INSULIN AND HYPERTENSION: A PHARMACOLOGICAL PERSPECTIVE

by

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ABSTRACT

Although considerable data lend support to the association between insulin resistance, hyperinsulinemia and hypertension, the precise nature of this relationship remains elusive. In the present study, we examined the proposition that these metabolic defects contribute causally to the development of high blood pressure. Essentially, if these metabolic abnormalities were responsible for an increase in blood pressure, then drugs that improve these metabolic defects should also attenuate hypertension. We, therefore, examined the effects of three drugs (that are known to enhance insulin action) on insulin sensitivity, plasma insulin levels and blood pressure in two established models of experimental hypertension: (a) the spontaneously hypertensive rat and (b) the fructose-hypertensive rat, where hypertension is induced in normotensive rats by feeding them a high fructose diet. The drug interventions were: (a) vanadyl sulfate, the (+IV) form of the trace element vanadium (b) bis(maltolato)oxovanadium(IV) (BMOV), an organic vanadium complex and (c) pioglitazone, a thiazolidinedione derivative that enhances peripheral insulin action.

In separate studies, 6 week old spontaneously hypertensive rats (SHR) and their Wistar Kyoto (WKY) controls were started on chronic oral treatment with vanadyl sulfate (0.4-0.6 mmol/kg/day), BMOV (0.35-0.45 mmol/kg/day) or pioglitazone (0.01-0.02 mmol/kg/day). All 3 drugs caused a sustained decrease in plasma insulin concentration in the hyperinsulinemic SHR without causing any change in the WKY. Surprisingly, all the drugs caused a marked decrease in systolic blood pressure in the SHR without causing any change in the WKY. Restoration of plasma insulin levels in the drug-treated SHR to levels that existed in the untreated SHR reversed the effect/s of the drugs on blood pressure. Low dose euglycemic insulin clamps (14 pmol/kg/min) conducted in conscious, fasted

rats revealed that insulin sensitivity, expressed as steady state glucose clearance per unit of plasma insulin, was higher in the untreated SHR as compared to the untreated WKY. Although BMOV further enhanced insulin sensitivity in the SHR, pioglitazone had no effect on insulin sensitivity in the SHR or WKY.

Fructose feeding induced hyperinsulinemia and increased blood pressure in normotensive Sprague Dawley rats. Vanadyl sulfate (0.4-0.6 mmol/kg/day) prevented the rise in plasma insulin and blood pressure in the fructose-fed rats. Again, restoration of plasma insulin concentration in the fructose-vanadyl treated rats to pre-treatment levels reversed the effects of vanadyl sulfate on blood pressure. Low dose insulin clamps demonstrated that insulin sensitivity was reduced in the fructose-fed rats. Vanadyl caused a marked enhancement in insulin sensitivity in the fructose-fed rats without any change in the control group.

In conclusion: (i) SHR are not insulin-resistant but rather are more insulin-sensitive than the WKY (ii) SHR are hyperinsulinemic and drug interventions that decrease hyperinsulinemia also attenuate hypertension in the SHR (iii) The effect of the drugs on blood pressure can be reversed by restoring plasma insulin levels in the drug-treated SHR to those observed in their untreated counterparts (iv) The antihypertensive effects of pioglitazone in the SHR are independent of its effects on insulin sensitivity, which suggests that hyperinsulinemia may be unrelated to insulin resistance in the SHR (v) Vanadyl sulfate completely prevents fructose-induced insulin resistance, hyperinsulinemia and hypertension. These data indicate that either hyperinsulinemia may contribute to the development of high blood pressure in both the SHR and the fructose-hypertensive rats or that the underlying mechanism is closely related to the expression of both these disorders.

