRs (Petersen and DiBona., 1996). In the only chronic study reported thus far, metformin elicited mild reductions in tail-cuff systolic blood pressure in obese, insulin-resistant rats accompained by decreases in plasma NE but not Epi levels (Kosegawa *et al.*, 1996).

Metformin and Blood Pressure: Plasma Insulin Levels

As a final possibility, metformin may reduce blood pressure by decreasing insulin resistance and plasma insulin levels (Verma *et al.*, 1994b). Consistent with this hypothesis, studies with both experimental animal models and humans showed metformin-induced reductions in both plasma insulin levels and blood pressure (Dorella *et al.*, 1996;Giugliano *et al.*, 1993a;Landin *et al.*, 1991;Verma *et al.*, 1994a;Verma *et al.*, 1994b). Although these findings are intriguing, the relationship between metformin, insulin, and blood pressure has not been consistent across all experiments. In human studies, for example, chronic metformin reduced blood pressure without affecting plasma insulin in two studies (Calle-Pascual *et al.*, 1995;Fanghanel *et al.*, 1996), whereas metformin reduced plasma insulin with no effect on blood pressure in a number of other experiments (Dorella *et al.*, 1996;Grant., 1996;Semplicini *et al.*, 1993).

Despite these inconsistencies, metformin-induced reductions in plasma insulin may mediate the reduction in vascular reactivity and the fall in sympathetic nerve activity discussed previously. Of these two possibilities, a relationship between insulin and vascular reactivity is least likely to explain the depressor effects of metformin. This is because insulin attenuates vascular smooth muscle contractions to pressor agents (Anderson and Mark., 1993;Standley *et al.*, 1991), and reductions in plasma insulin, by metformin, would therefore be expected to enhance vascular smooth muscle contractility instead of the observed reductions.

Metformin, on the other hand, may reduce plasma insulin and thereby inhibit sympathetic neural outflow. It is well known that insulin infusion during euglycemic clamp conditions causes increases in sympathetic nerve activity (Anderson and Mark., 1993;Muntzel *et al.*, 1994). If insulin elicits elevated sympathetic neural outflow, then metformin-induced hypo-insulinemia could cause sympathetic withdrawal. In acute studies, however, intravenous injection of metformin caused immediate reductions in renal sympathetic nerve activity, indicating direct effects of the drug that is not secondary to slower changes in plasma insulin. Whether long-term reductions in insulin, by metformin, cause sympathoinhibition has yet to be determined.

In summary, metformin may reduce plasma insulin levels, which, in turn, could lower sympathetic activity and blood pressure. Reductions in insulin are not likely to explain metformin-induced attenuations in vascular reactivity. These putative mechanisms make it critical to measure insulin levels during long-term studies of metformin on cardiovascular system regulation.

Preliminary Studies:

As discussed previously, earlier work by Petersen and colleagues (Petersen and DiBona., 1996) demonstrated that acute metformin injections caused decreases in both blood pressure and renal sympathetic nerve activity in SHRs. To determine whether these decreases were, in fact, secondary to sympathetic withdrawal, Dr. Muntzel's laboratory examined blood pressure responses to metformin in the presence or absence of an intact sympathetic nervous system. They found that whereas β-adrenergic blockade did not affect the hypotensive response, α-adrenergic blockade or ganglionic blockade abolished the acute decrease in blood pressure to intravenous metformin. Furthermore, metformin elicited much greater decreases in blood pressure in SHRs compared with controls, Wistar-Kyoto (WKY) rats. Therefore, these preliminary findings suggest that SHRs are genetically susceptible to the hypotensive actions of metformin, and that acute intravenous injections of the drug lower blood pressure, at least in part, by reducing sympathetic nerve activity.

Although these experiments provide good evidence that acute metformin reduces blood pressure through sympathetic withdrawal, it is unclear whether these findings reflect responses observed during chronic treatment. In fact, this evidence suffers from the same weakness observed in the vascular reactivity studies. Neither the sympathetic activity experiments nor the vascular reactivity studies tested long-term metformin effects on the cardiovascular system. In addition, no previous study has examined the possibility that the drug may simultaneously reduce sympathetic neural outflow and vascular reactivity. Therefore, the current experiments will test whether metformin produces long-term decreases in blood pressure accompanied by decreases in sympathetic nerve activity and vascular smooth muscle reactivity. In addition, we will attempt to determine the relative importance of these two systems in producing the blood pressure fall to metformin.

Preliminary work in my lab using the Data Sciences International (DSI) radiotelemetry system has demonstrated that metformin causes a long-term reduction in blood pressure in unrestrained SHRs. The proposed studies will use this model to investigate the mechanisms of this hypotensive effect.

Research Design and Methods

General Methods

Animals. Male SHRs, weighing 150-175 grams, were purchased from Taconic Laboratories (Germantown, NY). The animals were initially caged in groups of three in a temperature controlled colony room illuminated on a 12:12 light-dark cycle and given a normal 0.3% NaCl diet and tap water *ad libitum* until the start of the experiment. At the time of randomization into their experimental groups, the rats were individually housed and placed on special drinking regimens as specified in the experimental protocol section.

Implantation and use of radiotelemetry devices. Rats were anesthetized by subcutaneous injection of ketamine (40 mg/kg, i.m.) supplemented with xylazine (5 mg/kg, i.m.). The animals were then prepared for surgery using sterile techniques. Briefly, a midline incision was made and the abdominal aorta was carefully exposed caudal to the renal arteries. The aorta was ligated and the tip of the catheter from the radiotelemetry device was inserted through a hole made by a 23 gauge needle. The catheter was fixed to the artery as not to occlude the flow of blood using a polyester disk and tissue-glue. The body of the transducer was fixed to the abdominal wall with three silk sutures. The rats were then be given prophylactic penicillin 60,000 U, i.m. Mean arterial pressure and the heart rate was recorded continuously using a telemetric recording system (Data Sciences International, St. Paul, MN). This system consists of a radiotelemetry device (model TA11PA-C40), a receiver (model RLA1020), and a calibrated pressure analog adapter (model R11CPA) connected to a MacLab data acquisition system interfaced with a Macintosh computer.

Implantation of vascular catheters. For implantations of vascular catheters, rats were anesthetized with ketamine supplemented with xylazine or urethane. In certain experimental groups, a polyethylene catheter was inserted into the left jugular vein for drug administration, in other groups, a catheter was inserted into the carotid artery for collection of blood samples, and finally, other groups received both arterial and venous catheters. The free ends of the catheters were routed subcutaneously to exit through the dorsal surface of the neck. They were secured to the skull with stainless steel screws and dental acrylic, and then passed through a lightweight flexible spring attached to a 23 gauge hydraulic swivel. The tubing was filled with heparinized saline (75 U/ml) and plugged with stainless steel wire. Experiments were carried out 5 days following catheter instrumentation.

Catecholamine extraction from blood samples. Catecholamines were extracted from the blood samples with the aid of an Alpco catecholamine extraction kit. Both control (a known amount of catecholamine) and blood samples went through the extraction procedure. Once the extraction was completed, the samples were immediately injected into the HPLC-EC detector.

High pressure liquid chromatography (HPLC). Quantitative and qualitative catecholamine analysis of the extraction was analyzed by HPLC-EC detection. The internal standard, Epinine (Epinine; 200 pg) and 0.5 U of ascorbate oxidase were added to each sample before injecting it on to the HPLC.

Ascorbate oxidase clears the solvent front of ascorbic acid, and its presence does not affect the monoamines. The HPLC-EC included a reverse-phase ODS column (C18- 5μm particle size, Bioanalytical Systems) maintained at 32 °C, a Gynkotek HPLC Pump Series P 850, WISP 710 autoinjector, Decade electrochemical detector, and a Model 720 Data Module. The VT-03 electrochemical flowcell was set at a potential of + 0.70 volts versus a Ag/AgCl reference electrode. The mobile phase was a modification of that used by Kapoor and Chalmers (Kapoor and Chalmers., 1987) for hypothalamic dialysis and consisted of filtered, degassed 0.1 M KH_2PO_4 (pH to 3.17 using 9N citric acid) containing 0.3 mM EDTA, 0.5 mM octyl sodium sulfate and 7% methanol delivered at a flow rate of 1.3 ml/min. To establish the identity of peaks in the samples and to quantitate the amount of monoamine in each peak, retention times and peak heights were compared to those of known mixture of monoamine standards (100 pg of each monoamine). The height of the epinine peak in each sample was used to correct for any deterioration of monoamines between the time of sample collection and analysis by HPLC-EC. Data for each identified monoamine in each sample was converted to pg/fraction. The mixture of monoamine standards was run at the beginning and end of each day since peak retention times shift slightly over the course of the day.

Data Analysis. Most of the procedures described below have straightforward two-factor completely randomized designs. Results from the experimental groups were evaluated using sample Analysis of Variance (ANOVA), repeated-measures Analysis of Variance, and appropriate post-hoc tests (i.e. Tukey's) when necessary. Differences between groups were considered statistically significant at values of $p < 0.05$ level. Established software packages (SUPERANOVA) were used to perform these analyses. Analog values of heart rate and blood pressure were digitized on-line at a rate of 100 Hz by the MacLab data acquisition system.

For the catecholamine studies a one-way ANOVA was used to compare the control versus metformin-treated groups. The ganglionic blockade studies utilized a one, two and three-factor designs with repeated measures when necessary, for data collected during the 18 days of vehicle or metformin treated rats. The second factor is the time (diurnal) of observation and it was used for the measurements of body weight, heart rate, and blood pressure. The third factor was days of observation. For analysis of the blood pressure fall to ganglionic blockade, a one-way ANOVA was adequate. Responses to phenylephrine and angiotensin II were evaluated using 2 (groups) by 3 (doses) ANOVA with repeated measures for doses.

Cathecholamine level detection--Procedure. Upon arrival, all rats were placed on a 0.3% normal NaCl diet (Harlan-Teklad, Madison, WI) and given drinking water ad libitum. After a week of habituation, the rats were randomized into two experimental groups: the control group received normal water and a 0.3% NaCl diet, and the metformin group received the same diet and metformin (500 mg/kg/day) in the drinking water. Body weight and fluid intake were recorded continuously in all rats for a period of 23 days.

After the 18th day of vehicle or metformin treatment, arterial catheters were implanted and the rats were allowed two days recovery period. The rats were tethered for approximately 3 hours and were familiarized with the home cages to avoid problems of transposition. These precautions were necessary to provide blood pressure and plasma catecholamine measurements during non-stress conditions.

On the 3rd day following catheter implantation, the rats were attached to the overhead tether, and were allowed 2 hours of rest before data collection. Following this habituation period, blood pressure and heart rate were sampled for one hour using a Statham P23XL pressure transducer coupled to a model 7E polygraph (Grass Instruments, Quincy, MA) and to a Macintosh computer equipped with the MacLab data acquisition system. These data provided correlation between blood pressure and plasma catecholamines.

On the 4th day following catheter implantation, the rats were attached to the overhead tether and to a withdrawing syringe. The animals were allowed 2 hours of habituation before blood sampling. All blood collections were made between 3 and 5 pm to avoid the confounding influence of diurnal variations in plasma NE and Epi. Blood samples (1000 μl) were collected from the arterial catheter and placed in chilled test tubes containing EGTA and reduced glutathione. The samples were spun (~12000 rpm) at 4°C for 15 min and the plasma was stored at –20°C until analysis. Plasma NE and Epi were estimated by high-performance liquid chromatography with electrochemical detection.

At the end of the 4th day, the rats were put on an over-night fast. Then, on the 5th day, arterial blood samples (200 μl) were again collected, processed, and analyzed for plasma insulin levels using a rat insulin enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Ganglionic blockade – Procedure. After a week of habituation, a new group of SHRs underwent surgery for implantation of the radiotelemetry devices. The animals were allowed one week of post-surgery recovery followed by a week-long baseline period, during which body weight and water intake were monitored daily. In addition, blood pressure and heart rate was recorded continuously using the Data Sciences telemetry and MacLab interface. At the end of the baseline period, the rats were randomized into two experimental groups: the control group received normal water and a 0.3% NaCl diet, and the metformin group received metformin (500 mg/kg/day) in the drinking water and a 0.3% NaCl diet. Body weight, fluid intake, heart rate, and blood pressure were recorded continuously in all rats for 18 days.

To assess the sympathetic contribution to changes in blood pressure, ganglionic blockade was performed after the initial 18-day treatment period. To do this, venous catheters were implanted and the animals were allowed three days of recovery and habituation to the overhead tethering. The effectiveness of ganglionic blockade was evaluated by observing heart rate baroreceptor responses to phenylephrine before and after hexamethonium. Pre-blockade baroreflex sensitivity was ascertained on the fourth day by injecting 0.5 μg/kg phenylephrine and recording the blood pressure and heart rate responses using the radiotelemetry system. On the fifth post-surgery day, the venous catheter was attached to the hexamethonium syringe and the animal was permitted to sit quietly for two hours. Hexamethonium (30 mg/kg, iv) was injected as a bolus and the blood pressure and heart rate responses were determined by injecting 0.5 µg/kg phenylephrine. Complete blockade following hexamethonium is expected to abolish the baroreceptor-mediated bradycardia observed during phenylephrine-induced blood pressure elevations.

Pulsed Doppler Blood Flow Measurement -- Procedure: Detection of flow with the pulsed Doppler system is dependent on changes in the emitted ultrasonic frequency caused by the reflection of the signal moving off blood cells. The change in frequency, referred to as Doppler shift is expressed as a change in voltage on the polygraph and is proportional to the velocity of the blood cells in the vessel, given that the angle of the crystal to the vessel and the velocity of sound through the fluids and surrounding tissue remain constant. Then, assuming the cross-sectional area of the vessel adjacent to the probe remains constant, the Doppler shift is also proportional to the actual blood flow to the organ. This linear relationship between volume flow and Doppler shift exists for both continuous-wave Doppler flowmeter and the pulsed flowmeter. However, differences may exist from animal to animal in the actual Doppler shift for a given blood flow. This may be related to the orientation of the probe to the vessel, of the intrinsic characteristics of each probe.

Vascular reactivity -- Procedure. New groups of SHRs were placed into control and metformin groups as described above for a total of 23 days. At the end of this period, they were anesthetized with ketamine and xylazine and implanted with jugular arterial and venous catheters. Immediately after catheterization, a midline laparotomy was performed, and miniature pulsed Doppler flow probes were placed around the left renal artery, and the right iliac artery. The probes were sutured in place; the leads from the flow probes and catheters were tunneled subcutaneously and exteriorized between the nape. To protect the probe wires and catheter tubing while allowing animals unrestricted movement during experimental testing, the free ends of the catheters and Doppler leads were anchored to the lead and led through the spring coil tether assembly described in the general methods section. The wire leads from the flow probes were soldered to an ultraminiature receptacle, which was then anchored to the skull using the dental cement also described in the general methods. An ultraminiature plug with shielded miniature twisted pair cables was connected the Doppler flow meter to the flow-probe wires. The rats were allowed three days of recovery before cardiovascular testing.

Blood pressure and regional vascular reactivity was assessed in rats before and after ganglionic blockade to remove the confounding influences of baroreceptor responses to transient increases in blood pressure. Three doses each of phenylephrine (0.5, 1.0, and 2.0 μg/kg) and angiotensin II (2, 5, and 8 ng/kg) were administered to evaluate the possibility that metformin reduces blood pressure, heart rate, and regional vascular reactivity to α_1-adrenergic stimulation or to angiotensin II receptor stimulation. They were then given hexamethonium (30 mg/kg) and tested once more with phenylephrine and angiotensin II.

Sufficient time was allowed between injections for the recorded parameters to return to control levels. These data were recorded continuously before and during drug injection using the MacLab and Macintosh computer interface.

Results

Metformin lowered Blood Pressure

SHRs were given metformin treatment or vehicle beginning at 3 wks of age. After twenty-three days of continuous treatment, the animals underwent surgery to implant the radiotelemetry devices. Following a week of recovery, mean arterial blood pressure (MAP) was monitored for 4 days. As shown in figure 1, a 3-way analysis of variance (ANOVA) (Drug*Diurnal variation*Days) revealed that metformin lowered blood pressure in SHRs by 10 mmHg (main effect: F 1,8 = 9.503, $p<0.05$). Blood pressure values during the entire experiment showed marked diurnal oscillations (figures 2 & 3) with a significant increase at night (main effect diurnal: F 1,8 = 18.156, $p<0.05$). Moreover, a main effect for the 4 days of monitoring was also significant (F 3,24= 3.59, $p < 0.05$) (Figure 4 & 5). These data reflect a gradual fall in blood pressure over the 4 days of observed in both control and metformin groups that is independent of drug. There were no interactions.

Metformin lowered Heart Rate

Figure 6 shows metformin's heart rate lowering effects in SHRs monitored for 24-hour intervals that continued for 4 days. A 3-way ANOVA (Drug*Diurnal variation*Days) revealed a significant (10 bpm) fall in HR due to metformin (main effect drug: F 1,8 = 17.167, $p <0.05$). However, when day and night HR analyzed separately, the analysis revealed a slight but not significant HR lowering by metformin treatment. As in blood pressure, it was also observed

Figure 1:

4 day 24-hr MAP response in conscious rats.

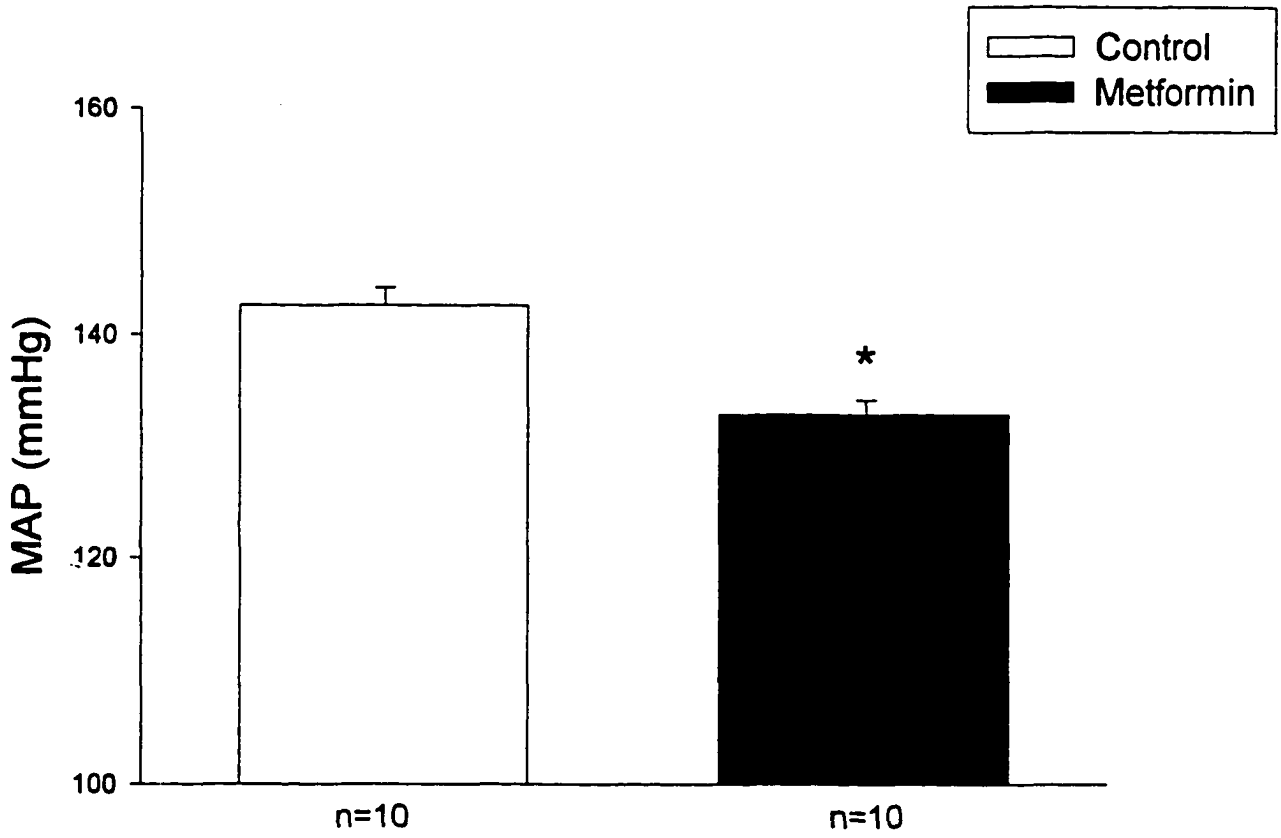

Control
Metformin
160
140
120
100
MAP (mmHg)
*
n=10
n=10

Figure 2:

4 day light cycle MAP response in conscious rats.

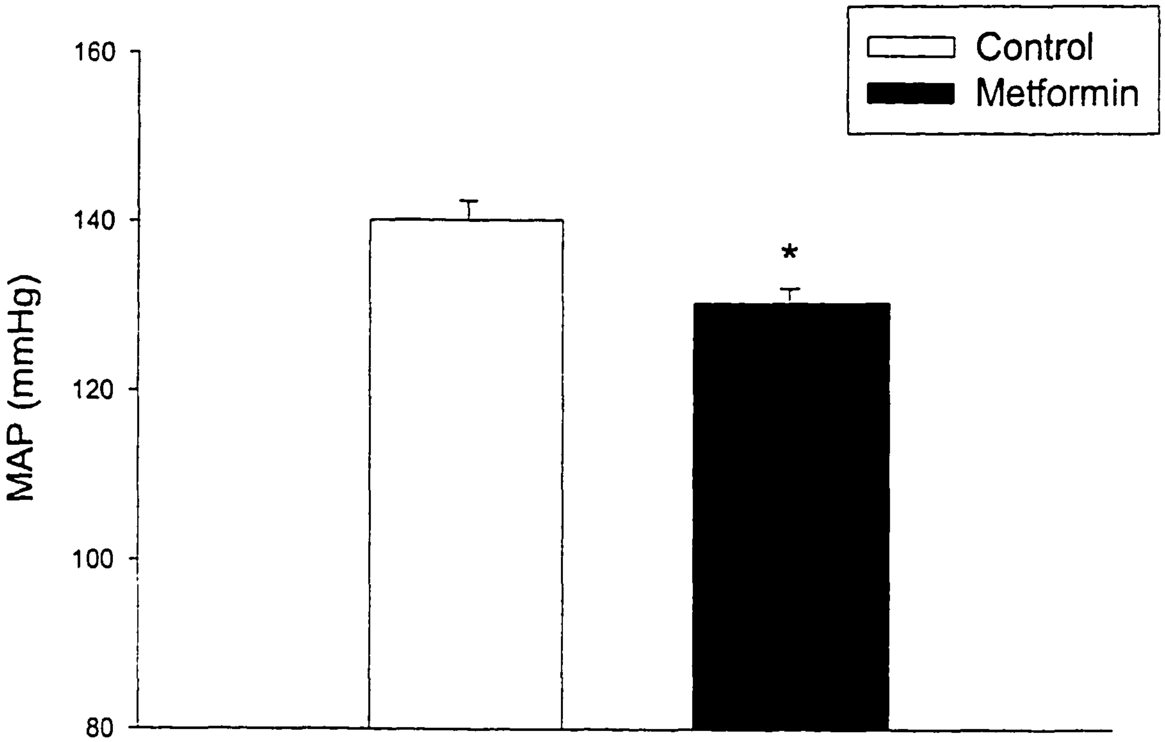

160
140
120
100
80
MAP (mmHg)
*
Control
Metformin

Figure 3:

4 day dark cycle MAP response in conscious rats.

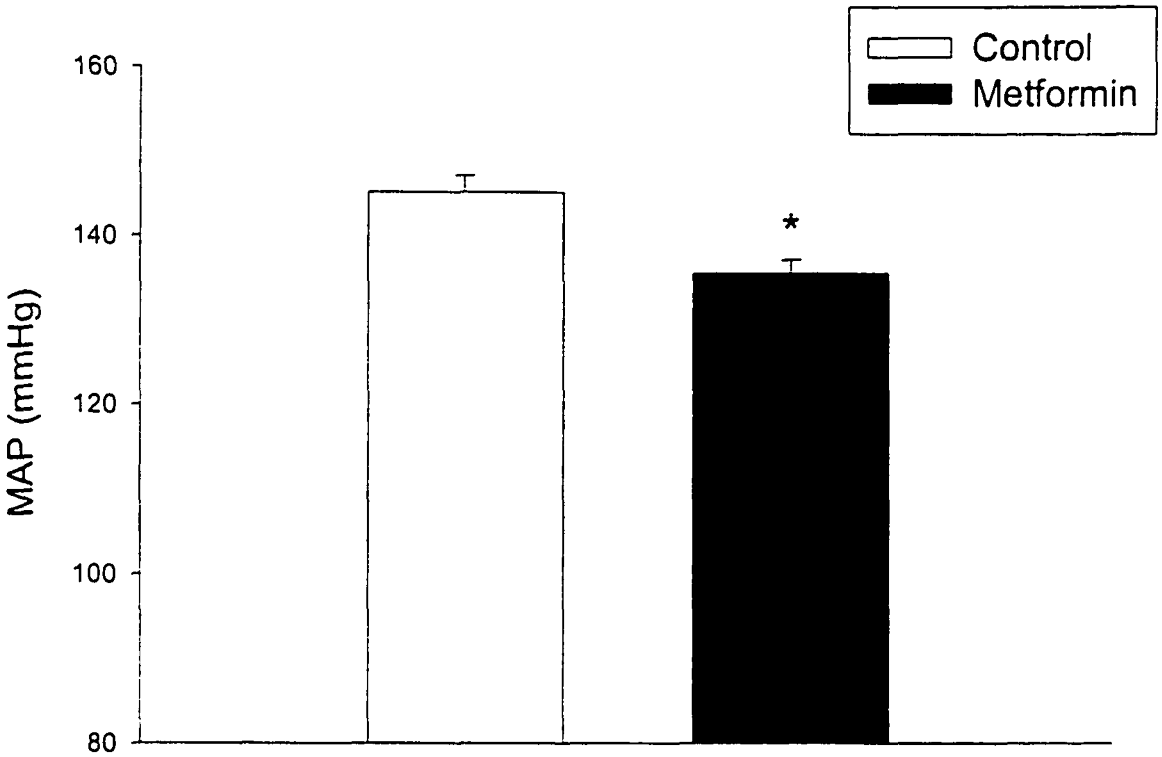
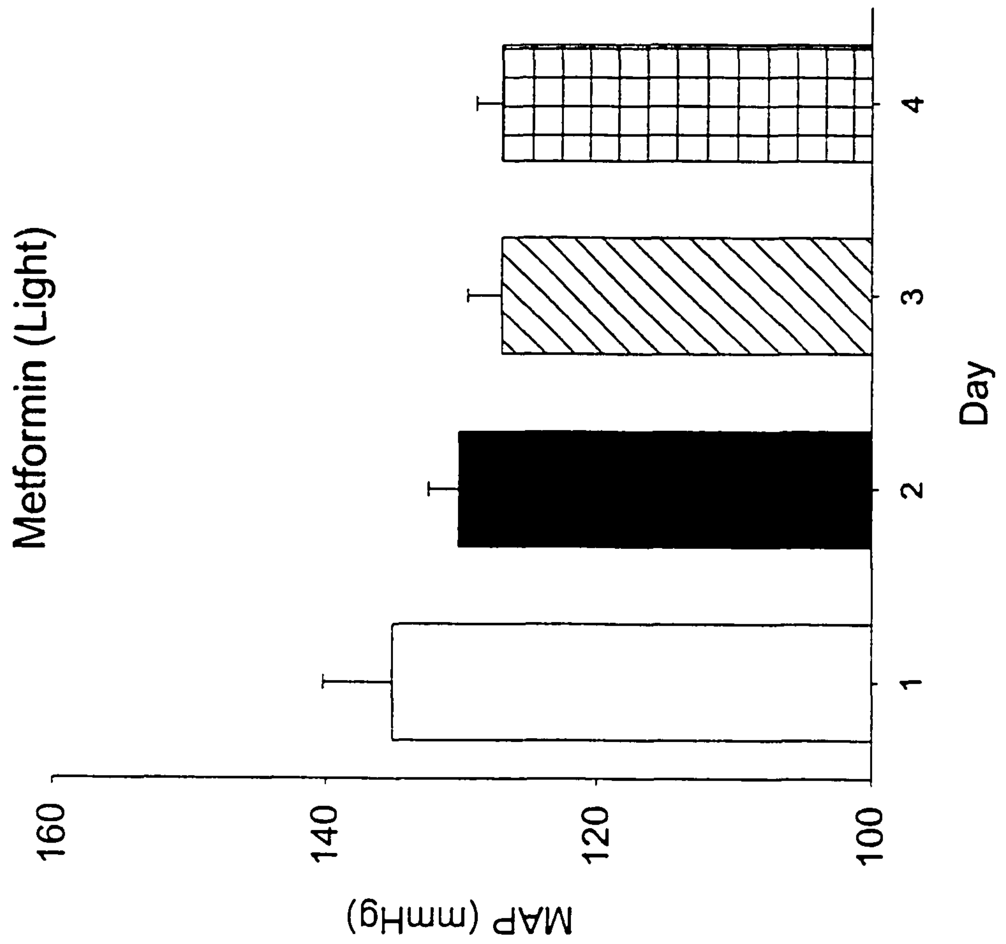

Control
Metformin
160
140
120
100
80
MAP (mmHg)
*

Figure 4:

4 day day-time MAP response in conscious rats.


Metformin (Light)
MAP (mmHg)
160
140
120
100
1
2
3
4
Day

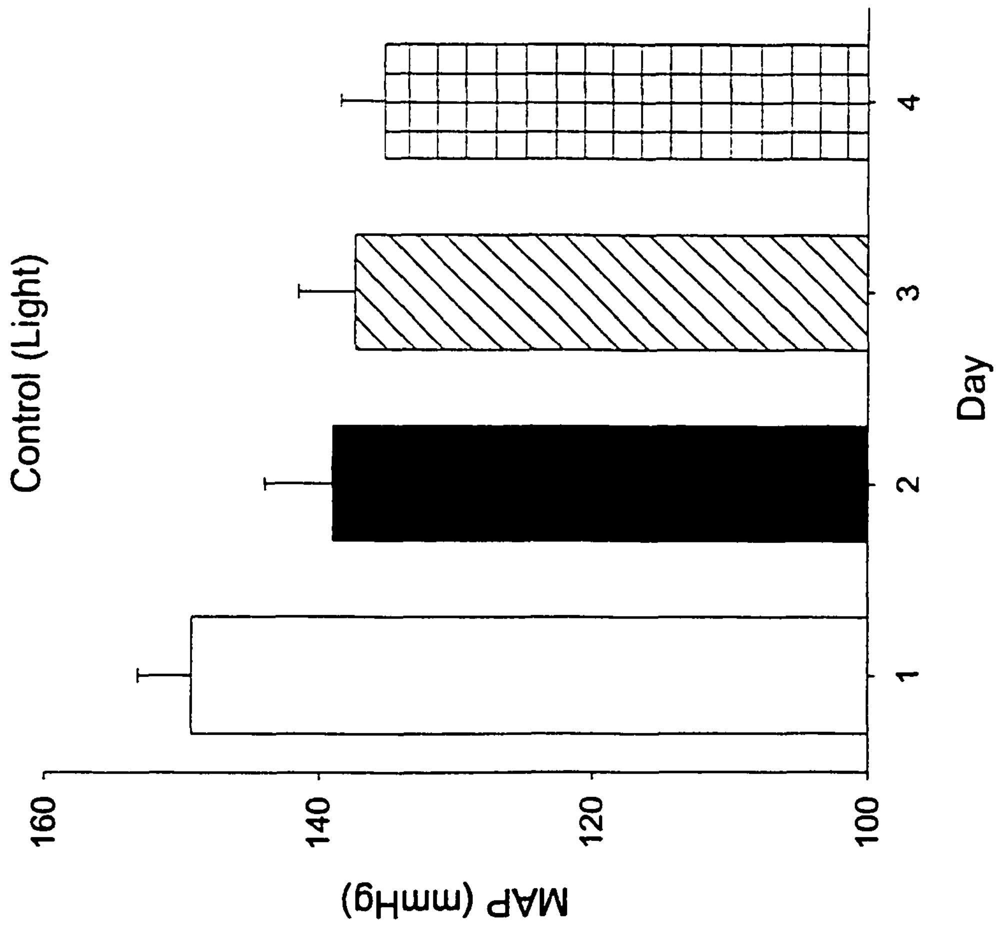
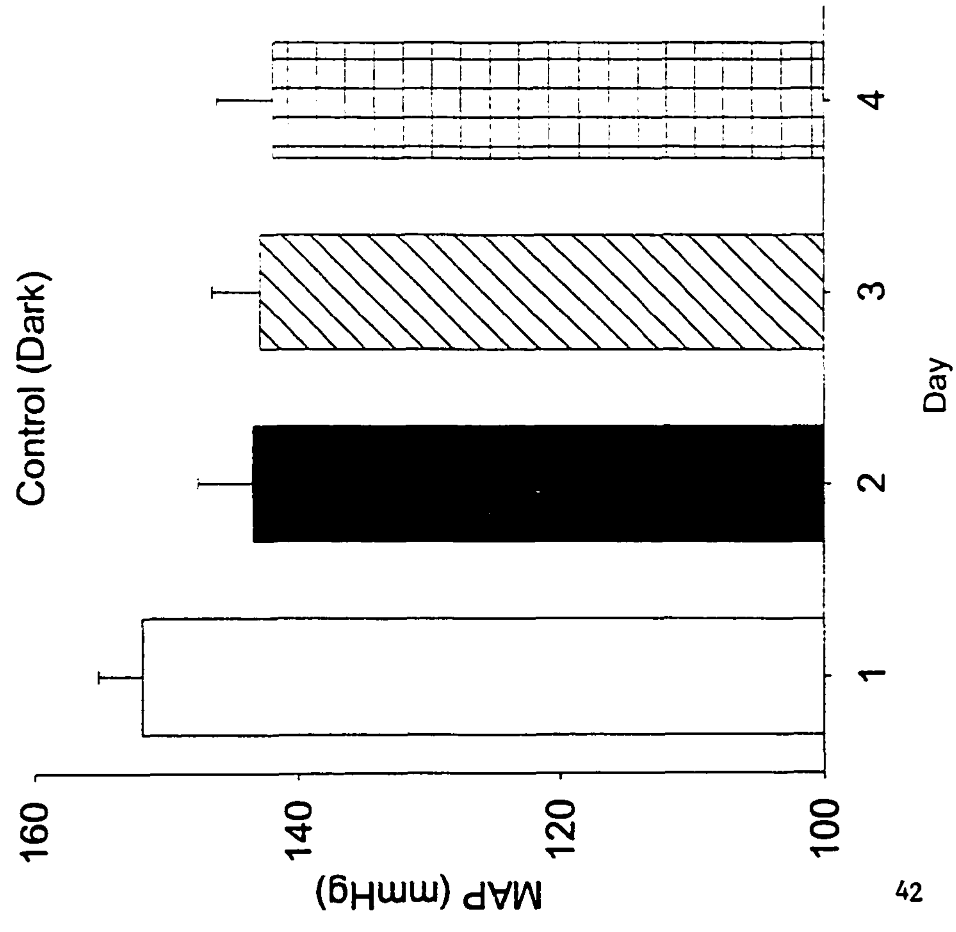

Control (Light)
MAP (mmHg)
160
140
120
100
1
2
3
4
Day

Figure 5:

4 day night-time MAP response in conscious rats.


Control (Dark)
MAP (mmHg)
160
140
120
100
1
2
3
4
Day

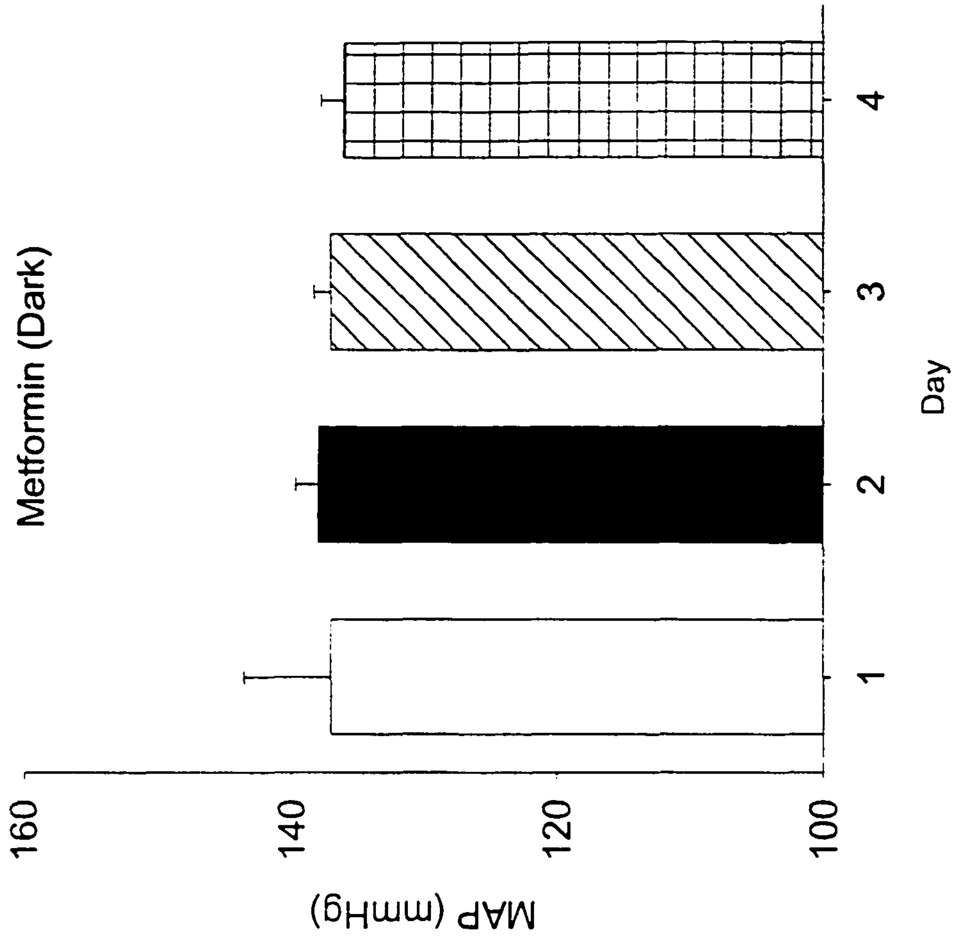
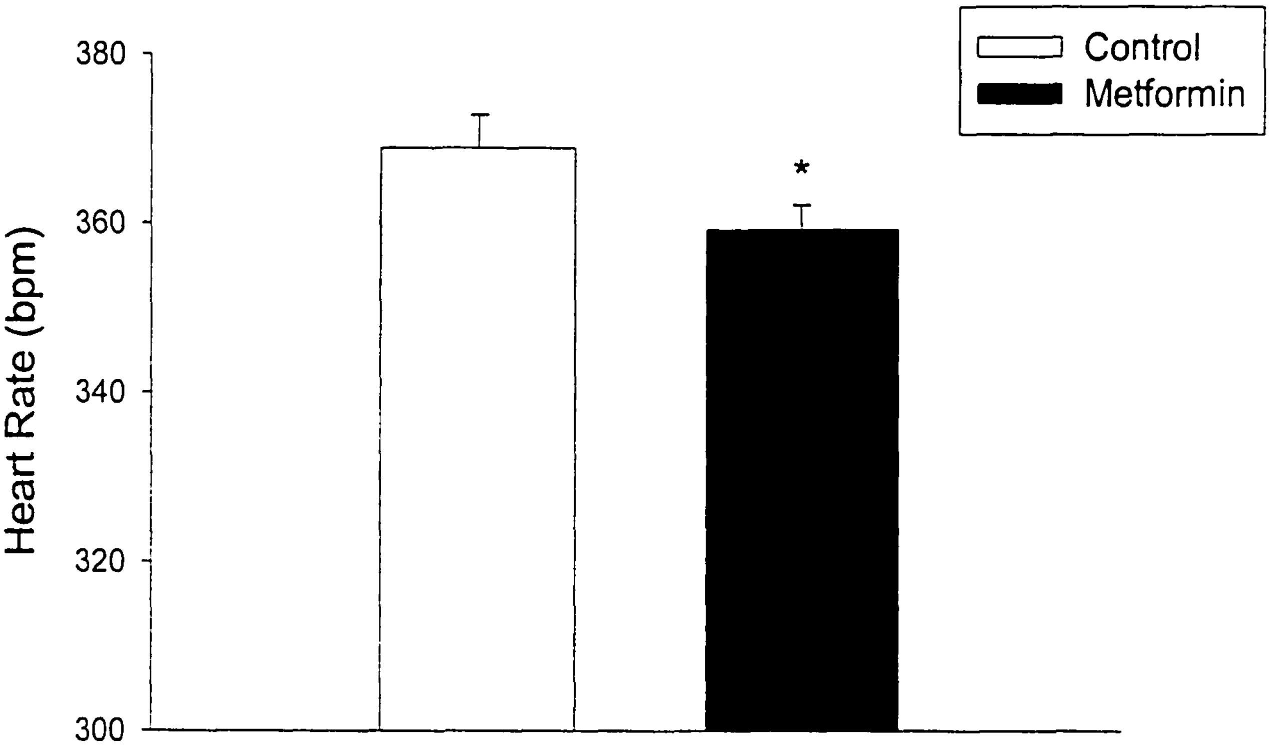

Metformin (Dark)
MAP (mmHg)
160
140
120
100
1
2
3
4
Day

Figure 6:

4 day 24-hr Heart Rate response in conscious rats.


380
360
340
320
300
Heart Rate (bpm)
*
Control
Metformin

throughout the entire experiment that there were marked diurnal oscillations. Figures 7 and 8 show a main diurnal effect that was significant, reflecting high HRs at night compared with day (F 1,8 = 6.4, $p < 0.05$). A main effect for the 24-hr/4 days of monitoring was not significant, reflecting no decrease in HR over the 4 days of monitoring.

Metformin had no effect on Body Weight

Figure 9 shows that metformin did not have a significant effect on body weight. A 2-way ANOVA (Drug*Days) demonstrated no significant main drug effect. The metformin treated animals continued to gain weight at the same rate that the control animals gained weight, as demonstrated by the main effect for days of metformin treatment (F 3,3 = 0.057, $p > 0.05$).

Figure 7:

4 day day-time Heart Rate response in conscious rats.

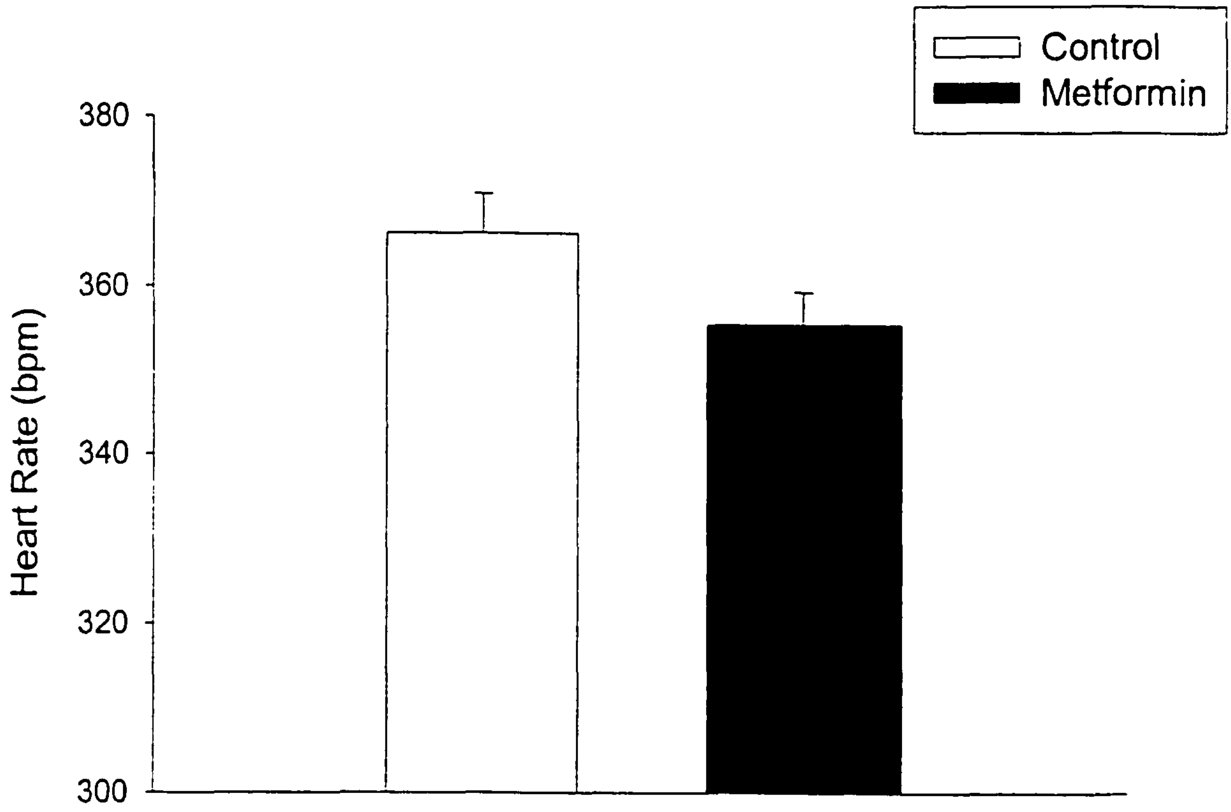

Control
Metformin
Heart Rate (bpm)
380
360
340
320
300

Figure 8:

4 day night-time Heart Rate response in conscious rats.