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LIST OF ABBREVIATIONS

ATPase	adenosine triphosphatase
BMOV	bis(maltolato)oxovanadium(IV)
BP	blood pressure
Ca^{+2}	calcium
cGMP	guanosine 3'-5'-cyclic monophosphate
DNA	deoxyribonucleic acid
EC_{50}	half-maximally effective concentration
ED_{50}	half-maximally effective dose
EGTA	ethylene glycol bis(b-aminoethyl ether-P-N,N,N',N'-tetraacetic acid)
GINF	glucose infusion rate
GOT	glutamic oxaloacetic transaminase
GPT	glutamic pyruvic transaminase
HGO	hepatic glucose output
K^+	potassium
LD_{50}	dose that is lethal to 50% of the treated animals
Na^+	sodium
NIDDM	non insulin dependent diabetes mellitus
PE	polyethylene
R_a	rate of glucose appearance
R_d	rate of glucose utilization = rate of total glucose disposal
SHR	spontaneously hypertensive rats
STZ	streptozotocin
VSM	vascular smooth muscle
WKY	Wistar-Kyoto rats

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DEDICATION

To my grandfather Mr. L. R. Sharma.....

.....who was my pillar of strength and source of inspiration

.....who brought joy and happiness to all those he knew

.....whose love, warmth and affection will always be cherished

INTRODUCTION

(I) ESSENTIAL HYPERTENSION: A METABOLIC DISEASE

One of the paradoxes in medicine is the inability of effective antihypertensive drugs to reduce the increased risk of coronary artery disease in hypertensive subjects (Collins *et al.,* 1990; Koerner *et al.,* 1982; Wikstrand *et al.,* 1988). Several reports indicate that although lowering BP reduces the incidence of cerebrovascular disease, it does not alter the mortality and morbidity associated with coronary artery disease. MacMahon et al., by applying the technique of meta-analysis, reviewed many of these studies (MacMahon *et al.,* 1989) and found that treating hypertension reduced all cause mortality by 11% and non fatal stroke by 39%, both of which were statistically significant reductions. However, the incidence of coronary artery disease decreased by a mere 8%, a difference that did not attain statistical significance. Although this apparent paradox has received considerable attention, most of it has focused on the notion that conventional antihypertensive therapy is associated with adverse side effects on lipid metabolism that could accentuate the risk for coronary artery disease (MacMahon *et al.,* 1985; Weinberger, 1986). Although this contention is based on good evidence, it probably addresses only one aspect of a multifaceted problem.

A finding that was overlooked until the last decade is that essential hypertension per se is associated with multiple metabolic abnormalities in carbohydrate and lipid metabolism (Ferrannini and Natali, 1991; Reaven 1991b). The metabolic defects associated with hypertension include insulin resistance, hyperinsulinemia and dyslipidemia (DeFronzo and Ferrannini 1991; Ferrannini *et al.*, 1987; Reaven 1991a,b), all of which have been shown to increase the risk for

coronary ischemic events (Kannell *et al.*, 1991; MRFIT research group 1986; Sowers *et al.*, 1991). What arouses particular concern are findings suggesting that these metabolic abnormalities persist and even worsen with conventional antihypertensive agents, particularly the thiazide diuretics and the beta adrenergic blockers (Lind *et al.,* 1994; Pollare *et al.,* 1989; Skarfors *et al.,* 1989; Weinberger 1986). Of these metabolic defects, two that seem to be frequently associated with hypertension are insulin resistance (or resistance to the glucoregulatory effects of insulin) and hyperinsulinemia (Ferrannini and Natali, 1991; Reaven 1994; Weidmann *et al.,* 1993). These defects in glucose metabolism are associated with an atherogenic risk profile and evidence suggests that they may play a role in the development of hypertension, dyslipidemia and atherosclerosis (DeFronzo and Ferrannini, 1991; Haffner *et al.,* 1992; Reaven 1988; Reaven 1994).

Lending further support to this hypothesis are recent reports indicating that insulin resistance and hyperinsulinemia are present in the normotensive offspring of patients with hypertension as early as the second decade of life (Allemann and Weidmann 1995; Grunfeld *et al.,* 1994). These changes, therefore, antedate the increase in BP and are not found in patients with secondary forms of hypertension (Shamiss *et al.,* 1992). The observation that insulin resistance and hyperinsulinemia occur not only in untreated human hypertensives (Ferrannini *et al.,* 1987; Shen *et al.,* 1988), but also in several rodent models of hypertension (Kotchen *et al.,* 1991; Mondon and Reaven 1988; Reaven *et al.,* 1991a) strengthens the contention that these abnormalities are intrinsically linked with hypertension and are not mere coincidental findings. Studies indicate that hyperinsulinemia is an independent risk factor for coronary artery disease (Ducimetiere *et al.,* 1980; Pyorala 1979) and that even a small degree of glucose intolerance significantly increases the risk of developing coronary heart disease