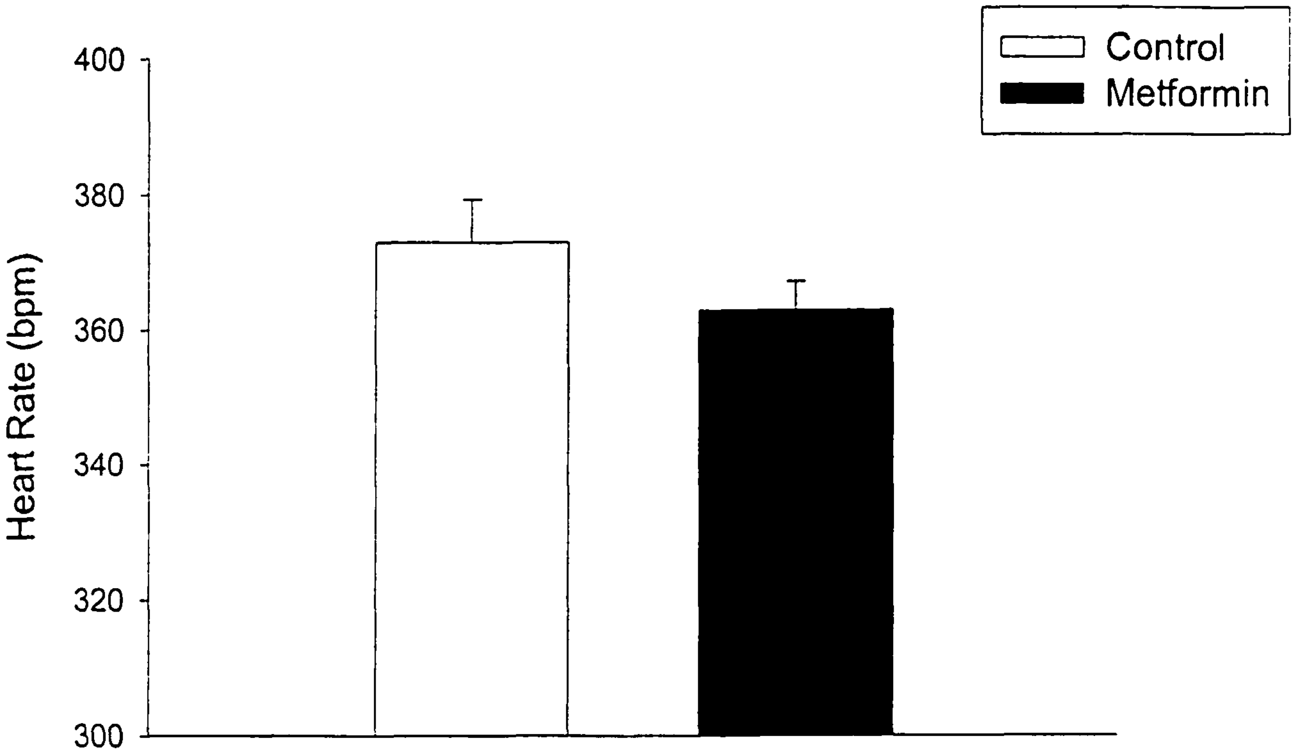
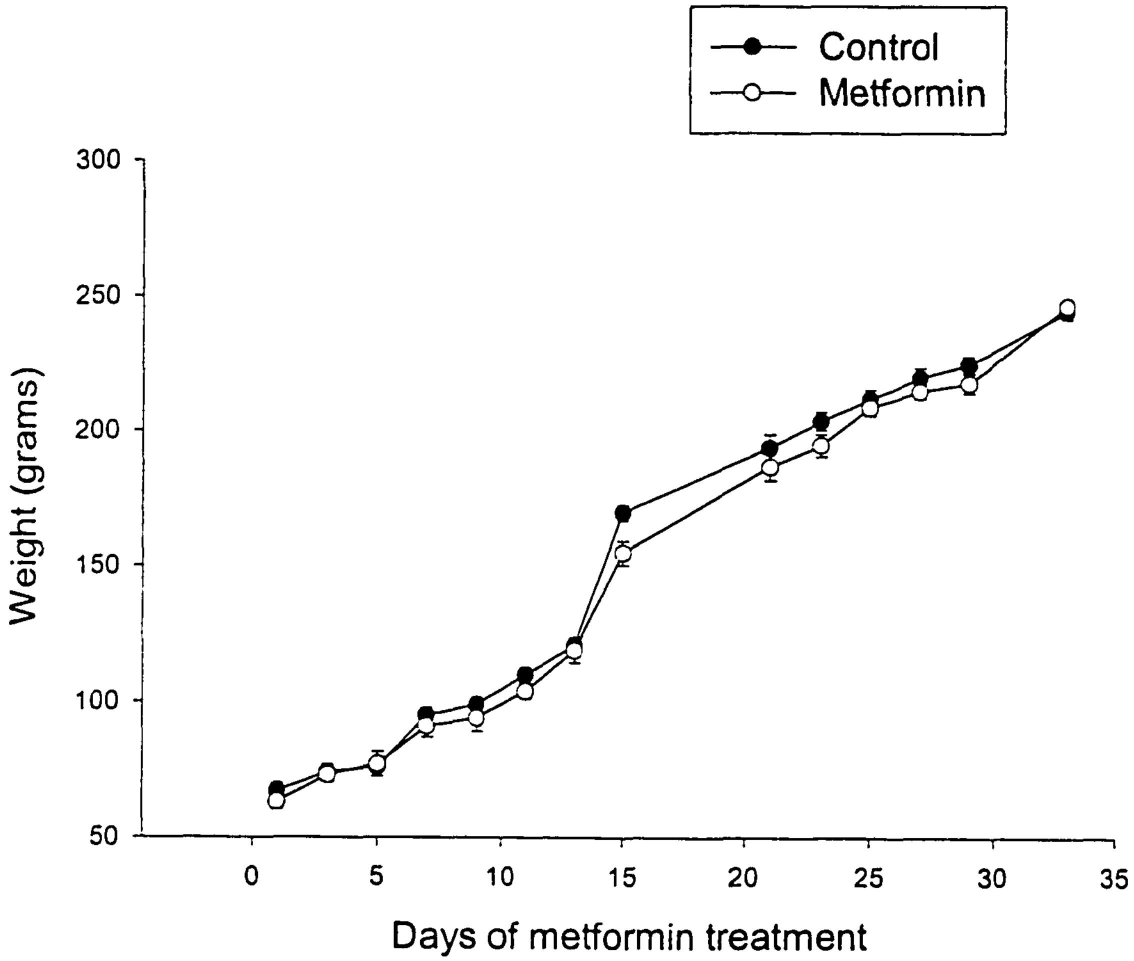

Control
Metformin
Heart Rate (bpm)
400
380
360
340
320
300

Figure 9:

Chronically administered metformin has no effect on weight.


Control
Metformin
300
250
200
150
100
50
Weight (grams)
0
5
10
15
20
25
30
35
Days of metformin treatment

Metformin changed Plasma Catecholamines levels

Blood taken from chronically metformin treated animals was collected a week after implantation of a catheter into the carotid artery. Figure 10a a representative graph of the NE plasma collection. Figure 10b shows that metformin lowered the concentration of NE found in plasma. Figure 11 shows Epi plasma concentration increases mediated by metformin. Figure 12 shows the concentration of plasma Dop which was not affected by metformin treatment. For NE, a 1 way-ANOVA revealed a significant main effect for drug (F 1,16 = 110.04, $p < 0.0001$). For Epi, a 1 way-ANOVA revealed a significant main drug effect (F 1,16 = 8.02, $p < 0.05$). However, for Dop, a 1 way-ANOVA revealed no main drug effect.

Metformin had no effect on Blood Pressure when Hexamethonium bromide (Hex) was used

The blood pressure response to Hex was utilized as an indirect measure of basal sympathetic activity contributing to vasoconstrictor tone. When rats are infused with Hex, an initial maximal fall in pressure is usually observed followed by a slightly higher plateau phase during which blood pressure is stable for many minutes. This plateau phase generated the data that are depicted in figure 13. In the present experimental setting, the initial maximal fall (nadir) was not observed. The metformin treated group showed a fall to the ganglionic blockade that was slightly attenuated but not significantly different from the control group. A 1-way ANOVA revealed no significant difference due to a main effect of drug.

Figure 10a:

Representative chromatogram of NE plasma levels.

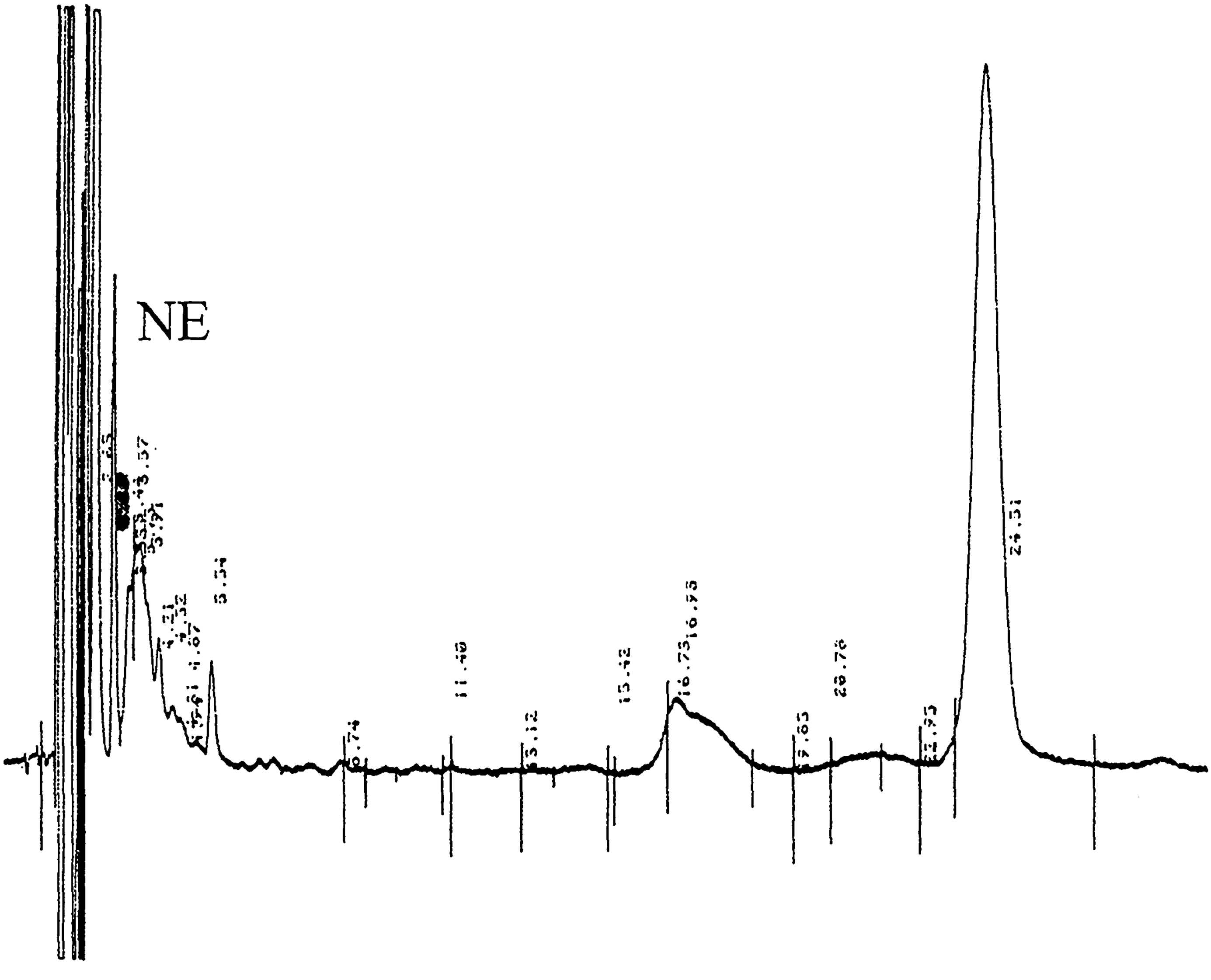

NE
3.57
3.71
4.21
4.32
4.67
5.54
11.40
13.12
15.42
16.73
16.93
20.76
24.51

Figure 10b:

Effects of metformin on plasma NE concentration.

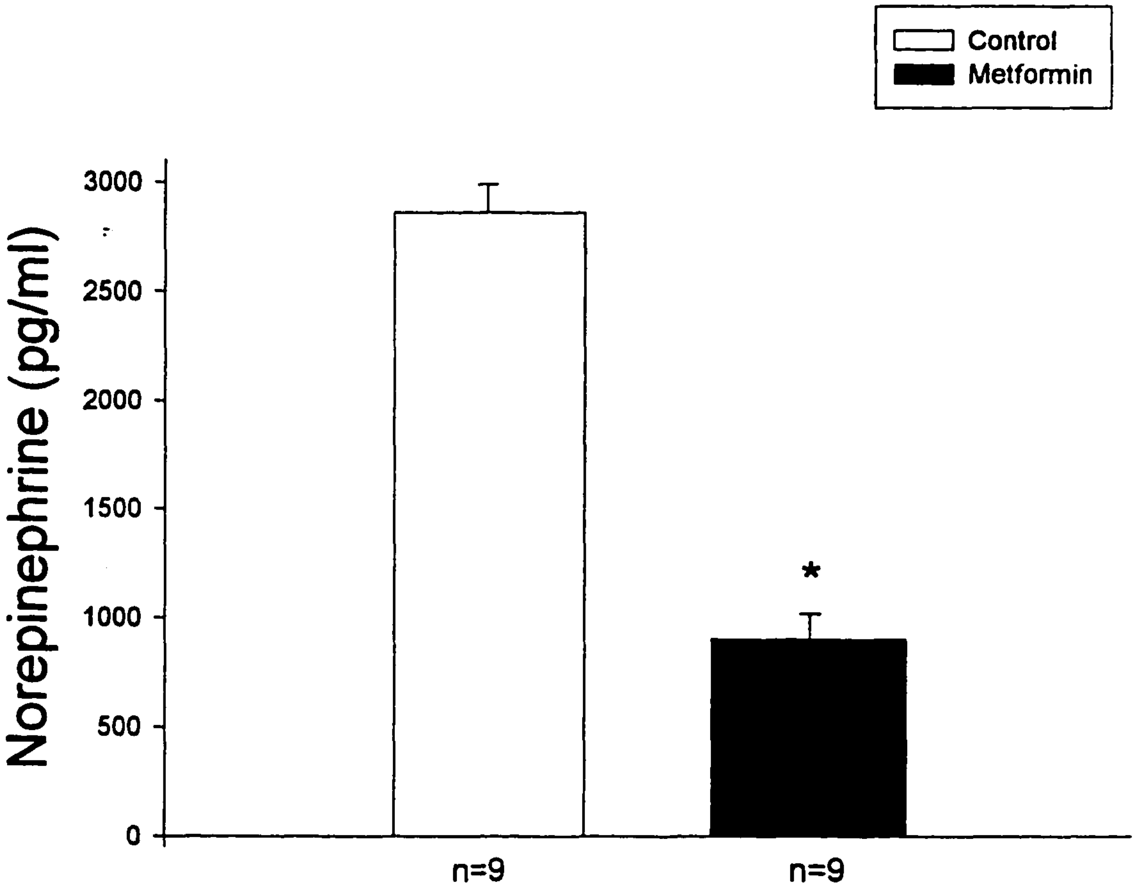
Control
Metformin
Norepinephrine (pg/ml)
3000
2500
2000
1500
1000
500
0
*
n=9
n=9

Figure 11:

Effects of metformin on plasma Epi concentrations.

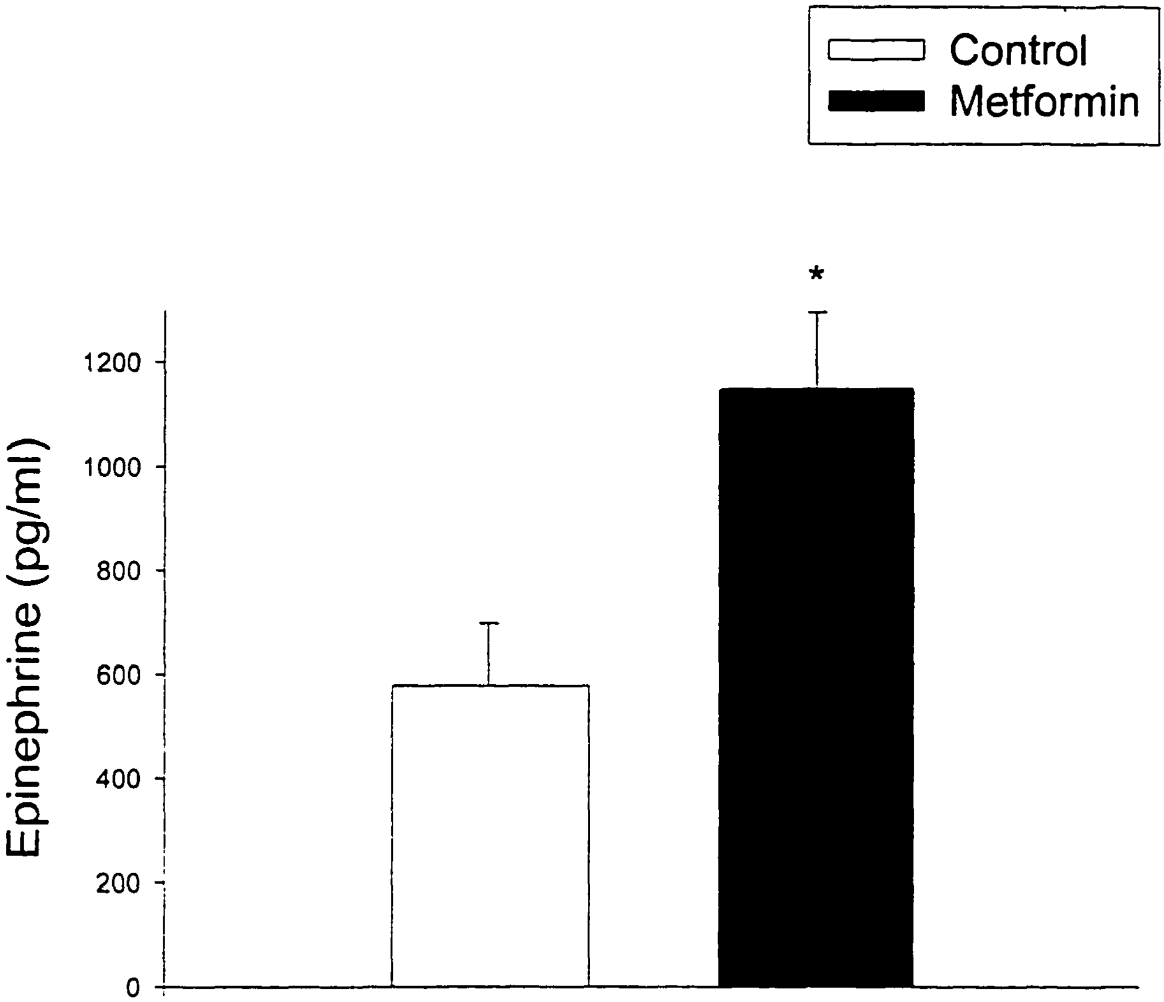

Control
Metformin
*
Epinephrine (pg/ml)
1200
1000
800
600
400
200
0

Figure 12:

Effects of metformin on plasma

Dop concentrations.

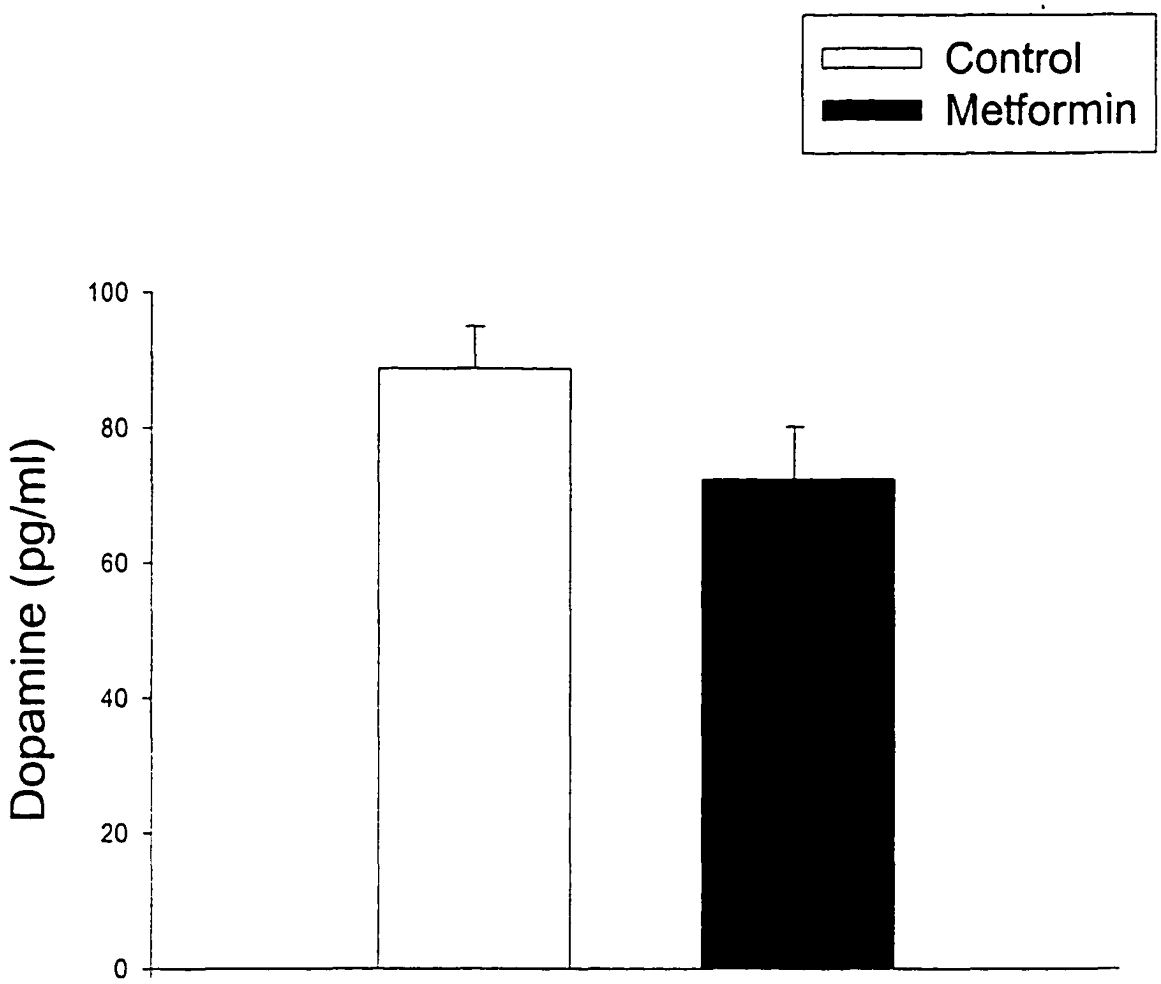
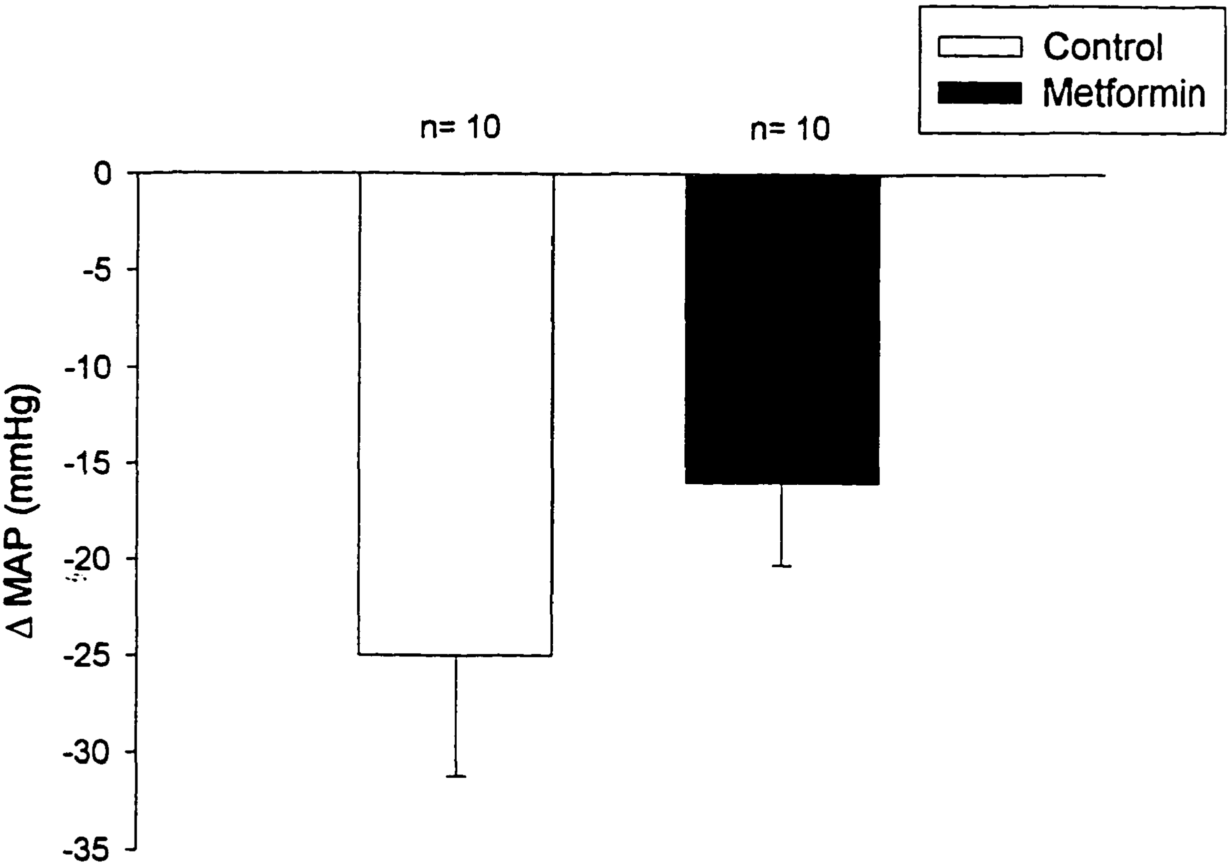

Control
Metformin
Dopamine (pg/ml)
100
80
60
40
20
0

Figure 13:

Effects of metformin on MAP decline to ganglionic blockade.


Control
Metformin
n= 10
n= 10
0
-5
-10
-15
-20
-25
-30
-35
Δ MAP (mmHg)

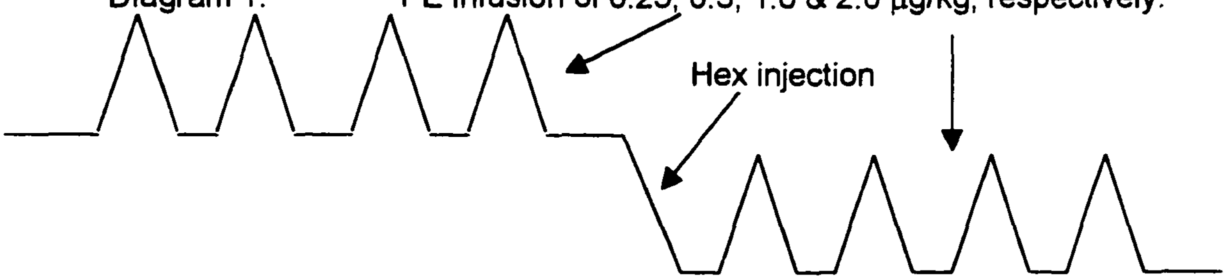
Metformin had no effect on Basal blood pressure but lowered heart rate in urethane-anesthetized SHR rats

Chronically metformin or vehicle treated animals underwent surgery to implant catheters in the carotid artery and jugular vein. Basal MAP in these anesthetized animals showed no effect of metformin. As shown in figure 14, a 1-way ANOVA revealed that the basal MAP values were not significantly lower due to a main drug effect. As shown in figure 15, basal HR was lowered in the unconscious rats by metformin treatment. A 1-way ANOVA revealed a significant lowering of HR due to a main drug effect (F 1,10 = 14.95, $p < 0.01$).

Metformin had no effect on Blood Pressure response to phenylephrine (PE) in urethane-anesthetized SHR rats

Chronically metformin and vehicle treated animals were given bolus injections of PE at four concentrations (0.25, 0.5, 1.0 & 2.0 µg/kg). After the animals were injected with PE, bolus injections of Hex were administered. Hex, a nicotinic ganglionic antagonist, abolishes sympathetic and parasympathetic postganglionic nerve activity. The Hex injection was followed by injecting the same four PE doses (Refer to diagram 1).

Diagram 1:



PE infusion of 0.25, 0.5, 1.0 & 2.0 µg/kg, respectively.

Hex injection

Figure 14:

Effects of metformin on MAP

in unconscious rats.

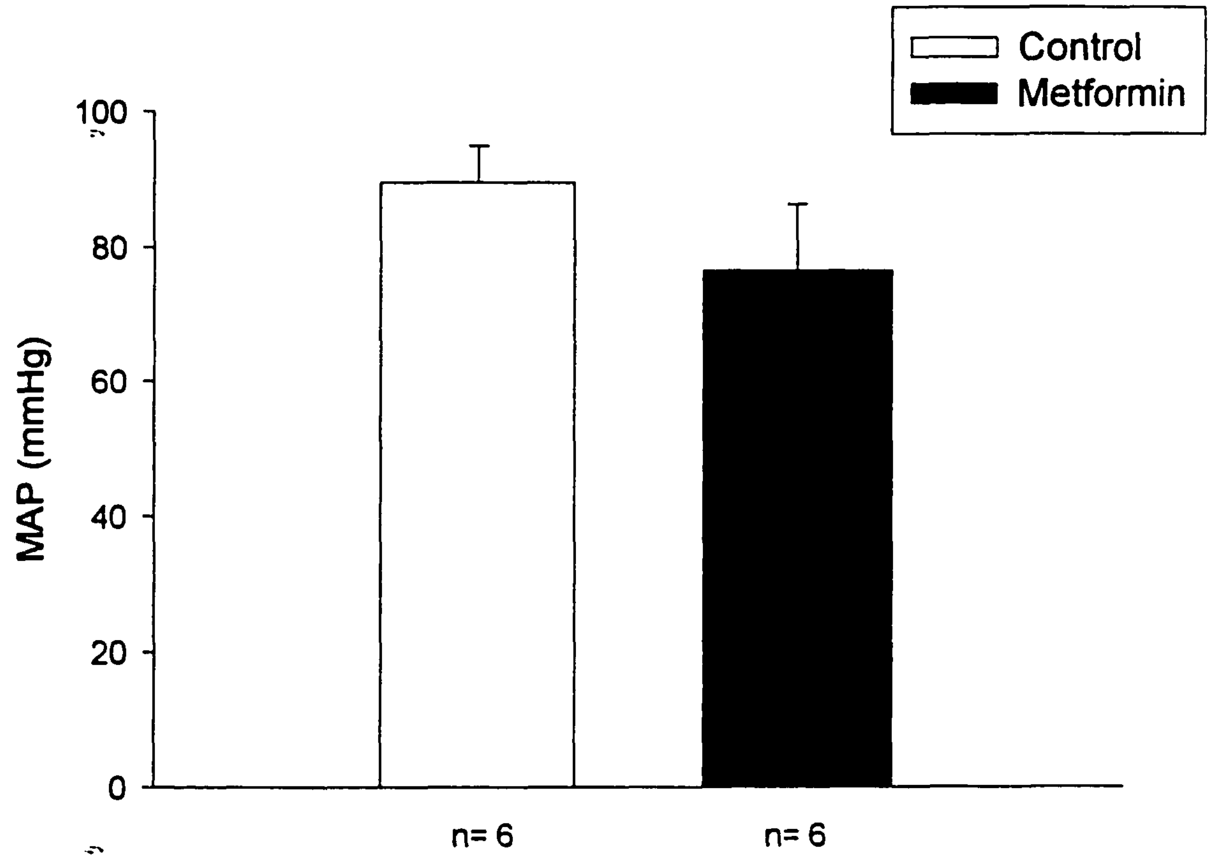
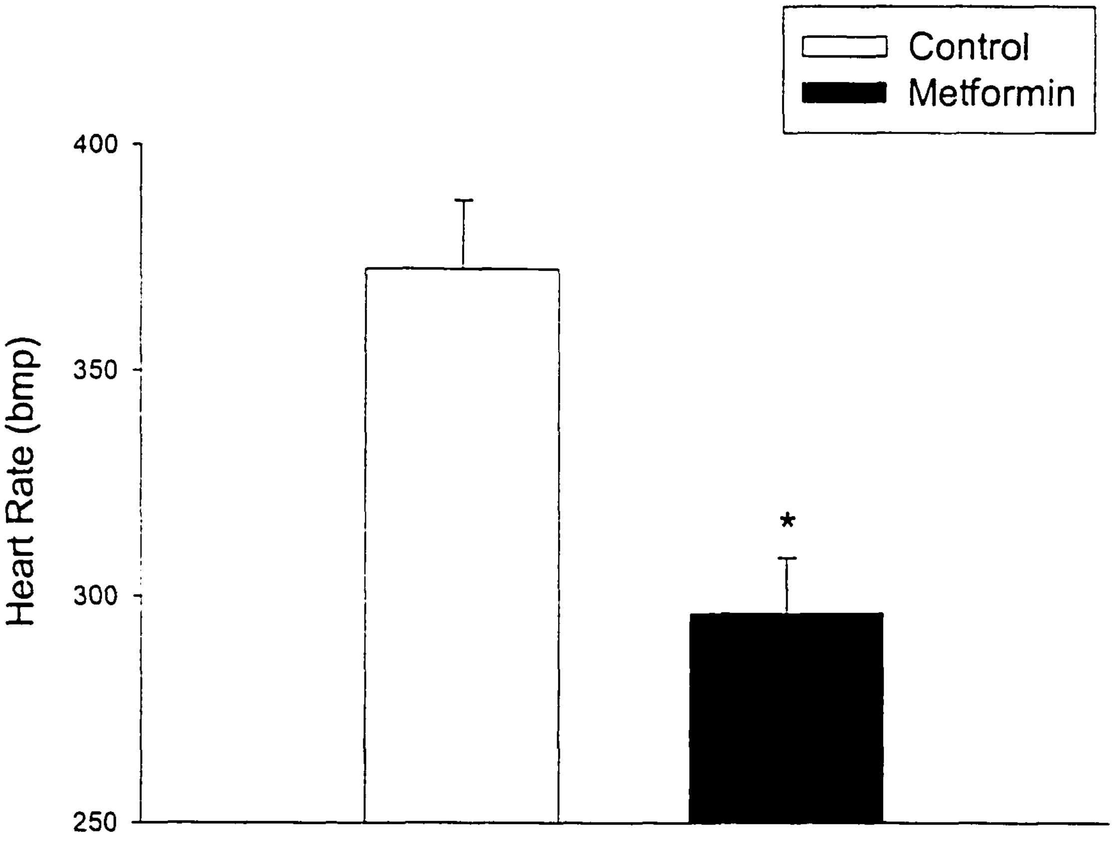

Control
Metformin
MAP (mmHg)
100
80
60
40
20
0
n= 6
n= 6

Figure 15:

Effect of metformin on Heart Rate in unconscious rats.

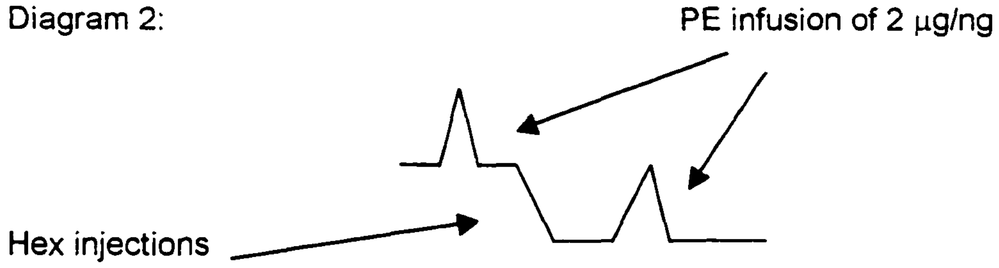

Control
Metformin
Heart Rate (bmp)
400
350
300
250
*

As shown in figure 16, a 3-way ANOVA (Drug*Hex treatment*Concentration) revealed that metformin increased MAP responses to PE both before and after Hex treatment ($F_{1,10} = 18.3$, $p < 0.05$). MAP responses to PE were significantly higher after treatment with Hex independent of drug ($F_{1,10} = 12.8$, $p < 0.05$). There was also a significant dose response to PE ($F_{3,30} = 55.3$, $p < 0.05$).

Metformin had no effect on Blood pressure response to PE in conscious SHR rats

In the conscious BP studies discussed previously, the animals were challenged with PE in order to compare conscious vs. unconscious responses. Conscious SHRs were challenged with a bolus injection of PE (2 µg/kg) followed by a Hex bolus injection and then a second PE (2 µg/kg) bolus injection (Refer to diagram 2). As shown in figure 17, metformin did not affect the MAP responses in conscious animals.

Diagram 2:



A 2-way ANOVA (Drug*Hex treatment) revealed that there was no significant change in MAP responses due to a main drug effect. There was a significant effect in MAP response due to Hex treatment. Once the animals were treated with Hex, the MAP response was significantly higher in both metformin

Figure 16:

Blood pressure response to phenylephrine in unconscious control & metformin treated rats.

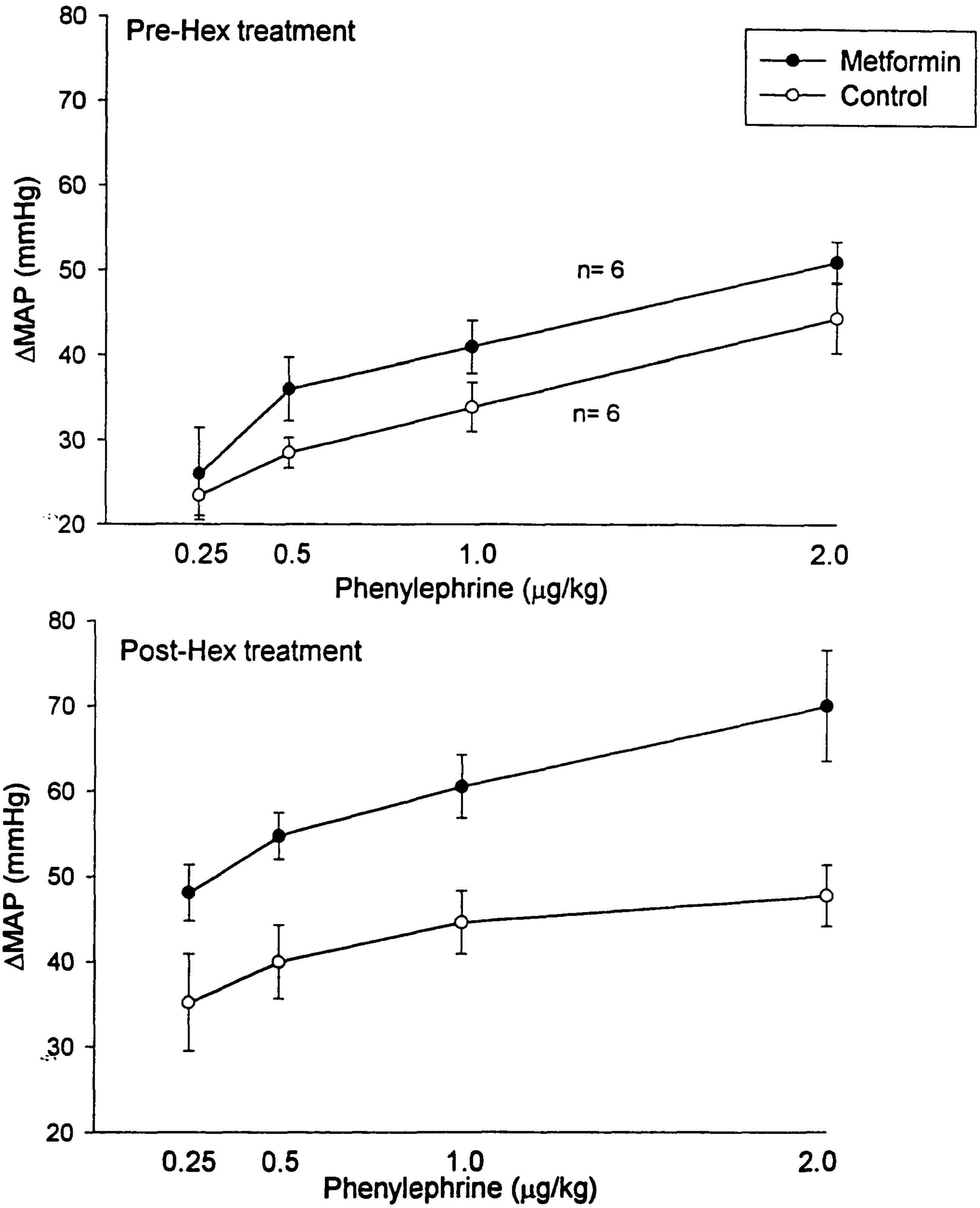
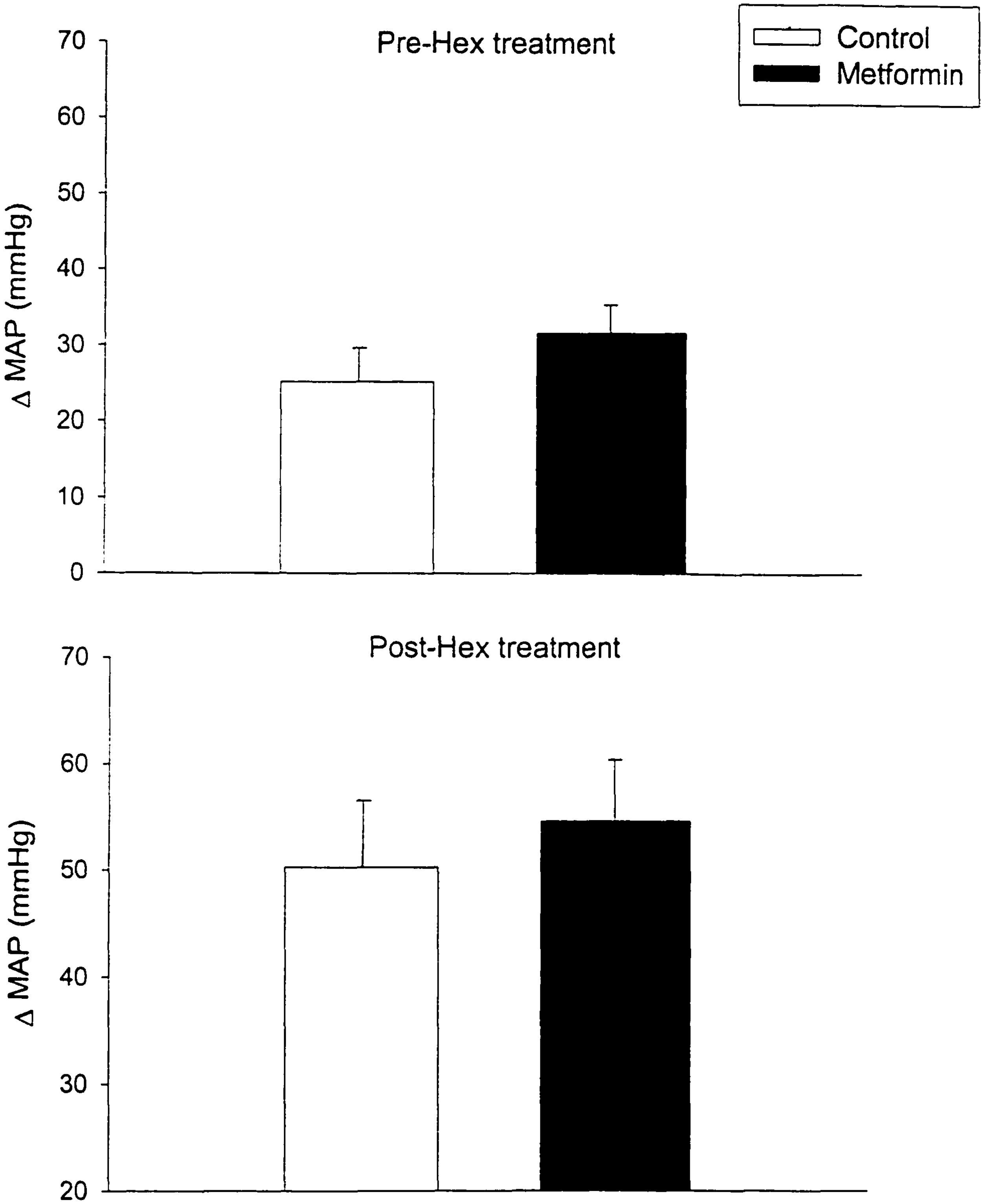

Pre-Hex treatment
Metformin
Control
n= 6
n= 6
ΔMAP (mmHg)
80
70
60
50
40
30
20
0.25
0.5
1.0
2.0
Phenylephrine (µg/kg)
Post-Hex treatment
ΔMAP (mmHg)
80
70
60
50
40
30
20
0.25
0.5
1.0
2.0
Phenylephrine (µg/kg)

Figure 17:

Blood pressure response to phenylephrine in conscious control & metformin treated rats.


Pre-Hex treatment
Control
Metformin
Δ MAP (mmHg)
70
60
50
40
30
20
10
0
Post-Hex treatment
Δ MAP (mmHg)
70
60
50
40
30
20

and control groups to the bolus injection of PE, which was independent of drug treatment (F 1,8= 14.811, $p<0.05$).

Metformin had no effect on Renal blood flow response to PE in urethane-anesthetized SHR rats

A renal doppler flow probe was implanted to monitor changes in blood flow at this particular arterial bed. Immediately following the placement of the renal doppler, the animals were given bolus injections of PE at four concentrations (0.25, 0.5, 1.0, 2.0 μg/kg). After the animals were injected with PE, a bolus injection of Hex was administered. This was followed by the same four PE concentrations (Refer to diagram 1). Figure 18a, shows representative increases in renal arterial doppler shift to PE, reflecting increases in blood velocity which are directly proportional to increases in blood flow. As shown in figure 18b, in this paradigm, metformin did not affect the blood flow response to PE in the renal arterial bed.