(Fuller *et al.,* 1980). Another finding that strongly supports this intriguing association is that patients with hypertension who have microvascular angina are insulin-resistant and hyperinsulinemic when compared to hypertensive subjects without ischemic heart disease (Botker *et al.,* 1993; Dean *et al.,* 1991; Fuh *et al.,* 1993). Such findings are provocative and further research efforts are needed to define these inter-relationships precisely. Given the fact that we still do not have a satisfactory answer to the coronary disease paradox, such issues deserve special consideration. The primary objective of the current investigation was to explore the relationship between insulin resistance, hyperinsulinemia and hypertension. Specifically, the proposition that these metabolic abnormalities may be causally related to hypertension was examined. Before addressing the specific research problem, it is important to review some of the epidemiological evidence linking insulin to high BP, which is discussed in the following section.

(II) INSULIN AND HYPERTENSION: EPIDEMIOLOGY

(i) Studies supporting the link between insulin and hypertension

Welborn et al. were the first to suggest an association between defective insulin action and hypertension (Welborn *et al.,* 1966). They examined 19 subjects with essential hypertension (10 of whom were untreated) and found higher fasting and postprandial insulin levels in both treated and untreated hypertensive patients when compared to control, normotensive subjects. This initial finding was subsequently confirmed in several large epidemiological studies. Berglund et al. screened middle aged Swedish hypertensive men who had no other overt clinical disease and who were not taking any antihypertensive therapy (Berglund *et al.,* 1976). They reported that both fasting insulin and glucose concentrations were higher in hypertensive patients as compared to their

normotensive controls. These results were confirmed in another extensive study from Israel (Modan *et al.,* 1985). Results from the study by Modan et al., which involved about 2500 subjects, demonstrated that hypertensive patients exhibited fasting and postprandial hyperinsulinemia independent of obesity, age or magnitude of glucose tolerance. This observation has since been confirmed by several epidemiological and clinical studies (Lucas *et al.,* 1985; Manicardi *et al.,* 1986; Swislocki *et al.,* 1989), which have documented the presence of irregularities in carbohydrate metabolism in young, non obese subjects with untreated, uncomplicated essential hypertension. In a different approach, Singer et al. compared the day long insulin and glucose profiles in response to standard meals in untreated hypertensive patients and found that the insulin response after each meal was markedly increased in hypertensive patients (Singer *et al.,* 1985). Results from their study indicated that essential hypertensive subjects not only had an increased insulin response to a glucose challenge but that this exaggerated insulin response occurred after every meal that they consumed.

(ii) <u>Studies refuting the link between insulin and hypertension</u>

Not all hypertensive patients are hyperinsulinemic and several studies demonstrate a weak correlation between insulin and high BP (Asch *et al.,* 1991; Collins *et al.,* 1990). In addition, it was reported that insulinemia was not related to the prevalence of hypertension in Pima Indians after controlling for obesity and drug treatment (Saad *et al.,* 1990). Mbanya et al. demonstrated that plasma insulin levels were similar between hypertensive and normotensive subjects and that insulin levels were increased only in those hypertensive patients who also had NIDDM (Mbanya *et al.,* 1988). In many studies, the correlation between plasma insulin levels and hypertension became weak after accounting for obesity

(Meehan *et al.,* 1993b). Due to a virtual flurry of reports examining the relationship between insulin and hypertension, this area has attracted considerable discussion and debate. In an effort to resolve this problem, Denker and Pollock performed a meta-analysis on the various studies reported between the years 1983-1991. They included only those studies that were conducted in untreated hypertensives or in which an adequate washout period was included as part of the study protocol and in which the subjects were neither glucose intolerant or diabetic (Denker and Pollock, 1992). The results of their meta-analysis indicated that fasting serum insulin concentration was strongly correlated with both systolic and diastolic BP when data from all the studies were pooled together to yield a statistically meaningful result.