A 3-way ANOVA (Drug*Hex treament*Concentration) revealed no significant change of blood flow in the renal arterial bed due to a main drug effect. The renal doppler shift response to PE was not significantly higher after treatment with Hex regardless of drug treatment. There was no significant main effect of PE concentration response.

Figure 18a:

Raw data representation of renal blood flow increases in response to phenylephrine in unconscious rats.

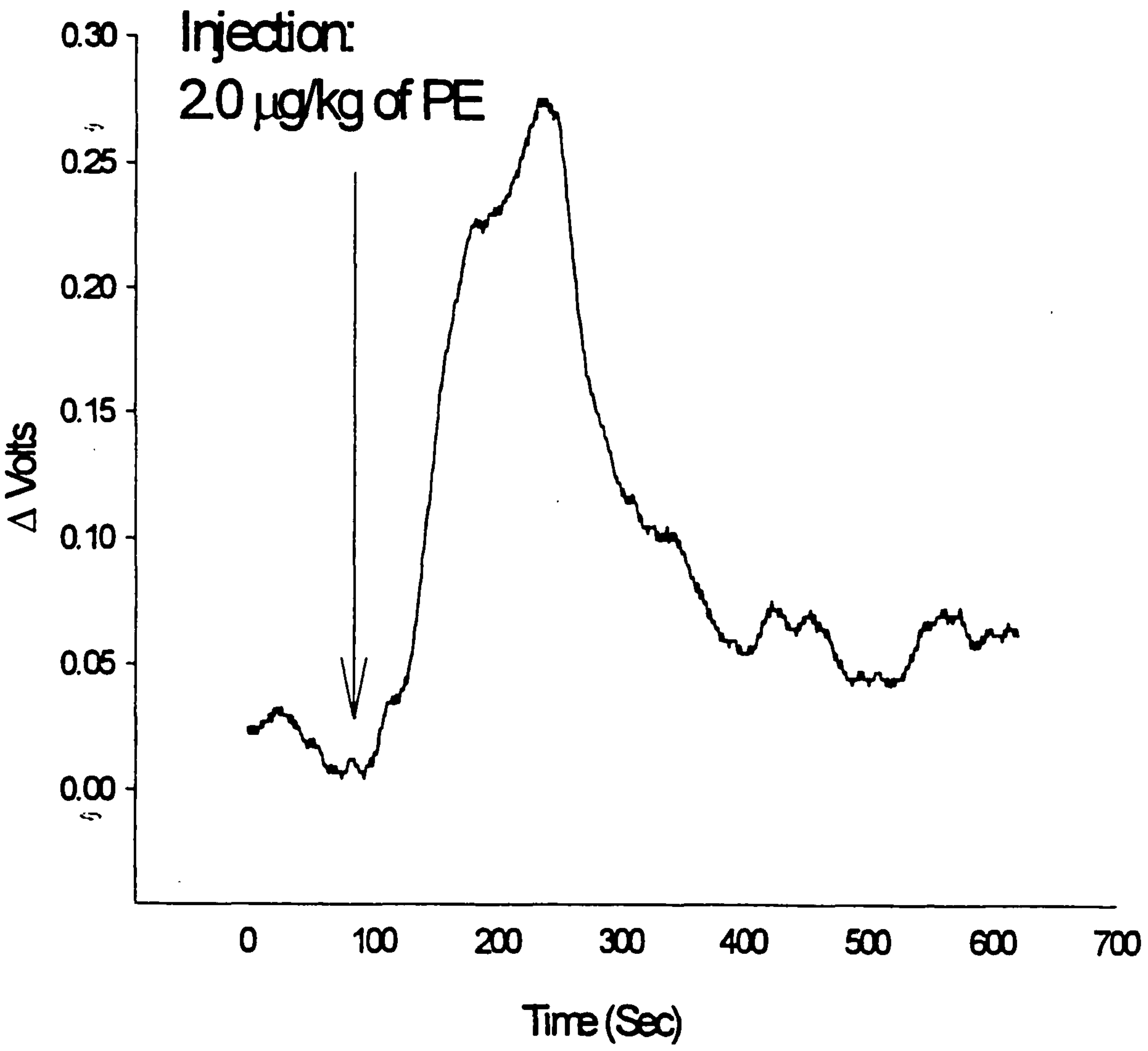
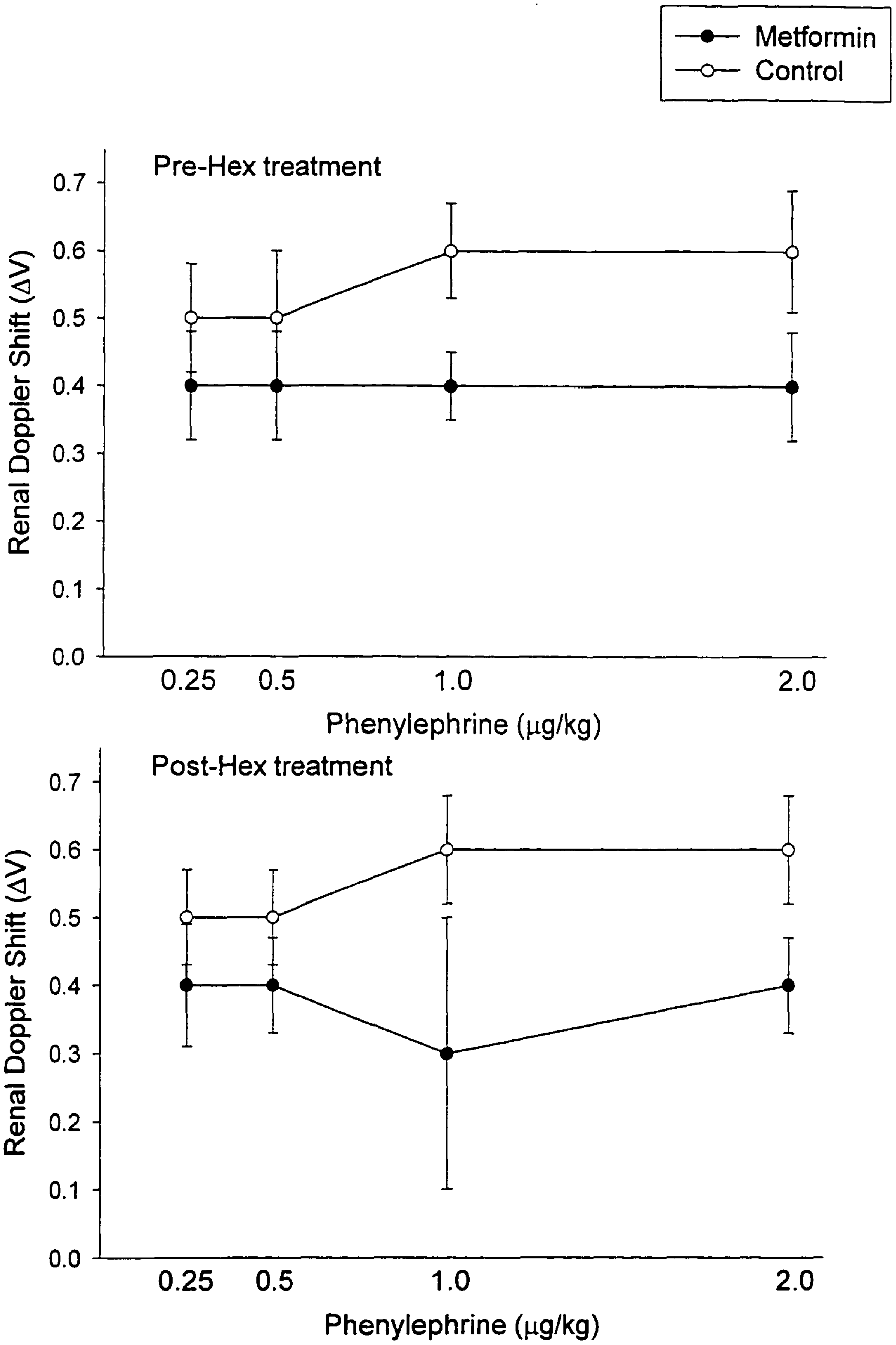

Injection:
2.0 µg/kg of PE
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Δ Volts
0
100
200
300
400
500
600
700
Time (Sec)

Figure 18b:

Renal blood flow increases in response to PE in unconscious rats.


Metformin
Control
Pre-Hex treatment
Renal Doppler Shift (ΔV)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.25
0.5
1.0
2.0
Phenylephrine (μg/kg)
Post-Hex treatment
Renal Doppler Shift (ΔV)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.25
0.5
1.0
2.0
Phenylephrine (μg/kg)

Metformin had no effect on Iliac blood flow response to PE in urethane-anesthetized SHR rats

An iliac doppler was implanted to monitor changes of blood flow at this particular arterial bed. Immediately following the placement of the iliac doppler, the animals were given bolus injections of PE at four concentrations (0.25, 0.5, 1.0, 2.0 µg/kg). After the animals were injected with PE, bolus injections of Hex were administered. This was followed by the same four concentrations PE (Refer to Diagram 1). Figure 19a, shows representative increases in iliac arterial doppler shift to PE, reflecting increases in blood velocity which are directly proportional to increases in blood flow. As shown in figure 19b, metformin did not affect the blood flow response to PE in the iliac arterial bed. A 3-way ANOVA (Drug*Hex treatment*Concentration) revealed no significant change in the iliac blood flow response due to a main drug effect. The iliac doppler shift response to PE was not significantly higher after treatment with Hex. Furthermore, there was no significant dose response to PE in the iliac arterial bed.

Metformin had no effect on Blood Pressure response to angiotensin II in urethane-anesthetized SHR rats

Metformin and vehicle treated animals were injected with three subsequent concentrations of angiotensin II (25, 50, and 100 ng/kg). After the animals were injected with angiotensin II, a bolus injection of Hex was administered. This was followed by injecting the same three concentrations of angiotensin II (Refer to Diagram 3).

Figure 19a:

Raw data representation of iliac blood flow increases in response to PE in unconscious rats.

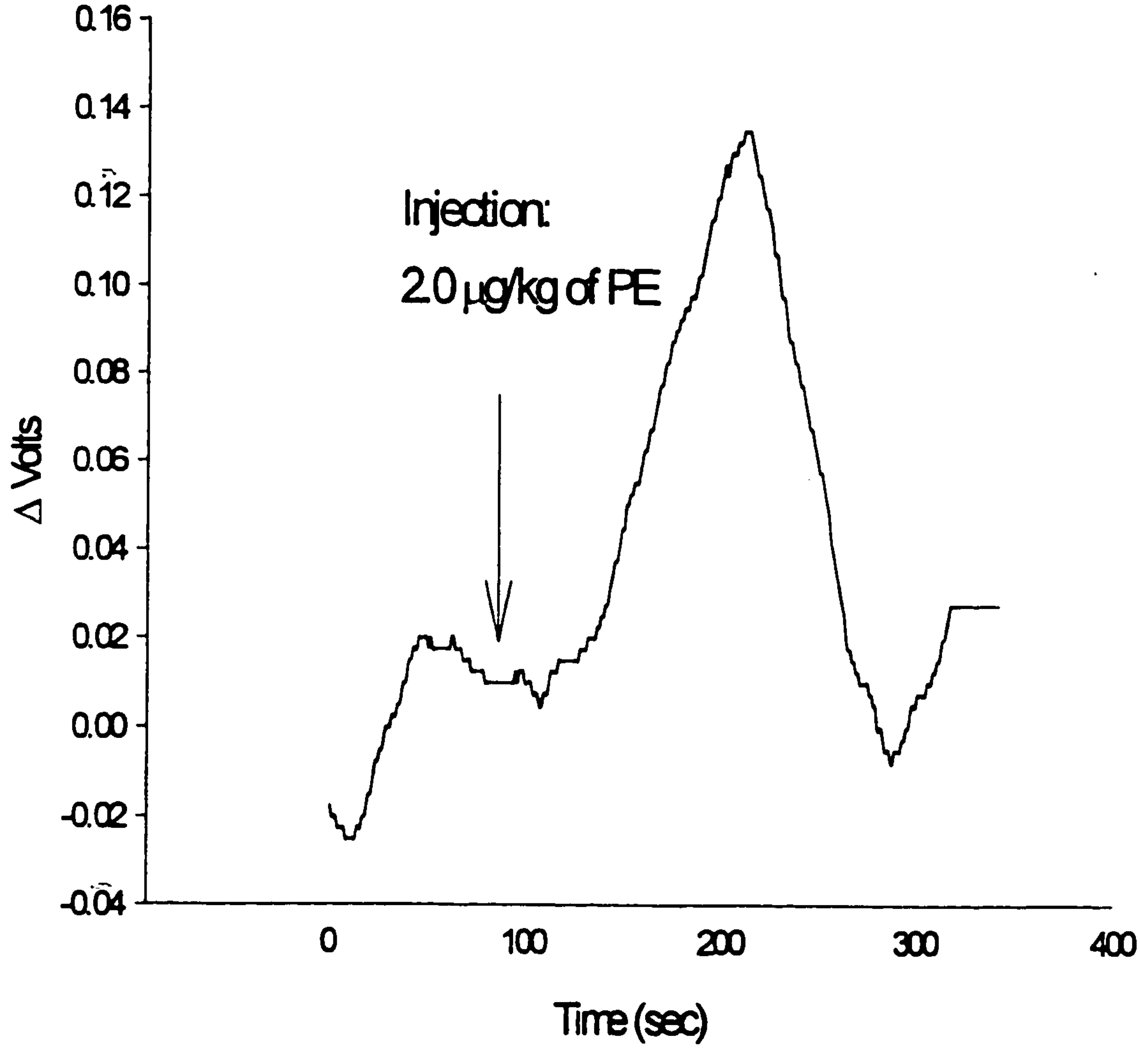

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
Δ Volts
Injection:
2.0 μg/kg of PE
0
100
200
300
400
Time (sec)

Figure 19b:

Iliac blood flow increases in response to PE

in unconscious rats.

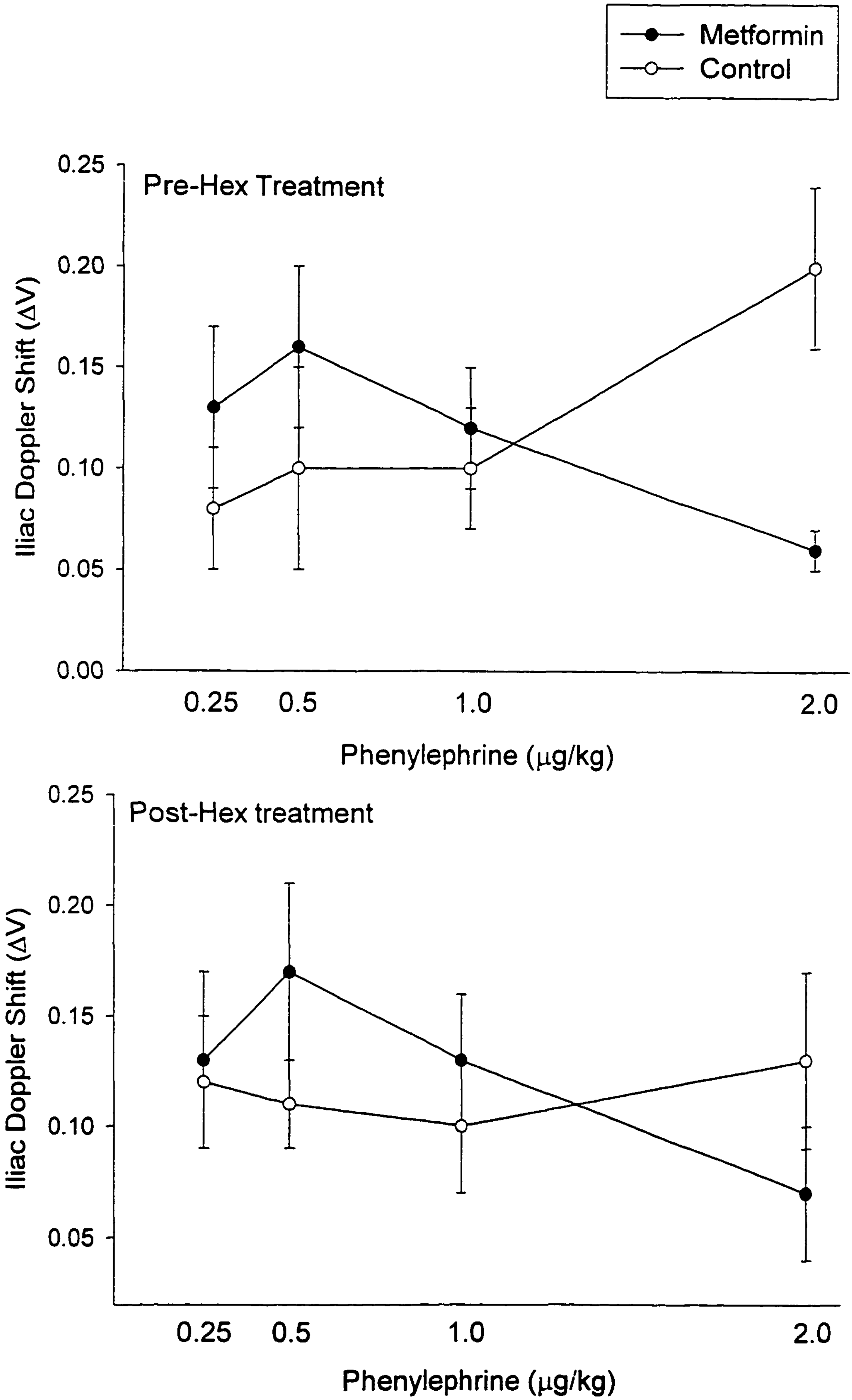
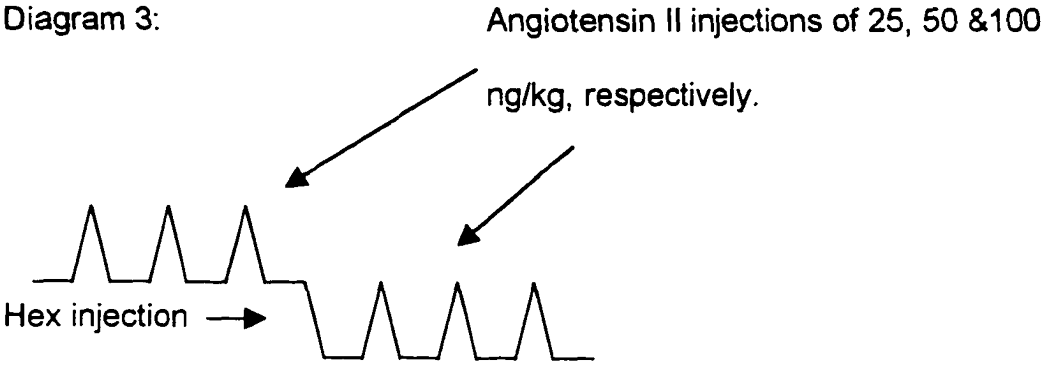

Metformin
Control
Pre-Hex Treatment
Iliac Doppler Shift (ΔV)
0.25
0.20
0.15
0.10
0.05
0.00
0.25
0.5
1.0
2.0
Phenylephrine (μg/kg)
Post-Hex treatment
Iliac Doppler Shift (ΔV)
0.25
0.20
0.15
0.10
0.05
0.25
0.5
1.0
2.0
Phenylephrine (μg/kg)

Diagram 3:



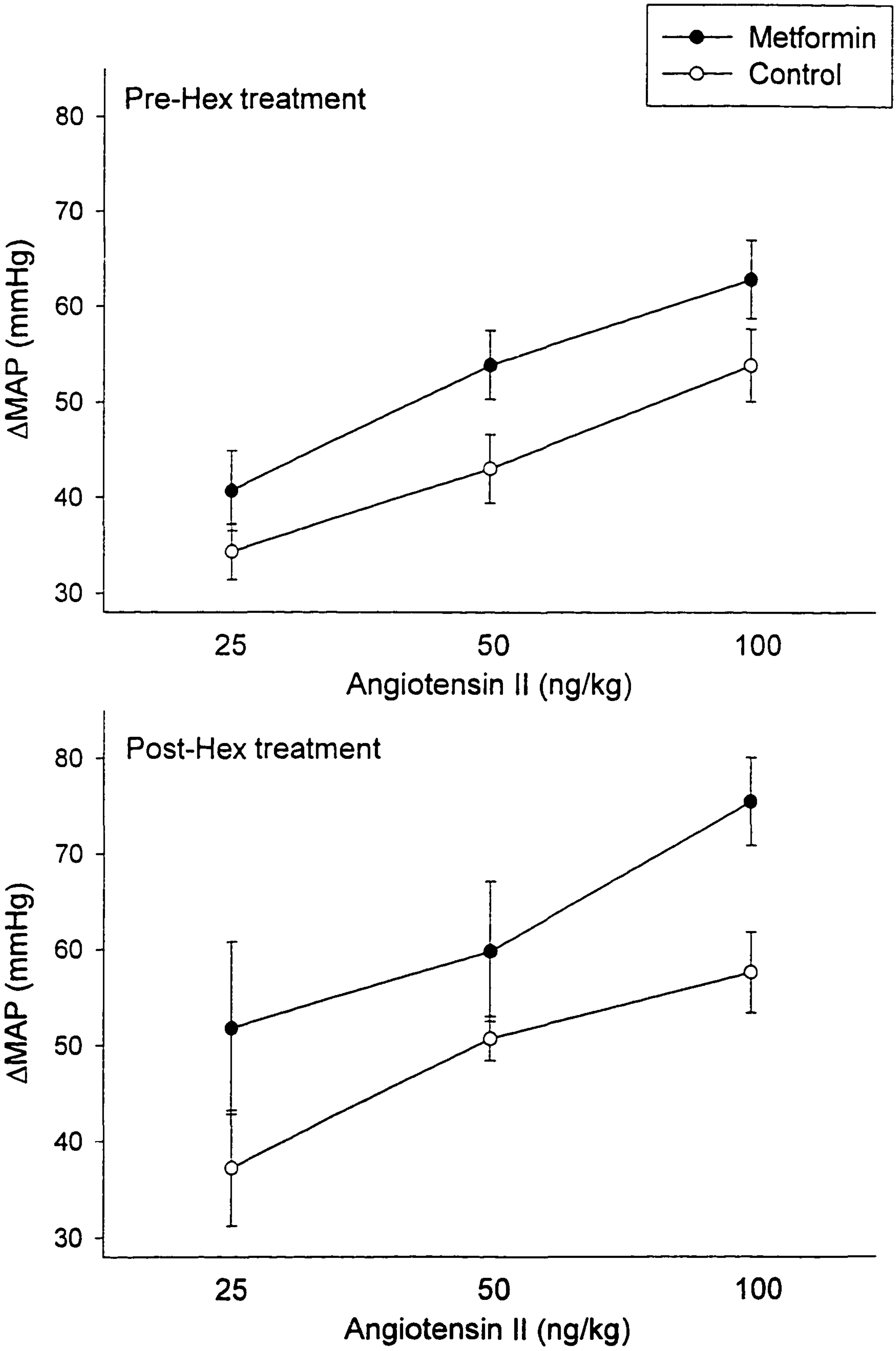
As shown in figure 20, metformin increased MAP responses to bolus injections of angiotensin II. A 3-way ANOVA (Drug*Hex treatment*Concentration) revealed significantly increased MAP responses due to a main drug effect (F 1,10 = 5.5, $p < 0.05$). MAP responses to angiotensin II were significantly higher after treatment with Hex in both control and metformin treated animals, and this response was equivalent in metformin and control rats (F 1,10 = 5.1, $p < 0.05$). Moreover, there was also a significant dose response to angiotensin II (F 2,20 = 42.7, $p < 0.05$).

Metformin had no effect on Renal blood flow response to angiotensin II in urethane-anesthetized SHR rats

A renal doppler was implanted to monitor changes of blood flow at this particular arterial bed. Immediately following the placement of the renal doppler,

Figure 20:

Blood pressure response to angiotensin II in unconscious control and metformin treated rats.


Metformin
Control
Pre-Hex treatment
ΔMAP (mmHg)
80
70
60
50
40
30
25
50
100
Angiotensin II (ng/kg)
Post-Hex treatment
ΔMAP (mmHg)
80
70
60
50
40
30
25
50
100
Angiotensin II (ng/kg)

the animals were given bolus injections of angiotensin II of at three concentrations (25, 50 and 100 ng/kg). After the animals were injected with angiotensin II, a bolus injection of Hex was administered. This was followed by the same three concentrations of angiotensin II injected prior to Hex treatment (Refer to Diagram 3). Angiotensin II evoked a biphasic response in the renal arterial bed. That is, there was an initial increase in blood flow immediately followed by a decrease in blood flow. Figure 21a, shows representative increases and decreases in renal arterial doppler shift to angiotensin II, reflecting increases and decreases in blood velocity which are directly proportional to increases and decreases in blood flow. Figure 21b and 21c illustrates the increasing and lowering effects mediated by angiotensin II, respectively. Metformin had no effect on the renal blood flow increase or decrease responses when challenged with angiotensin II.

For the blood flow increase responses to angiotensin II, a 3-way ANOVA (Drug*Hex treatment*Concentration) revealed no significant change in the renal arterial flow due to a main drug effect. Renal doppler shift responses to angiotensin II were not significantly changed after Hex treatment. However, there was a significant dose response to angiotensin II ($F_{2,20} = 3.6$, $p < 0.05$). There was an interaction between drug and Hex treatment ($F_{1,10} = 5.09$, $p < 0.05$). This interaction reflected an increased reactivity to angiotensin II, across all doses, in the metformin group prior to Hex treatment in contrast to no effect of metformin on reactivity in the metformin group after Hex treatment.

Figure 21a:

Raw data representation of renal blood flow increases and decreases in response to angiotensin II in unconscious rats.

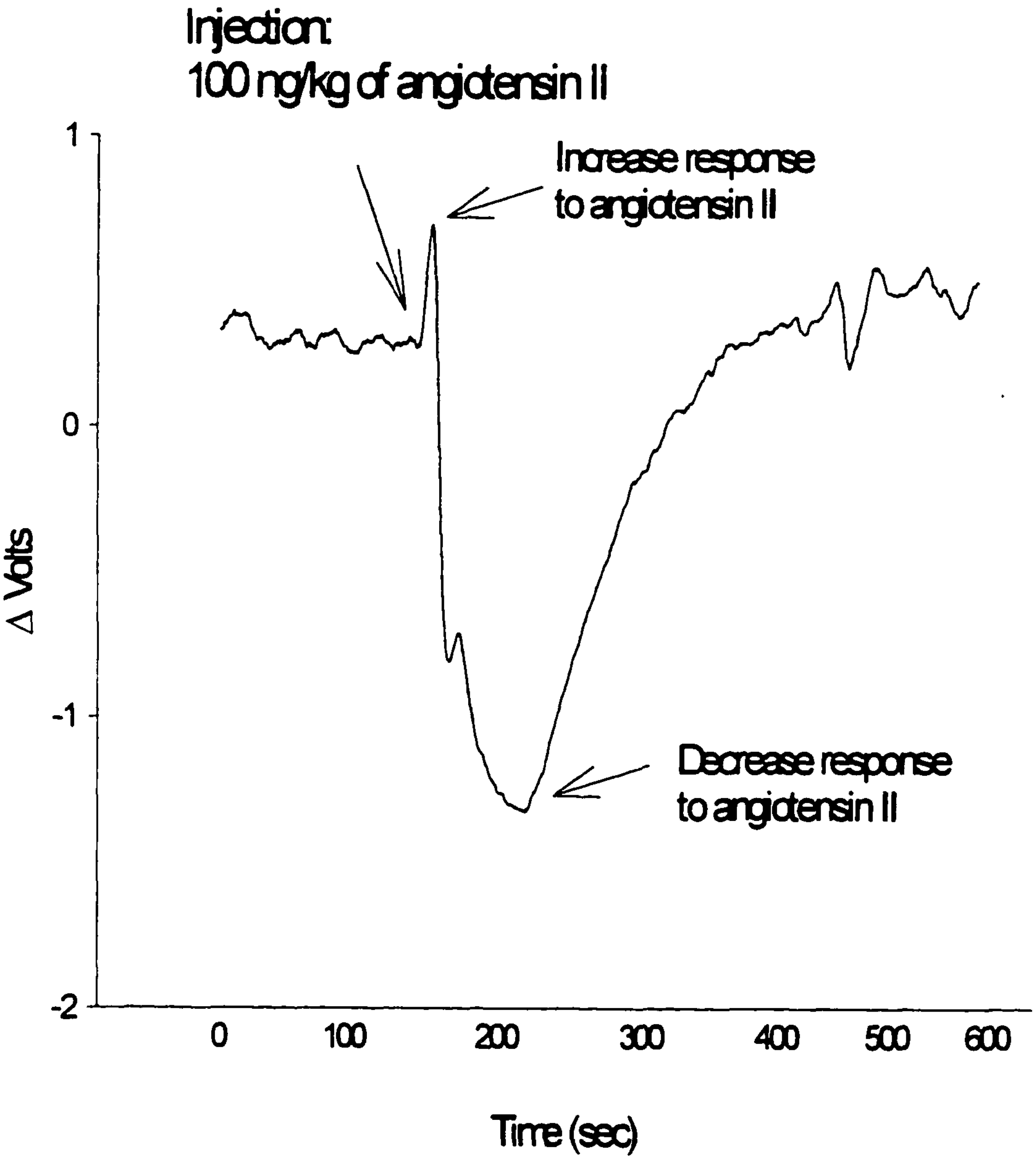

Injection:
100 ng/kg of angiotensin II
Increase response to angiotensin II
Decrease response to angiotensin II
1
0
-1
-2
Δ Volts
0
100
200
300
400
500
600
Time (sec)

Figure 21b:

Renal blood flow increases in response to angiotensin II in unconscious rats.

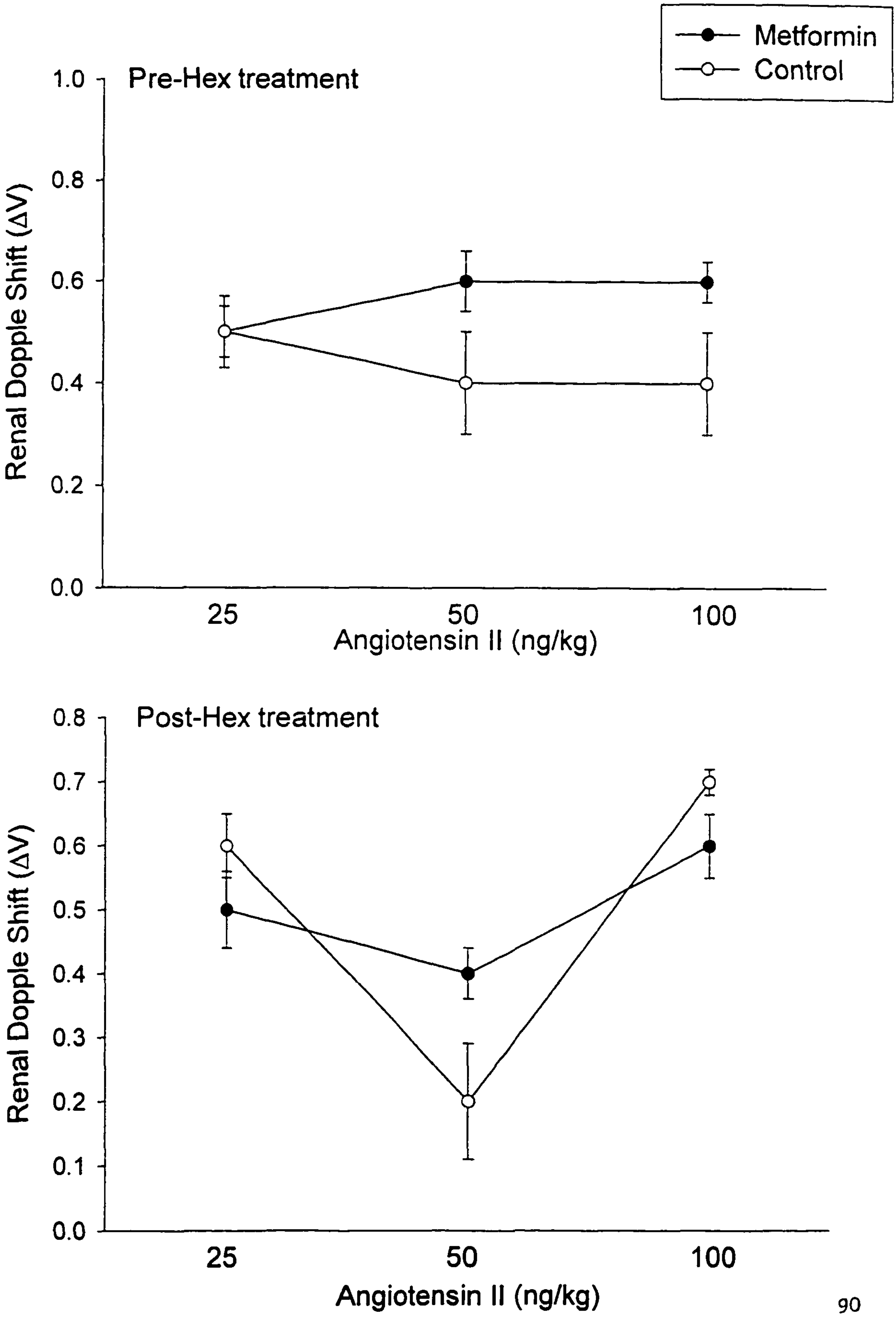
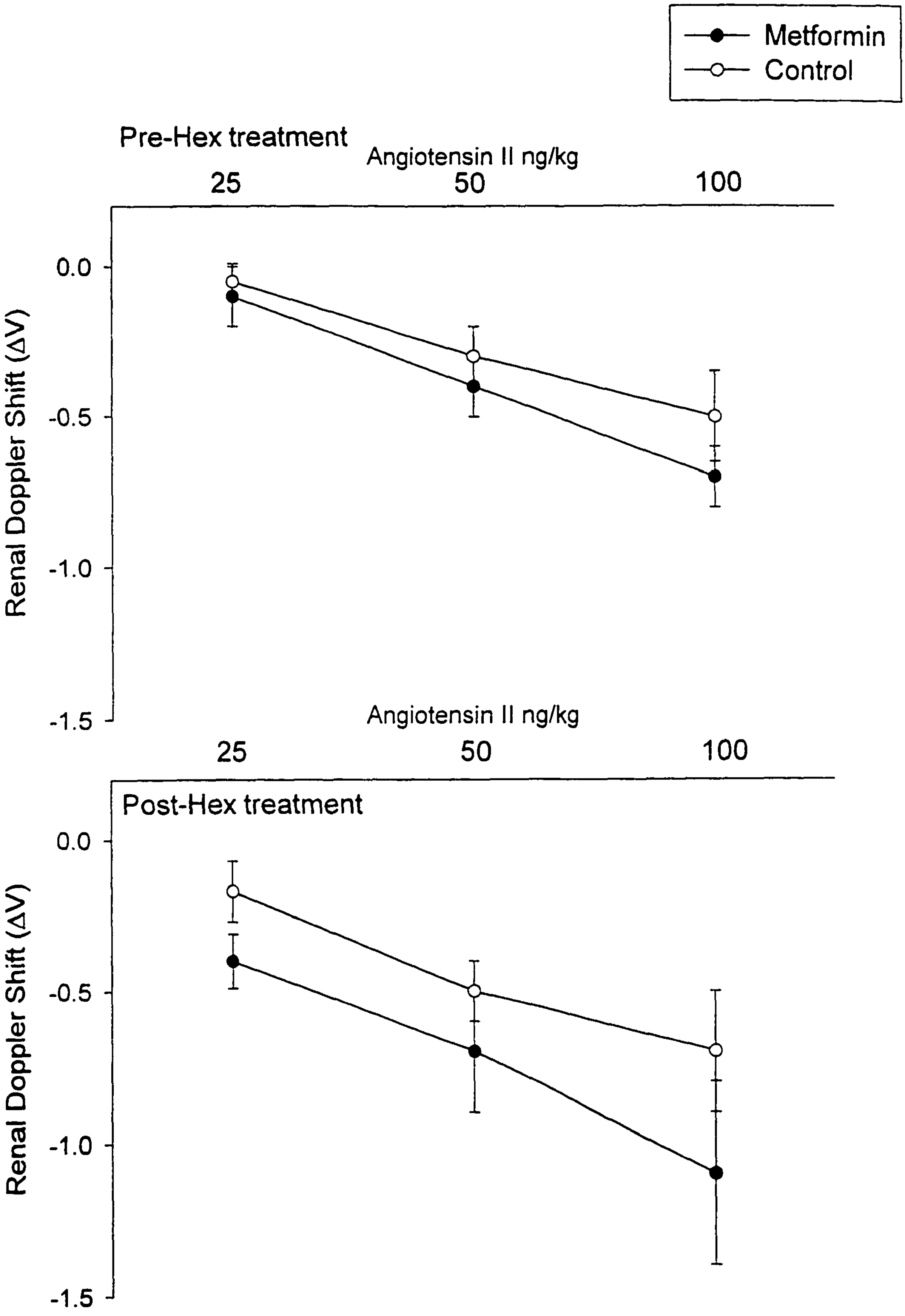

Metformin
Control
Pre-Hex treatment
Renal Dopple Shift (ΔV)
1.0
0.8
0.6
0.4
0.2
0.0
25
50
100
Angiotensin II (ng/kg)
Post-Hex treatment
Renal Dopple Shift (ΔV)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
25
50
100
Angiotensin II (ng/kg)

Figure 21c:

Renal blood flow decreases in response to angiotensin II in unconscious rats.


Metformin
Control
Pre-Hex treatment
Angiotensin II ng/kg
25
50
100
Renal Doppler Shift (ΔV)
0.0
-0.5
-1.0
-1.5
Angiotensin II ng/kg
25
50
100
Post-Hex treatment
Renal Doppler Shift (ΔV)
0.0
-0.5
-1.0
-1.5

For the blood flow decreased responses to angiotensin II, a 3-way ANOVA (Drug*Hex treatment*Concentration) revealed no significant change due to a main drug effect. The renal doppler shift response to angiotensin II was not significantly changed after Hex treatment. However, there was a significant dose response to angiotensin II (F 2,20 = 44.2, $p < 0.05$).

Metformin had no effect on Iliac blood flow response to angiotensin II in urethane-anesthetized SHR rats

An iliac doppler was implanted to monitor changes of blood flow at this particular arterial bed. Immediately following the placement of the iliac doppler, the animals were given bolus injections of angiotensin II at three concentrations (25, 50 and 100 ng/kg). After the animals were injected with angiotensin II, a bolus injection of Hex was administered. This was followed by the same three concentrations of angiotensin II (Refer to diagram 3). Figure 22a, shows representative increases in iliac arterial doppler shift to angiotensin II, reflecting increases in blood velocity which are directly proportional to increases in blood flow. As shown in figure 22b, metformin did not affect the blood flow increase responses in the iliac arterial bed when challenged with angiotensin II.

A 3-way ANOVA (Drug*Hex treatment*Concentration) revealed no significant change in iliac blood flow responses due to a main drug effect. Iliac doppler shift response to angiotensin II was not significantly changed after Hex treatment. Moreover, there was no significant dose response to angiotensin II. However, there was a significant interaction between metformin and doses

Figure 22a:

Raw data representation of iliac blood flow increases in response to angiotensin II in unconscious rats.

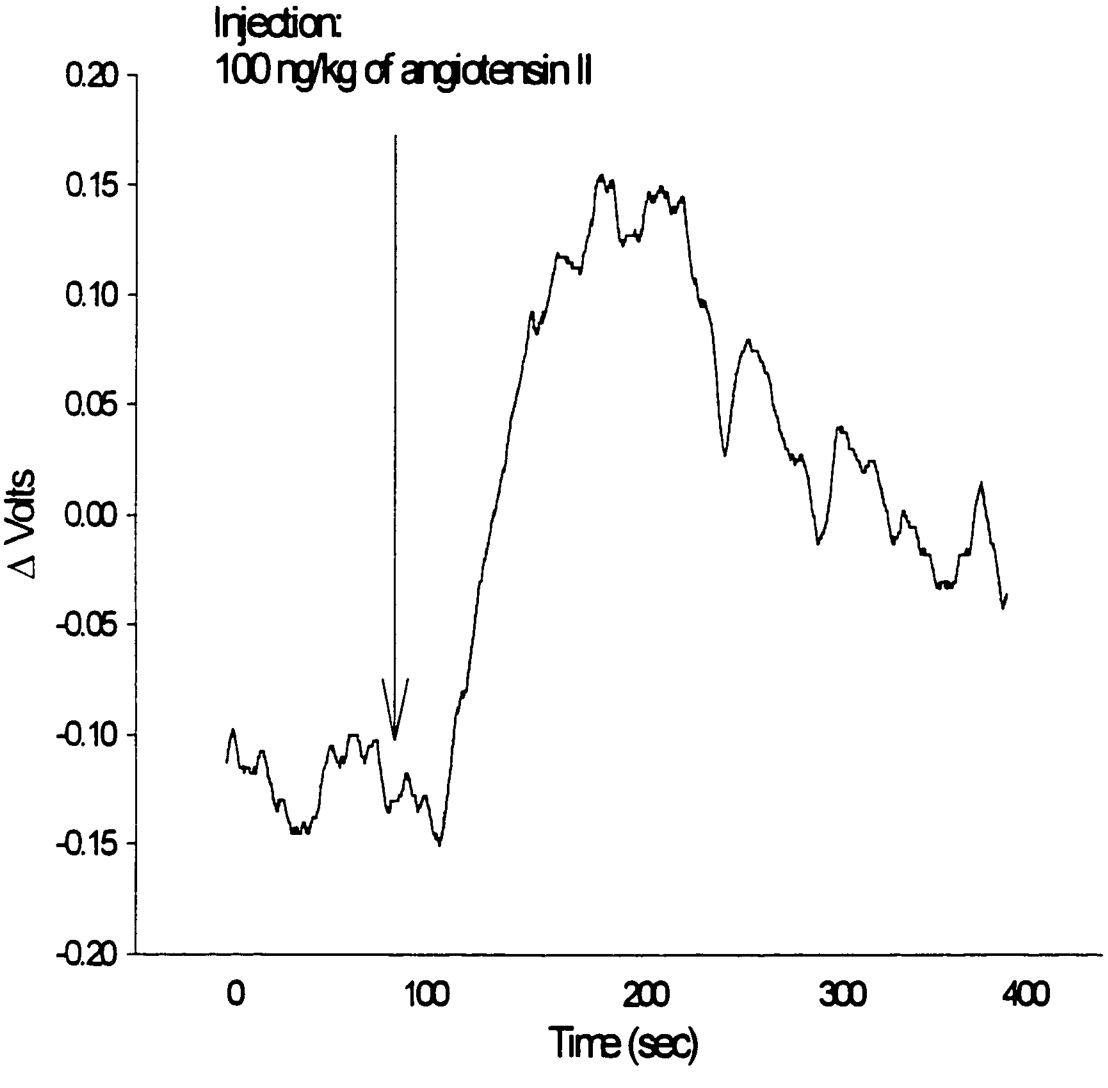
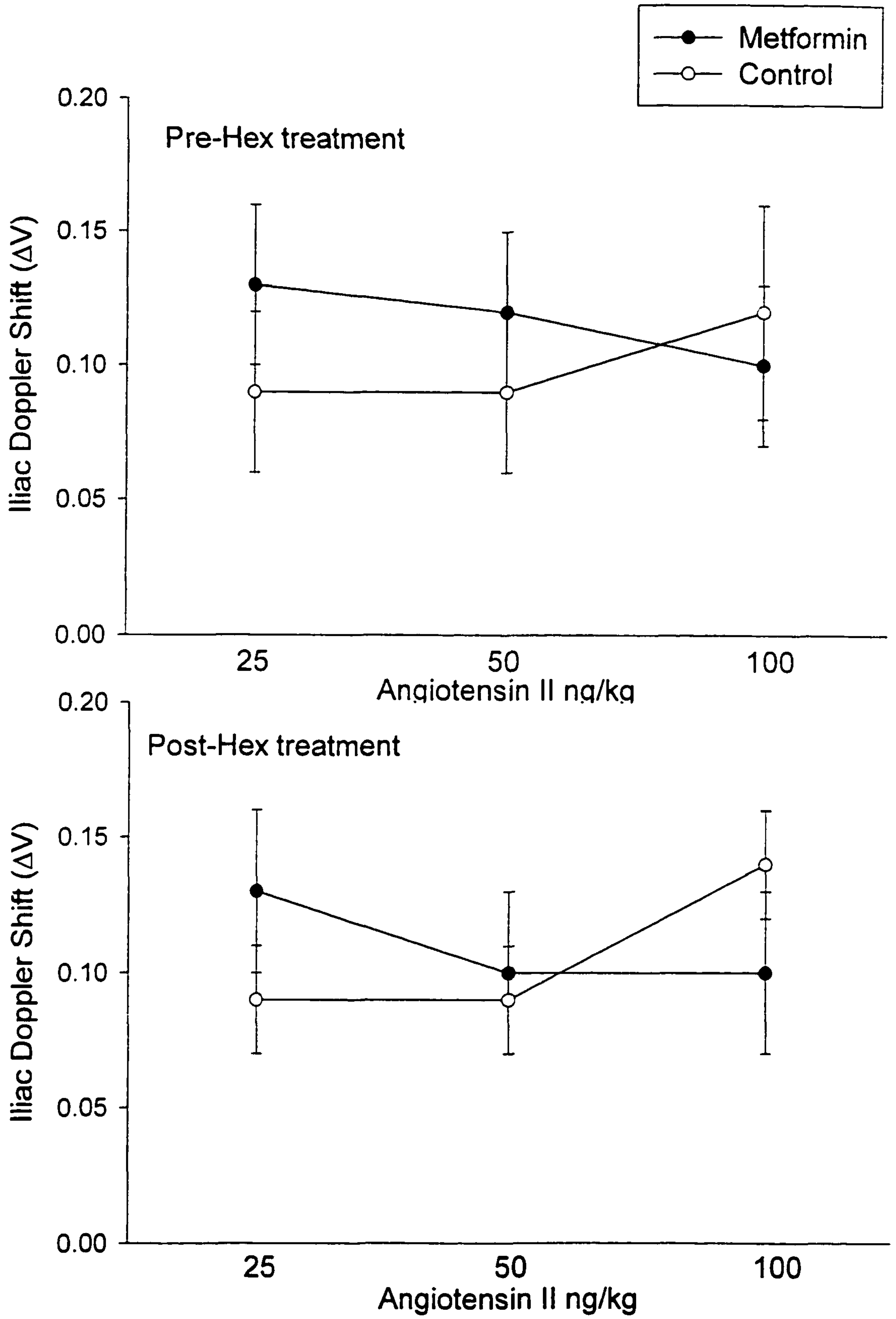

Injection:
100 ng/kg of angiotensin II
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
Δ Volts
0
100
200
300
400
Time (sec)

Figure 22b:

Iliac blood flow increases in response to angiotensin II in unconscious rats.