Despite the controversy that exists due to the disparate results of almost 50 different studies reported in the last 10 years, several consistencies have emerged regarding this association. First, as many as 50% of hypertensive patients appear to be insulin-resistant and hyperinsulinemic when compared to age and weight matched normotensive controls (Denker and Pollock 1992; Zavaroni *et al.,* 1992). There is ethnic variation in this relationship such that significant correlations between these metabolic defects and hypertension exist in Caucasian, Hispanic White and Japanese populations but not in Afro-Americans or Pima Indians (Miura *et al.,* 1995; Saad *et al.,* 1991). However, what must be considered is the fact that correlations do not prove causality. A lack of correlation in an epidemiological study does not rule out a role of insulin in the development of hypertension in that population. For example, although Saad et al. found that insulin concentration and hypertension were not correlated in Afro-Americans, subsequent studies demonstrated that hypertensive Afro-Americans

were both insulin-resistant and hyperinsulinemic when compared with normotensive controls (Falkner *et al.,* 1990; Falkner *et al.,* 1993).

Second, it was reported in several studies that the correlation between insulin and BP became weak or even non significant after accounting for obesity. This led some investigators to propose that obesity may be the primary factor that causes or modulates insulin resistance in hypertensive subjects. Although several studies had indicated that insulin resistance was present even in lean, untreated hypertensive patients, the role of hypertension in modulating insulin resistance in obese subjects remained undefined. It was not clear whether the insulin resistance observed in obese, hypertensive subjects was due to obesity per se or high BP or both. The answer to this dilemma came from a recent study in which insulin resistance was quantified in 5 different groups of subjects: normotensive-obese, normotensive-nonobese, hypertensive-obese, hypertensive-nonobese and hypertensive-obese with NIDDM (Maheux *et al.,* 1994). Their results unequivocally demonstrated that the effects of obesity, hypertension and NIDDM on insulin resistance are additive and that each one of these diseases independently contributes towards resistance to insulin's glucoregulatory effects. Taken together, current evidence indicates that insulin resistance and hyperinsulinemia are present in a substantial proportion of hypertensive patients. Furthermore, this relationship is stronger among some ethnic groups than in others and is independent of age, obesity or drug treatment.

(iii) <u>Prospective studies in normotensive offsprings of hypertensive subjects</u>

Although insulin resistance and hyperinsulinemia correlated positively with high BP in a considerable proportion of hypertensive patients, it was not clear whether these defects were primary or whether they were a consequence of

hypertension. In the first report that dealt with this issue, insulin sensitivity and plasma insulin levels in normotensive offsprings of essential hypertensive parents were compared with those obtained from age and weight matched normotensive subjects with no parental history of hypertension (Ferrari *et al.,* 1991). It was found that young, lean, normotensive adults (mean age about 24 years) who had a positive family history of hypertension were insulin-resistant and hyperinsulinemic when compared to normotensive controls without a hypertensive parent. Insulin sensitivity was decreased by about 28% and insulin levels were increased by about 15% in subjects with a parental history of hypertension, which indicated that these defects antedate the increase in BP and are not secondary to hypertension. Almost identical results were reported by Facchini et al. one year later; in their study the majority of subjects were female as opposed to the study by Ferrari et al. in which the subjects were predominantly male (Facchini *et al.,* 1992). These results indicated that the link between insulin and hypertension had a genetic basis that was independent of age, gender or body weight.

Subsequent studies extended these observations and demonstrated that the reduced insulin sensitivity that antedated the increase in BP in normotensive adults with a positive family history of hypertension was also accompanied by a higher platelet intracellular calcium concentration (Ohno *et al.,* 1993). This suggested that disturbed intracellular calcium metabolism was also an inherited trait and raised the possibility that this intracellular defect could be a link between insulin and hypertension. However, it was not clear whether the abnormal calcium metabolism was a cause or consequence of insulin resistance, a question that remains unanswered to date. A recent study demonstrated that hyperinsulinemia was present even in young children (mean age about 14 years) who were normotensive, normolipidemic but had a positive family history of

hypertension, which strongly suggests that defects in insulin action antedate most of the metabolic and hemodynamic abnormalities seen in hypertensive subjects (Grunfeld *et al.,* 1994). The observation that insulin resistance can be modified by environmental influences such as body weight or physical exercise (DeFronzo and Ferrannini, 1991) suggests that the final phenotypic expression of these defects is probably a combination of both genetic and acquired influences. Nevertheless, the studies discussed above clearly indicate that insulin resistance is a genetically inherited trait and is not simply a consequence of increased BP.