Metformin
Control
Pre-Hex treatment
Iliac Doppler Shift (ΔV)
0.20
0.15
0.10
0.05
0.00
25
50
100
Angiotensin II ng/kg
Post-Hex treatment
Iliac Doppler Shift (ΔV)
0.20
0.15
0.10
0.05
0.00
25
50
100
Angiotensin II ng/kg

response (F 2,20 = 3.84, $p < 0.05$). In general, this reflects increased responses of blood flow in the control group to the highest concentration of angiotensin II versus a decreased response of blood flow responses in the metformin group. However, it is our belief that this interaction has no physiological significance.

Discussion:

Effects of Metformin on BP:

Research in diabetic treatment continues to be of interest for an ever-growing population. Metformin, in addition to giving another choice of drug to the diabetic population, has been reported to have hypotensive properties. As previously mentioned in this report, metformin was tarnished by earlier reports of causing lactic acidosis. These fears were put to rest with new studies suggesting that this severe side effect occurred rarely and if monitored correctly, can be completely prevented. Now, metformin has been shown to lower blood glucose and blood pressure. However, reports that metformin-treatment lowers blood pressure in both animals (Morgan *et al.*, 1992;Petersen *et al.*, 1997;Verma *et al.*, 1994b) and humans (Chan *et al.*, 1993;Giugliano *et al.*, 1993a;Haupt *et al.*, 1991;Landin *et al.*, 1991;Velazquez *et al.*, 1994) lack the mechanism by which it exerted its hypotensive effects.

In the present study, blood pressure (BP) levels were lowered in chronically metformin-treated SHRs in both conscious and urethane-anesthetized animals. In the conscious study, monitoring of the rats for 24-hour periods for 4 days showed significantly lower blood pressures in metformin SHRs when compared to the control group. In the urethane-anesthetized study, the lowering effect was present, but it was not significantly lower when compared to controls. Hypotensive properties of metformin, we think, were attenuated by the anesthetic and its known effects on the cardiovascular system (Gilman *et al.*, 1990). Also, in the conscious study the metformin group showed significantly lower BP levels during both the inactive (day-time) and active periods (night-time) when compared to the control group.

The observation that metformin lowered BP in SHRs was expected (Bhalla *et al.*, 1996;Muntzel *et al.*, 1997;Petersen and DiBona., 1996;Verma *et al.*, 1994b). Other groups have reported hypotensive action by metformin when given to SHRs at the same dose and route (orally and intravenous) as in the present study (Verma *et al.*, 1994b) or as a smaller dose administered subcutaneously (Bhalla *et al.*, 1996). In addition, in 1997, Andersen's group reported that central infusion of metformin (specifically to the lateral ventricle) resulted in BP decreases. Intravenous infusion of metformin has also been reported to decrease BP (Muntzel *et al.*, 1997;Sterne., 1969).

Metformin exerts variable effects on different strains of rats. The best example to illustrate this discrepancy is the WKY rat, which is the parent strain to the SHR. WKYs are considered by some scientists to be the control for effects observed in the SHR. This is because WKYs were selectively inbred by Okamoto and Aoki (Okamoto and Aoki., 1963) to generate the SHR strain, a line of rats that would model hypertension. In 1996, Bhalla et al., reported a BP decrease in metformin-treated SHRs; the WKY-metformin-treated group also showed lowered of BP levels but these levels were not significantly different when compared to the WKY control group. Furthermore, in the study by Muntzel et al., (1997), it was reported that bolus administration of metformin evokes greater depressor responses in SHRs than in WKYs. Muntzel's findings are supported by previous reports showing hypotensive actions of oral or interperitoneal metformin in SHRs but not in WKYs or Sprague-Dawley rats (Morgan *et al.*, 1992;Verma *et al.*, 1996). Another commonlymployed strain is the Sprague-Dawley (SD) rat. This strain when treated with metformin has, in general, responded with decreased BP after metformin treatment (Petersen *et al.*, 1997;Verma *et al.*, 1994b), but in these reports metformin was acutely administered, or decreases in BP were only reported in fructose-fed SD rats. In other studies, metformin had no effect on this strain of rats (Jansson., 1995). In summary, although metformin lowers BP in a variety of strains, its hypotensive actions are strongest and have been well established in SHRs.

Another parameter that appears to be important for BP effects is the age that animals are started on metformin treatment. SHRs start to show signs of hypertension between weeks 4-6 (~ 100 grams) of their development (Okamoto et al., 1969). In the present study, SHRs were initially placed under metformin at 3 weeks of age (~60 grams), which raises the possibility that the drug attenuates hypertension development only in young SHRs. Bhalla et al. (1996) and Verma et al. (1994) started treatment in SHRs at about 6 –8 wks (80-100 grams). Their observations, similar to the present study, showed a lowering of BP under metformin treatment. However, in adult SHRs (175-200 grams), Muntzel et al.,(1999) found no lowering of BP levels under metformin treatment(Muntzel *et al.*, 1999). Altogether, these findings indicate that metformin lowers BP levels in young SHRs but may have little effects in adult SHRs with established hypertension.

In previous work by Verma et., al., (1994), Kosegawa et al., (1995) and Muntzel et al., (1999) a decrease in body weight gain was reported in the metformin-treated SHRs. In the present study, we report no change in body weight relative to the control group. It must be highlighted, that the metformin group was consistently lower in weight, by an average of a few grams, yet a 2 way ANOVA with repeated measure at the weight level shows no significant difference. Thus, we observed a lowering effect of BP due of metformin treatment in the absence of weight loss. Therefore, we postulate that the effects on BP by metformin are independent of weight loss. In agreement, other studies in rats showed metformin-induced reductions in BP before decreases in weight were observed (Kosegawa *et al.*, 1996;Verma *et al.*, 1994a;Verma *et al.*, 1994b). In clinical studies, administration of metformin also lowered BP levels without changes in body weight (Giugliano *et al.*, 1993a;Landin *et al.*, 1991) or body mass index (Giugliano *et al.*, 1993b), which, again, suggest that weight reduction is not a major parameter for BP lowering that is mediated by metformin. In summary, the lower BP to metformin is independent of body weight change.

Effects of Metformin on Sympathetic Nerve Activity:

The literature supporting the lowering of BP by metformin in a number of papers including this work has been equally contradicted by the same number of papers showing no effect of the drug. When one examines the human studies that reported metformin mediated BP lowering effects, including studies with lean hypertensives (Landin *et al.*, 1991), obese hypertensive (Giugliano *et al.*, 1993a;Giugliano *et al.*, 1993b), hypertensive and normotensive individuals with

NIDDM (Chan *et al.*, 1993;Giugliano *et al.*, 1993b;Haupt *et al.*, 1991), and normotensive women with polycystic ovary syndrome (Velazquez *et al.*, 1994), they share the common denominator of insulin resistance.

The ever growing body of literature on insulin resistance suggests that individuals who suffer from obesity, essential hypertension and NIDDM are often diagnosed with a higher than normal sympathetic nerve activity during the developmental stage of their hypertension (Anderson and Mark., 1993;Tuck., 1992). In addition, SHRs show an increase of sympathetic nerve activity between 3-9 wks of age (Pfeffer and Frohlich., 1973). It is also reported that insulin infusion during euglycemic clamp conditions causes increases in sympathetic nerve activity (Anderson and Mark., 1993;Muntzel *et al.*, 1994). Due to these reports, it was postulated in the present study that the antihypertensive effects of metformin are mediated by the lowering of basal sympathetic nerve activity.

A fall in sympathetic nerve activity can originate from a metformin-induced reduction in plasma insulin levels (Dorella *et al.*, 1996;Giugliano *et al.*, 1993a;Landin *et al.*, 1991;Verma *et al.*, 1994a;Verma *et al.*, 1994b). However, these findings are not consistent. There are reports of metformin-induced BP decrease without effects on plasma insulin levels (Calle-Pascual *et al.*, 1995;Fanghanel *et al.*, 1996), and also reports of reduced plasma insulin levels with no change on BP (Dorella *et al.*, 1996;Grant., 1996;Semplicini *et al.*, 1993).

Another mechanism is a direct effect of metformin to lower sympathetic nerve activity. For example, reductions in BP and plasma NE levels in hypertensive, obese women (Giugliano *et al.*, 1993b) were reported when treated with metformin. However, this lowering of NE plasma concentration mediated by metformin was not found in insulin-resistant hypertensive men (Gudbjornsdottr *et al.*, 1994). Another study reports of a blunting effect of metformin on the rise of NE caused by insulin infusion during glucose clamp (Dorella *et al.*, 1996). In animal models, metformin consistently causes a decrease in sympathetic nerve activity, at least in acute studies. Petersen et al., (1996) reported that acute intravenous administration of metformin produced a dose-dependent reversible decrease in efferent renal sympathetic nerve activity and blood pressure in SHRs (Petersen and DiBona., 1996). Muntzel et al., (1997) showed an abolishment of the metformin-mediated lowering of BP by pretreating with Hex, a ganglionic blocker, or by pretreating with an α-pressor blocker. In 1999, Muntzel et al., (Muntzel *et al.*, 1999) acutely injected animals with metformin and showed a decrease in lumbar sympathetic nerve activity. In summary, evidence that metformin lowers sympathetic nerve activity is conflicting in humans, yet in rats, metformin consistently lowers sympathetic nerve activity, but so far only in acute studies.

One goal of the present study was to examine if metformin causes chronic decreases in sympathetic nerve activity. One method of assessing long-term changes in sympathetic nerve activity is examining plasma catecholamine levels. In the present report, a decrease in NE plasma levels was detected after animals were treated with metformin for 23 consecutive days beginning at the age of 3 weeks. This result is consistent with the long-term studies by Kosegawa et al.,

(1996) which showed a decrease of NE levels in Otsuka Long-Evans Tokushima fatty (OLETF) rats. In the present study, we also found a significant increase of Epi plasma concentration and no change in Dop levels in the metformin group. This data is divergent from the Kosegawa report, in that there was no change in Epi plasma concentration. Kosegawa's study differs in a number of parameters from the present study: they used OLETF rats, the concentration of metformin was 5 times less than the one use in the present study, and the age of the rat at the time of the study was 12 weeks old. The rat model employed, OLETF, is a new model to study NIDDM with mild obesity, hyperinsulinemia, and hypertriglyceridemia, and needs to be further characterized for an accurate comparison to be made. Nevertheless, the metformin induced decreased levels of NE in the study by Kosegawa et al.,(1996) and in the present study is strong evidence that metformin causes a decrease in chronic sympathetic nerve outflow.

To explain the increase in Epi in our study, it is postulated that metformin had an effect on the production or availability of phenylethanolamone N-methyltransferase (PNMT), which converts NE to Epi in the adrenal medulla. This enzyme is directly affected by high levels of glucocorticoids and may be similarly affected by metformin. Glucocorticoids have been reported to increase transcription of the PNMT gene (Evinger *et al.*, 1992) and to reduce the rate of PNMT degradation due to enhanced levels of the cofactor S-adenosylmethionine (Ciaranello., 1978;Wong *et al.*, 1982), thereby increasing Epi levels. Thus, metformin by increasing PNMT activity may also increase Epi levels.□□The increase in Epi levels could be explained by an alternate mechanism. In 1997, Peuler(Peuler., 1997) reported that arterial plasma NE and Epi levels were increased in the metformin treated group before and after using a ganglionic blockade. This finding, Peuler suggested, is evidence that metformin causes a direct release of NE and Epi that is independent of sympathetic neuroral activity. Therefore, in our study the increase of Epi levels detected could have been caused by metformin stimulating a release of Epi independent of sympathetic nerve activity.

The concentrations of the catecholamines in the present study are from plasma but they originate from different tissues. Epi and Dop spill over is from the adrenal medulla (considered by some to be a specialized sympathetic ganglion) and NE spill over is from the sympathetic nerve terminals from throughout the body. The storage and release mechanisms of catecholamine in this system are then important to address. Catecholamine storage has been extensively studied in adrenal chromaffin cells and in the sympathetic neurons. In addition to the catecholamines found in the chromaffin granules, we also find nucleotides (mainly ATP), ascorbic acid, Ca^{+2} ions, and a family of glycoproteins called chromogranins, a small number of DA β-hydroxylase (DHB), enkephalin peptides and another peptide called neuropeptide Y. DHB and ascorbic acid are necessary for NE synthesis and the remaining granule constituents are still being investigated. ATP synthesized in mitochondria is transported into the granules, along with DA synthesized in the cytoplasm of the cell. DA is converted to NE within the chromaffin granules. In Epi secreting chromaffin cells, NE must return to the cytoplasm in order to be N-methylated by PNMT (Corcoran *et al.*, 1984). The resulting Epi is then taken back up into the granule by the same mechanism that transports DA. As a note of interest, sympathetic neurons posses two types of dense-core vesicles: large and small. The large vesicles seem to contain most, if not all, of the same substances present within chromaffin granules (Winkler *et al.*, 1987).

It is possible that the elevated Epi levels in the metformin group may have caused the observed decrease in BP. Epi can exert a decrease in BP by activation the β_2-adrenergic receptors on arteries, which are more than a hundred-fold more potent than NE activation of these specific receptors. Activation of the β_2-adrenergic receptors causes relaxation of arteries. However, by activating the β_1-receptors in the heart, Epi increases the cardiac output at the same time as the decrease in peripheral resistance resulting in no real change in BP (Gilman *et al.*, 1990). In summary, the decrease NE level is evidence that metformin causes chronic decrease in sympathetic nerve. Epi may be increased by metformin by perhaps enhancing PNMT. However, the increase of Epi would not explain the fall in BP. In addition, it is realized that the combination of a decrease in NE and an increase in Epi is unusual. This finding should be replicated in future metformin studies.

A second method to assess sympathetic activity is the indirect quantification of sympathetic vasoconstriction by using Hex. It should be emphasized that this method does not estimate the level of central neural outflow passing through the sympathetic nerves, but rather indicates whether the overall vasoconstrictor function of the sympathetic nervous system is reduced in one group relative to the other. This study found a decrease in the BP fall response to Hex in both conscious and urethane-anesthetized metformin-treated rats when compared to the control group. That is, the fall in BP to the ganglionic blocker was greater in the control group than in the metformin treated group. Nevertheless, the fall in BP was not significantly different between the two groups. We expected that the fall in BP to Hex in the metformin group would be less then the control group because that is evidence of decrease sympathetic nerve activity. However, it is well known that difference in the reduction in BP after administration of ganglionic blockade between groups of animals may be due to mechanisms other than efferent sympathetic nerve acitivity. For example, it was possible that increased vascular responsiveness partially opposed a reduction in sympathetic nerve activity, yielding a smaller change from resting blood pressure. In agreement with this possibility, we did observe an increase pressor response to NE reactivity. Employing Hex raises the additional concern that the changes in BP reflect blocking both sympathetic and parasympathetic activity to the heart. The work by Fink and Ploucha (1996) suggest that this does not appear to be a factor in SHRs, because vagal blockade by itself had no effect on cardiac output or on arterial pressure in conscious rats. Furthermore, the vessels in SHRs are reportedly to be predominantly under sympathetic influence. In summary, the Hex results support the NE findings suggesting that metformin decreases sympathetic nerve activity and ultimately lowers BP.

Metformin not only lowered BP but also lowered Heart rate (HR) in both conscious and urethane-anesthetized SHRs. This finding is supported by Muntzel et al.,(1999) and Petersen et al., (1996). Petersen's study (1996), in which metformin was delivered by bolus injection, documented a bradycardia response in Saffan-anesthetized SHR rats. The Muntzel study (1999), which delivered metformin orally, also reported a bradycardia in conscious rats. In the urethane-anesthetized group from the present study, the lowering effect of metformin, we think, was enhanced by the anesthetic treatment. It should also be mentioned, however, that Petersen et al.,(1997) and Muntzel et al.,(1997) both reported a tachycardia response to metformin that is contradictory to the present finding. In these studies that reported tachycardia, metformin was delivered in a bolus injection. These reports illustrate the complex interaction of the anesthetic drugs and possible interactions with the drug, in this case metformin effect. Another consideration to the difference in result is that bolus injections will generate an atrial sinus reflex and/or a bainbridge reflex. In brief, both of these reflexes are triggered by an increase in blood volume registered in the atrium or in the ventricle of the heart resulting in a rapid but short-lived increase in HR. These reflexes compensate for brief increases in the load that the heart is to pump; this change in load must be compensated by an increase in HR which can explain the tachycardia reported in the Petersen et al., (1997) and Muntzel et al., (1997).

Altogether, these findings indicate that acute bolus metformin treatment has inconsistent effects on HR. However, long-term metformin treatment has consistently decreased HR (Muntzel *et al.*, 1999;Petersen *et al.*, 2000). A decrease in HR is commonly sited as evidence of decrease sympathetic nerve activity to the heart. Thus, the decrease in HR in these studies and in the present study is the third piece of evidence that suggests a suppression of sympathetic nerve activity by metformin. We can not, however, disregard the possibility that an increase in the parasympathetic system can cause a similar response.

Effects of metformin on Vascular Smooth muscle reactivity:

The goal of this study was to explain whether metformin decreases BP by lowering sympathetic nerve activity or by reducing vascular reactivity. Most of the previous vascular reactivity studies that examined metformin effects utilized arterial strip preparations treated with metformin for 2-24 hours (Bhalla *et al.*, 1996;Chen *et al.*, 1997;Dominguez *et al.*, 1996;Peuler *et al.*, 1997;Sharma and Bhalla., 1995). These studies reported decreases in vascular reactivity in metformin treated animals when challenged with pressor agents. For example, treatment with metformin caused reductions in contractile responses to NE (Peuler *et al.*, 1997;Verma *et al.*, 1996), PE (Chen *et al.*, 1997), and KCl (Peuler *et al.*, 1997).

The mechanism of inhibition is most likely related to calcium regulation since metformin reduced transient increases in intracellular calcium to PE (Chen *et al.*, 1997), arginine vasopressin (Bhalla *et al.*, 1996;Dominguez *et al.*, 1996), thrombin (Bhalla *et al.*, 1996;Dominguez *et al.*, 1996), and to angiotensin II

(Sharma and Bhalla., 1995). These inhibitory actions of metformin were observed in both cultured smooth muscle (Bhalla *et al.*, 1996;Dominguez *et al.*, 1996) and in arterial ring segments (Chen *et al.*, 1997). A series of studies by Chen's lab (Chen *et al.*, 1997) reported that metformin caused repolarization of PE-constricted rat tail artery segments. These effects were associated with decreases in intracellular calcium and reductions in isometric force, suggesting that metformin-induced relaxation is caused by repolarization and subsequent reductions in calcium influx (Chen *et al.*, 1997). The repolarization, in turn, may be secondary opening of K^+ channels since metformin-induced relaxations of PE-contracted arteries were attenuated by treatment with tetraethylammonium, a non-specific blocker of K^+ channels (Smith *et al.*, 1998).

Because all of these studies were performed in the acute setting, it is not clear whether alterations in contractility are related to long-lasting BP reductions with chronic metformin treatment. A more physiologically relevant study by Verma et al., (1994) showed a reduction in contractions to NE in the mesenteric arteries that were removed from rats that had been treated with metformin for 10 weeks. In the present study, we report findings in intact whole animals under metformin treatment for 3 wks. In contrast to the finding of Verma et al. (1994), metformin treated in the animals present study showed a significant increased BP response to PE and angiotensin II in a dose dependent manner before and after Hex treatment in urethane-anesthetized rats.

Our results contradict the prior body literature. In other words, the studies mentioned previously found a reduction of vascular reactivity in metformin-treated animals versus the present study that reports an increase in vascular reactivity. The increase response of BP in response to pressor agents may be explained by the catecholamine findings. Because NE levels were decreased in the metformin treated animals, we postulate that a state of supersensitivity is developed in those rats. Classical examples of such cases are abundant in the scientific literature. Sympathectomy by chemical (guanethidine, anti-NGF and 6-Hydroxydopamine) and surgical techniques consistently generate an increase BP response to NE compared with non-sympathectomy rats (Johnson and Manning., 1984). Several mechanisms are known to contribute to supersensitivity, but the extent and mechanism of the phenomenon varies from system to system. Mechanisms such as proliferation of receptors, loss of mechanisms for transmitter removal and increased postjunctional responsiveness are thought to play a role in this particular matter. In brief, proliferation of the adrenergic receptors has been observed in arteries from skeletal muscle, in which the number of receptors increases up to 20-fold or more after sympathectomy; the receptors are no longer restrictively localized on the endplate region of the fibers (Rang *et al.*, 1995). Loss of mechanisms for transmitter removal is another factor that can contribute to supersensitivity. The noradrenergic synapses that lose neuronal reuptake of NE contribute substantially to supersensitivity. Finally, an increase in postjunctional responsiveness occurs; in some instances postsynaptic cells become supersensitive without a corresponding increase in the number of receptors. Thus, smooth muscle cells become partly depolarized and hyperexcitable, and this phenomenon contributes to their supersensitivity. The mechanism for this change is not known.