(III) INSULIN AND HYPERTENSION: EXPERIMENTAL EVIDENCE

(i) Human studies

Ferrannini et al. provided the first direct evidence that essential hypertensive patients are insulin-resistant. By employing the euglycemic hyperinsulinemic clamp technique, they studied insulin sensitivity in young, lean, untreated hypertensive subjects and found that insulin sensitivity was markedly decreased (about 40%) in hypertensive patients when compared to age and weight matched controls (Ferrannini *et al.,* 1987). Furthermore, they also demonstrated that the insulin resistance was tissue specific in that it occurred mainly in the skeletal muscle and that insulin-induced suppression of hepatic glucose production was normal in hypertensive subjects. Another interesting observation that stemmed from their elegant study was that almost the entire reduction in insulin sensitivity could be accounted for by a decrease in nonoxidative glucose disposal (glycogen synthesis) whereas glucose oxidation and suppression of lipolysis were unaltered in hypertensive patients. This observation has subsequently been confirmed by several investigators (Cepaldo *et al.,* 1991; Pollare *et al.,* 1990; Rooney *et al.,* 1992).

Other studies have unequivocally demonstrated that not only is insulin resistance present in hypertensive subjects but that it is not improved by lowering BP with antihypertensive drugs (Pollare *et al.,* 1989; Skarfors *et al.,* 1989; Weinberger 1986). On the contrary, several studies indicate that antihypertensive therapy worsens insulin resistance and hyperinsulinemia in essential hypertensives (Lind *et al.,* 1994; Shen *et al.,* 1988). The view that insulin resistance is not secondary to an increase in BP is further supported by studies indicating that insulin-mediated glucose utilization and glucose stimulated insulin secretion is normal in patients with secondary hypertension such as reno-vascular hypertension and primary hyperaldosteronism (Shamiss *et al.,* 1992). More direct evidence for such a link has come from studies where it was observed that physical training lowered BP in obese patients (without any change in body weight), but only in those patients who were hyperinsulinemic before the start of the training program (Krotkiewski *et al.,* 1979).

However, it is important to remember that insulin resistance is not always associated with hypertension and vice versa. As was discussed earlier, there is ethnic variability in this relationship such that the link appears stronger in Caucasians and Hispanic Whites. Two other arguments that have been frequently cited as evidence against the hypothesis that insulin plays a role in the genesis of hypertension deserve mention. First, insulin when administered acutely is a vasodilator and does not cause an increase in BP (Anderson and Mark, 1993). The vasodilator actions of insulin will be addressed in detail in a subsequent section; however, the inability of insulin to increase BP acutely does not go against the view that insulin may modulate BP chronically. It rather suggests that if insulin is a vasoactive hormone, then resistance to its vasodilatory effects may manifest as an increase in peripheral vascular resistance and thereby

raise BP. The finding that BP falls when the dose of insulin is decreased in obese, hypertensive patients with NIDDM (Tedde *et al.,* 1989) and that there is an increase in BP when insulin treatment is started in NIDDM patients (Randeree *et al.,* 1992) strengthens the contention that chronic insulin therapy may exert pressor effects in humans. This view is further supported by a recent study indicating that troglitazone, a drug that improved insulin sensitivity, also lowered BP in essential hypertensive patients with diabetes mellitus (Ogihara *et al.,* 1995).

The second argument that is often advanced against the view that insulin modulates BP is that patients with insulinoma who are hyperinsulinemic are generally not hypertensive (Sawicki *et al.,* 1992). What is overlooked while advancing this argument is that patients with insulinoma do not have primary insulin resistance, since hyperinsulinemia in such patients is accompanied by marked hormonal counter-regulatory responses. In addition, patients with insulinoma lack the substrate for insulin (i.e. glucose) and it has been shown that insulin's effects on metabolic and vascular smooth muscle responses are dependent on the availability of glucose (Yanagisawa-Miwa *et al.,* 1990). Finally, in the study that addressed this issue (Sawicki *et al.,* 1992), hyperinsulinemia was present for a relatively short period (about 18 months) as opposed to the increased levels of insulin that are present throughout the life span of hypertensive patients.