Pharmacological reduction of sympathetic nerve activity mediated by metformin, if sustained for a few days, may cause a degree of supersensitivity of the target tissue. The smooth muscle cells in a supersensitive state, challenged by PE would result in the increase in BP responses (Rang *et al.*, 1995). The previous in vitro studies (Bhalla *et al.*, 1996;Chen *et al.*, 1997;Dominguez *et al.*, 1996;Peuler *et al.*, 1997;Sharma and Bhalla., 1995;Smith *et al.*, 1998) removed arteries from the rats. Once the arteries were removed, they were bathed with metformin for a short period of time. These arteries lack the sympathetic nerve activity input that generates supersensitivity. Therefore, a supersensitivity mechanism was not possible to develop in any of the in vitro studies. However, in the study by Verma et al.,(1994), the rats were treated with metformin for 10 wks and supersensitivity for NE should have occurred. However, when the arteries from the rats were challenged with NE, they exhibited a decreased response to the pressor agent. This discrepancy in results between their findings and the present study is difficult to explain. Verma's study examined SD rats versus our study that used SHRs. A second possibility that can help explain the divergent results is the fact that Verma examined only the mesenteric arteries, whereas we looked at BP increases that reflect responses from all of the arteries in the animal. For instance, it is possible that metformin decreased reactivity in the mesenteric arterial bed but at the same time increased reactivity in most of the other arterial beds of the animals, resulting in the observed increased of BP response to NE. In addition, Verma's study treated the animals with metformin for 10 wks, whereas, the rats in this study were under metformin treatment for 3 wks. This difference in time of treatment could lead to the divergent results.

Curiously, when the conscious rats were also challenged with PE, we found that the pressor response was again increased in the metformin treated group, but the difference was not significantly different. This difference in response between the conscious and urethane-anesthetized groups can be attributed to the anesthetic. Also by anesthetizing the animal, we are usually suppressing the baroreceptor response which contributes to any restraints that would after the response to pressor drugs (Gilman *et al.*, 1990).

The increases in blood pressure responses to angiotensin II stimulation are also difficult to explain. Sharma et al., (1995) observed a decrease in $[Ca^{+2}]_i$ transients in the metformin treated vascular smooth muscle cells when challenged with angiotensin II. This observation suggests that a decrease in vascular reactivity to the angiotensin II should be expected in the whole rat. In contrast, we reported an increase in vascular reactivity when the animals were challenged with angiotensin II. Sharma's study was performed on WKY rats that were treated with metformin for 1-24 hrs under in vitro settings. That is, once the vascular smooth muscle cells from the thoracic aorta were cultured for a week, the cells were then treated with metformin. Thus, although it is difficult to explain the discrepancy between Sharma's results and our results, the differences are again, related to the fact that they examined vascular strips, whereas, we examined the whole animal.

In summary, metformin increased vascular reactivity, specifically to PE and angiotensin II. This effect does not contribute to the long-term decreases of BP mediated by metformin.

Renal, iliac and mesenteric dopplers were employed to assess blood flow reactivity to pressor agents at specific arterial beds. As stated in the results section, dopplers are registering increases and/or decreases in blood velocity which are directly proportional to increases and/or decreases in blood flow. In our early experiments, our analysis of the mesenteric arterial blood flow was plagued with technical difficulties. The animals usually went into shock. Because of this, we stopped blood flow analysis of the mesenteric vascular bed. In pilot conscious studies, the rats appeared sickly and uncomfortable after implantation of the doppler blood probes. Therefore, we decided to perform all of our blood flow experiments in anesthetized rats.

The renal and iliac blood flow responses to PE and the iliac responses to angiotensin II were simply transient increases in blood flow. It will be recalled that blood flow in any specific vascular bed is determined by blood pressure divided by resistance in that specific bed. Therefore, the increase in blood flow elicited by PE and angiotensin II was probably caused by a relatively larger increase in blood pressure relative to resistance in that specific vascular bed. In contrast, the renal vascular bed had a different response to angiotensin II. We observed a biphasic response, that is, a rise in blood flow immediately followed by a fall in blood flow. At first, the response was similar to the PE response, an increased blood flow due to a higher blood pressure level relative to the resistance in the vascular bed. This was immediately followed by a decrease blood flow response to the pressor agent. This decrease is thought to be because angiotensin II caused vasoconstriction that is strongest in the kidneys and in the splanchnic vascular beds; blood flow in these regions falls sharply when angiotensin II is infused. Angiotensin II has differential effects on the tone of vascular beds throughout the circulatory system. For instance, angiotensin II-induced vasoconstriction is less in vessels skeletal muscle. In this region blood flow actually may increase, especially following small changes in the concentration of angiotensin II, because the relatively weak vasoconstrictor response in opposed by the elevated systemic blood pressure (Gilman *et al.*, 1990).

It will be recalled that we observed increased BP responses to PE in metformin rats relative to controls. The enhanced pressure response in metformin rats was presumably caused by increased contraction of arteries in the body. Because blood flow in any vascular bed is determined by BP divided by resistance to that specific vascular bed, we expected an enhanced resistance causing a decrease in blood flow elevations relative to the control rats.

In contrast to what was expected, there were no differences in blood flow responses between the metformin treated and the control groups in the renal or iliac arterial beds when challenged with PE. The same paradigm was performed with angiotensin II as the pressor agent to assess if metformin's vascular reactivity effects were specific to a single pressor system. The renal doppler response to angiotensin II elicited a biphasic response. This biphasic response was not significantly different between the metformin and control group. In the iliac arterial system, we found no significant difference in the metformin vs. control group to PE and angiotensin II. Overall, the absence in blood flow differences between the two groups, in the arterial beds assessed can be attributed to blood flow changes in other arterial beds branching off the descending aorta. In other words, the descending aorta has more branches than the renal and iliac arteries. These branches can mask the effects that occurred in the vascular beds that we were analyzing by either increasing or decrease blood flow leading to a lack in response in the vascular beds of interest. Another possibility is that the doppler method was not sufficiently sensitive to register small differences in blood flow between metformin and the control groups.

Summary-Conclusion:

In summary, conscious and urethane-anesthetized rats under chronic metformin treatment showed decreases in BP. This lowering effect was independent of weight loss. The purpose of this study was to examine if metformin's mediated BP decreases were through chronic decreases in sympathetic nerve activity, mediated by decreases in vascular reactivity, or by both mechanisms. We found that metformin decreased sympathetic nerve activity, but did not reduce vascular reactivity.

Sympathetic nerve activity was assessed by a direct measurement of NE spill over. NE plasma concentrations were found to be lower in metformin treated animals versus controls. We also assessed sympathetic nerve activity using Hex. It will be recalled that this method does not estimate levels of central neural outflow passing through the sympathetic nerves, but rather indicates whether the overall vasoconstriction function of the sympathetic nervous system is reduced in one group relative to the other group. The fall in BP to Hex tended to be less in the metformin treated group indicating reduced sympathetic nerve activity. As a further indicator of lower sympathetic nerve activity, we found that metformin caused chronic decreased HR.

In order to determine whether metformin lowered BP by an alternative mechanism, vascular reactivity was also assessed. In contrast to our expectations, metformin treated rats showed an increase in, vascular reactivity. This increase was attributed to supersensitivity generated by a decreased amount of neurotransmitter, specifically NE. The fact that metformin decreased vascular reactivity in *in vitro* studies but increased vascular reactivity *in vivo* (present study) is very interesting and merits further investigation. This increase in vascular reactivity may explain why the fall in BP to Hex in metformin treated rats was larger than expected.

Metformin, an antihyperglycemic agent, is gaining popularity in the United States. This is due to the many complications that can arise from the use of insulin (insulin insensitivity) and from the increasing population suffering from NIDDM. The reports of lowering blood pressure with metformin may increase this drug's demand. But with still so much to go in order to understand hypertension as a whole, I do not see metformin as a treatment for this specific ailment. We must keep in mind that this beneficial side effect is not reported in all patients under metformin treatment. This supports and implies that diabetes and hypertension are extremely complex by themselves, and if one tries to find a common factor between the two, she will be looking for a very long time, and during such a time many misdiagnoses can occur. If metformin was to be taken in combination with a cardiovascular drug, interactions between drugs are sure to occur. These interactions should be researched and reported for the benefit of the general diabetic population.

References

EA Anderson and AL Mark (1993). "The vasodilator action of insulin. Implications for the insulin hypothesis of hypertension [editorial] [published erratum appears in Hypertension 1993 May;21(5):745] [comment]." *Hypertension* **21**(2): 136-41.

CJ Bailey and C Day (1989). "Traditional plant medicines as treatments for diabetes." *Diabetes Care* **12**: 553-64.

CJ Bailey and RC Turner (1996). "Metformin." *N Engl J Med* **334**(9): 574-9.

R Beckmann (1969). "Resorption, Verteilung im Organismus und Ausscheidung von Metformin." *Diabetologia* **5**: 318-24.

RC Bhalla,KF Toth *et al.* (1996). "Vascular effects of metformin. Possible mechanisms for its antihypertensive action in the spontaneously hypertensive rat." *Am J Hypertens* **9**(6): 570-6.

D Bild and SM Teutsch (1987). "The control of hypertension in persons with diabetes: a public health approach." *Public Health Rep* **102**(5): 522-9.

AL Calle-Pascual,J Garcia-Honduvilla *et al.* (1995). "Comparison between acarbose, metformin, and insulin treatment in type 2 diabetic patients with secondary failure to sulfonylurea treatment." *Diabete Metab* **21**(4): 256-60.

IW Campbell,C Duncan *et al.* (1987). "The effect of metformin on glycaemic control, intermediary metabolism and blood pressure in non-insulin-dependent diabetes mellitus." *Diabet Med* **4**(4): 337-41.

JC Chan,B Tomlinson *et al.* (1993). "Metabolic and hemodynamic effects of metformin and glibenclamide in normotensive NIDDM patients." *Diabetes Care* **16**(7): 1035-8.

XL Chen,K Panek and CM Rembold (1997). "Metformin relaxes rat tail artery by repolarization and resultant decreases in Ca2+ influx and intracellular [Ca2+]." *J Hypertens* **15**(3): 269-74.

RD Ciaranello (1978). "Regulation of phenylethanolamine N-methyltransferase synthesis and degradation.I. Regulation by rat adrenal glucocorticoids." *Mol. Pharcacol.* (14): 478-489.

Copenhagen,Levin and Munksgaard (1937). The Papyrus Ebers: The greatest Egyptian Medical Document.

JJ Corcoran,SP Wilson and N Kirshner (1984). "Flux of catecholamines through chromaffin vesicles in cultured bovine adrenal medullary cells." *J. Biol. Chem.* (259): 6208-6214.

MP Czech (1995). "Molecular actions of insulin on glucose transport." *Annu Rev Nutr* **15**: 441-71.

LJ Dominguez,AJ Davidoff *et al.* (1996). "Effects of metformin on tyrosine kinase activity, glucose transport, and intracellular calcium in rat vascular smooth muscle." *Endocrinology* **137**(1): 113-21.

M Dorella,M Giusto *et al.* (1996). "Improvement of insulin sensitivity by metformin treatment does not lower blood pressure of nonobese insulin-resistant hypertensive patients with normal glucose tolerance." *J Clin Endocrinol Metab* **81**(4): 1568-74.

B Draznin,S Melmed and D LeRoth (1989). Molecular and Cellular Biology of Diabetes Mellitus. New York, Alan R. Liss, Inc. **3:**

M Epstein and JR Sowers (1992). "Diabetes mellitus and hypertension." *Hypertension* **19**(5): 403-18.

MJ Evinger,AC Towle *et al.* (1992). "Glucocorticoids stimulate transcription of the rat phenylethanolamine N-methyltransferase (PNMT) gene in vivo and in vitro." *Cell. Mol. Neurobiol.* (12): 193-215.

G Fanghanel,L Sanchez-Reyes *et al.* (1996). "Metformin's effects on glucose and lipid metabolism in patients with secondary failure to sulfonylureas." *Diabetes Care* **19**(11): 1185-9.

D Finch,G Davis,J Bower and K Kirchner (1990). "Effect of insulin on renal sodium handling in hypertensive rats." *Hypertension* **15**(5): 514-8.

RO Gans,L v.d. Toorn *et al.* (1991). "Renal and cardiovascular effects of exogenous insulin in healthy volunteers." *Clin Sci (Colch)* **80**(3): 219-25.

AG Gilman,TW Rall,AS Nies and P Taylor (1990). Goodman and Gilman's The Pharmacological Basis of Therapeutics 8th edition. Pergamon Press.

D Giugliano,N De Rosa *et al.* (1993a). "Metformin improves glucose, lipid metabolism, and reduces blood pressure in hypertensive, obese women." *Diabetes Care* **16**(10): 1387-90.

D Giugliano,A Quatraro *et al.* (1993b). "Metformin for obese, insulin-treated diabetic patients: improvement in glycaemic control and reduction of metabolic risk factors." *Eur J Clin Pharmacol* **44**(2): 107-12.

PJ Grant (1996). "The effects of high- and medium-dose metformin therapy on cardiovascular risk factors in patients with type II diabetes." *Diabetes Care* **19**(1): 64-6.

S Gudbjornsdottr,P Friberg *et al.* (1994). "The effect of metformin and insulin on sympathetic nerve activity, norepinephrine, spillover and blood pressure in obese, insulin resistant, normoglycemic, hypertensive men." *Blood pressure* **3**: 394-403.

MI Harris (1998). "Diabetes in America: epidemiology and scope of the problem." *Diabetes Care* **21 Suppl 3**: C11-4.

E Haupt,B Knick *et al.* (1991). "Oral antidiabetic combination therapy with sulphonylureas and metformin." *Diabete Metab* **17**(1 Pt 2): 224-31.

LS Hermann and A Melander (1992). Biguinades: basic aspects and clinical uses. International textbook of diabetes mellitus. A. K.G.M.M. ,R. A. Defronzo ,H. Keen and P. Zimmet. Chichester, England, Wiley, J. **1:** 773-95.

WA Hsueh and RE Law (1998). "Cardiovascular risk continuum: implications of insulin resistance and diabetes." *Am J Med* **105**(1A): 4S-14S.

Y Ido,A Vindigni *et al.* (1997). "Prevention of vascular and neural dysfunction in diabetic rats by C-peptide." *Science* **277**(5325): 563-6.

JT Ireland,WST Thomson and J Williamson (1980). Diabetes today, A handbook for clinical team. Springer Publishing Company.

L Jansson (1995). "Effects of the biguanide metformin on splanchnic blood flow in rats: Preferential and dose-dependent increases in islet blood flow." *Pharmacology* (51): 43-47.

EM Johnson and PT Manning (1984). "Guanethidine-Induced destruction of sympathetic neurons." *International Review of Neurology* **25**: 1-37.

BB Kahn (1992). "Alterations in glucose transporter expression and function in diabetes: mechanisms for insulin resistance." *J Cell Biochem* **48**(2): 122-8.

V Kapoor and JP Chalmers (1987). "A simple, sensitive method for the determination of extracellular catecholamines in the hypothalamus using in vivo dialysis." *J. Neuroscience Methods* **19**: 173-182.

T Kayano,CF Burant *et al.* (1990). "Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoform (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogene-like sequence (GLUT6)." *J Biol Chem* **265**(22): 13276-82.

GL King,AD Goodman *et al.* (1985). "Receptors and growth-promoting effects of insulin and insulinlike growth factors on cells from bovine retinal capillaries and aorta." *J Clin Invest* **75**(3): 1028-36.

I Kosegawa,S Katayama *et al.* (1996). "Metformin decreases blood pressure and obesity in OLETF rats via improvement of insulin resistance." *Hypertens Res* **19**(1): 37-41.

AJ Krentz,RE Ferner and CJ Bailey (1994). "Comparative tolerability profiles of oral antidiabetic agents." *Drug Safety* **11**: 223-41.

K Landin,L Tengborn and U Smith (1991). "Treating insulin resistance in hypertension with metformin reduces both blood pressure and metabolic risk factors." *J Intern Med* **229**(2): 181-7.

JM Lord,SI White *et al.* (1983). "Effect of metformin on insulin receptor binding and glycaemic control in type II diabetes." *Br Med J (Clin Res Ed)* **286**(6368): 830-1.

DA Morgan,CA Ray,TW Balon and AL Mark (1992). "Metformin increases insulin sensitivity and lowers arterial pressure in spontaneuosly hypertensive rats." *Hypertension* **20**: 421A.

M Muntzel,T Beltz,AL Mark and AK Johnson (1994). "Anteroventral third ventricle lesions abolish lumbar sympathetic responses to insulin." *Hypertension* **23**(6 Pt 2): 1059-62.

MS Muntzel,A Abe and JS Petersen (1997). "Effects of adrenergic, cholinergic and ganglionic blockade on acute depressor responses to metformin in spontaneously hypertensive rats." *J Pharmacol Exp Ther* **281**(2): 618-23.

MS Muntzel,B Nyeduala and S Barrett (1999). "High dietary salt enhances acute depressor responses to metformin." *Am. J. Hypertension* **12**(12 Pt 1-2): 1256-9.

S O'Rahilly,RC Turner and DR Matthews (1988). "Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes." *N Engl J Med* **318**(19): 1225-30.

M Odawara,T Kadowaki *et al.* (1989). "Human diabetes associated with a mutation in the tyrosine kinase domain of the insulin receptor." *Science* **245**(4913): 66-8.

K Okamoto and K Aoki (1963). "Development of a strain of spontaneous hypertensive rats." *Jap. Circ. J.* **27**: 282.

G Pagano,V Tagliaferro *et al.* (1983). "Metformin reduces insulin requirement in Type 1 (insulin-dependent) diabetes." *Diabetologia* **24**(5): 351-4.

E Peschke and D Peschke (1998). "Evidence for a circadian rhythm of insulin release from perifused rat pancreatic islets." *Diabetologia* **41**(9): 1085-92.

JS Petersen,D Andersen *et al.* (2000). "Intraventricular metformin attenuates salt-induced hypertension in spontaneously hypertensive rats." *Journal of Pharmacological and Exp. Ther.* **Submitted 01/00**:

JS Petersen and GF DiBona (1996). "Acute sympathoinhibitory actions of metformin in spontaneously hypertensive rats." *Hypertension* **27**(3 Pt 2): 619-25.

JS Petersen,W Liu,DR Kapusta and KJ Varner (1997). "Metformin inhibits ganglionic neurotransmission in renal nerves." *Hypertension* **29**(5): 1173-7.

RG Petty and JD Pearson (1992). "The influence of hypoglycaemic agents on the growth and metabolism of human endothelial cells." *Diabet Med* **9**(1): 30-7.

JD Peuler (1997). "Adrenergic depressor and pressor actions of metformin in spontaneously hypertensive rats (SHR)." *American Journal of Hypertension* **10**(4): 107A.

JD Peuler,JA Miller *et al.* (1997). "Disparate effects of antidiabetic drugs on arterial contraction." *Metabolism* **46**(10): 1199-205.

MA Pfeffer and ED Frohlich (1973). "Hemodynamic and myocardial function in young and old normotensive and spontaneously hypertensive rats." *Circ. Res.* **32-33**(Suppl I): 28.

KS Polonsky,BD Given *et al.* (1988). "Abnormal patterns of insulin secretion in non-insulin-dependent diabetes mellitus." *N Engl J Med* **318**(19): 1231-9.

HM Raabe,JH Hopner *et al.* (1998). "Biochemical and biophysical alterations of the 7S and NC1 domain of collagen IV from human diabetic kidneys." *Diabetologia* **41**(9): 1073-9.

RA Rabini,R Staffolani *et al.* (1998). "Decreased nitric oxide synthase activity in platelets from IDDM and NIDDM patients." *Diabetologia* **41**(1): 101-4.

HP Rang,MM Dale,JM Ritter and P Gardner (1995). Pharmacology. New York, Edingburgh, London, Melbourne, Tokyo, Churchill Livingstone.

S Seifter and S Englard (1990). Carbohydrate metabolism. In diabetes mellitus: theory and practice. H. Rifkin and D. J. Porte. New York: Elsevier Science Publishing Co., Inc. 1-40.

A Semplicini,S Del Prato *et al.* (1993). "Short-term effects of metformin on insulin sensitivity and sodium homeostasis in essential hypertensives." *J Hypertens Suppl* **11 Suppl 5**: S276-7.

RV Sharma and RC Bhalla (1995). "Metformin attenuates agonist-stimulated calcium transients in vascular smooth muscle cells." *Clin Exp Hypertens* **17**(6): 913-29.

JM Smith,JM Lee and JD Peuler (1998). "Effects of tetraethylamonium (TEA) on acute relaxation of adrenergic tension by metformin (MET)." *FASEB J.* **12**(4): A96.

PR Standley,F Zhang *et al.* (1991). "Insulin attenuates vasopressin-induced calcium transients and a voltage- dependent calcium response in rat vascular smooth muscle cells." *J Clin Invest* **88**(4): 1230-6.

J Sterne (1969). "Pharmacology and mode of action of hypoglycemic guanidine derivatives." *Med. Chem. Ser. Monogr.* **9**: 193-245.

RW Stout,EL Bierman and R Ross (1975). "Effect of insulin on the proliferation of cultured primate arterial smooth muscle cells." *Circ Res* **36**(2): 319-27.

T Temelkova-Kurktschiev,C Koehler *et al.* (1998). "Relationship between fasting plasma glucose, atherosclerosis risk factors and carotid intima media thickness in non-diabetic individuals." *Diabetologia* **41**(6): 706-12.

M Trovati and G Anfossi (1998). "Insulin, insulin resistance and platelet function: similarities with insulin effects on cultured vascular smooth muscle cells." *Diabetologia* **41**(6): 609-22.

ML Tuck (1992). "Obesity, the sympathetic nervous system, and essential hypertension." *Hypertension* **19**(1 Suppl): I67-77.

EM Velazquez,S Mendoza *et al.* (1994). "Metformin therapy in polycystic ovary syndrome reduces hyperinsulinemia, insulin resistance, hyperandrogenemia, and systolic blood pressure, while facilitating normal menses and pregnancy." *Metabolism* **43**(5): 647-54.

S Verma,S Bhanot and JH McNeill (1994a). "Antihypertensive effects of metformin in fructose-fed hyperinsulinemic, hypertensive rats." *J Pharmacol Exp Ther* **271**(3): 1334-7.

S Verma,S Bhanot and JH McNeill (1994b). "Metformin decreases plasma insulin levels and systolic blood pressure in spontaneously hypertensive rats." *Am J Physiol* **267**(4 Pt 2): H1250-3.

S Verma,S Bhanot and JH McNeill (1996). "Decreased vascular reactivity in metformin-treated fructose- hypertensive rats." *Metabolism* **45**(9): 1053-5.

LE Wagenknecht,RB D'Agostino, Jr. *et al.* (1998). "Impaired glucose tolerance, type 2 diabetes, and carotid wall thickness: the Insulin Resistance Atherosclerosis Study." *Diabetes Care* **21**(11): 1812-8.

H Winkler,M Sietzen and M Schober (1987). "The life cycle of catecholamine-storing vesicles." *Ann. N.Y. Acad. Sci.* (493): 3-19.

DJ Withers,JS Gutierrez *et al.* (1998). "Disruption of IRS-2 causes type 2 diabetes in mice." *Nature* **391**(6670): 900-4.

DL Wong,EL Zager and RD Ciaranello (1982). "Effects of hypophysectomy and dexamethasone administration on central and peripheral S-adenosylmethionine levels." *J. Neurosci.* (2): 758-764.

I Yokota,J Matsuda *et al.* (1998). "Comparison of GAD and ICA512/IA-2 antibodies at and after the onset of IDDM." *Diabetes Care* **21**(1): 49-52.