In summary, current evidence indicates that insulin resistance in hypertension is a primary defect (independent of obesity, diabetes or drug treatment) and is tissue specific (resides primarily in skeletal muscle) and pathway specific (involves glycogen synthesis). This defect in insulin-mediated glucose uptake may play a role in the development of hypertension or may chronically predispose a certain proportion of subjects with a specific

neurohumoral phenotype towards an increase in BP. However, the independent contribution of insulin resistance towards an increase in BP is probably smaller and more complex than is often emphasized and to assume that insulin is directly linked to a rise in BP in all hypertensive subjects is oversimplistic and incorrect.

(ii) Animal studies

The association between insulin and hypertension has also been documented in several models of rodent hypertension. These include the Dahl rat (Kotchen *et al.,* 1991), the spontaneously hypertensive rat (Mondon and Reaven 1988; Reaven, 1991a,b), the Milan hypertensive rat (Dall-Aglio *et al.,* 1991) and the fructose-hypertensive rat (Hwang *et al.,* 1987). All these hypertensive rat models, although unrelated to each other, exhibit common defects in glucose metabolism. In Dahl rats, insulin resistance and hyperinsulinemia occur in salt sensitive as well as salt resistant animals and are independent of the salt content of the diet (Reaven *et al.,* 1991). In spontaneously hypertensive rats, hyperinsulinemia precedes the development of hypertension (Reaven and Chang 1991); however, the presence of insulin resistance in this rat strain remains controversial (Buchanan *et al.,* 1992a; Buchanan *et al.,* 1992b; Frontoni *et al.,* 1992; Hulman *et al.,* 1993). Some very relevant findings have emerged from studies in which insulin resistance and hyperinsulinemia were induced in normotensive Sprague Dawley rats by giving them a fructose enriched diet (Hwang *et al.,* 1987). Induction of these metabolic defects was associated with a concomitant increase in blood pressure in these rats. Furthermore, exercise training (which resulted in improved insulin sensitivity) and somatostatin administration (which decreased hyperinsulinemia) to the fructose-fed rats attenuated the fructose-induced increase in BP in the animals (Reaven *et al.,*

1988; Reaven *et al.,* 1989b). Although these findings do not establish causality, they do support such a link.

Results obtained from studies conducted in dogs are in contrast to those reported in rats. Acute insulin infusion in dogs did not raise BP (Liang *et al.,* 1982), whereas it led to a dose-dependent increase in BP in rats (Edwards and Tipton, 1989). Furthermore, chronic insulin infusion in dogs for up to 4 weeks did not cause hypertension, although it increased plasma insulin levels almost six-fold (Hall *et al.,* 1990a). When experiments were repeated in dogs made susceptible to hypertension by partial nephrectomy coupled with a high salt intake, insulin still did not cause an increase in BP (Hall *et al.,* 1990b). In contrast, a chronic, physiological increase in plasma insulin concentration increased BP in rats (Brands *et al.,* 1991). Interestingly, when dogs were fed a high fat or a high fructose diet, they became insulin-resistant, hyperinsulinemic and hypertensive (Martinez *et al.,* 1994). Thus there are species differences with regard to the effects of insulin on BP, which may be a result of differential effects of insulin on the sympathetic, renal or cardiovascular systems. These disparate results between dog and rodent studies support the notion that hyperinsulinemia may increase BP only in conjunction with the contribution of other pressor systems and may have a different phenotypic expression in different animal species. Taken together, although results from animal studies appear contradictory, there is sufficient evidence to suggest that the link between insulin and hypertension is more than coincidental and that insulin may cause hypertension in certain animal species. The obvious issue that then needs consideration pertains to the possible mechanisms linking insulin to an increase in BP, which is addressed in the next section. Hyperinsulinemia in hypertension is a reflection of resistance to the peripheral uptake and utilization of glucose, with high levels of insulin needed to

maintain and sustain euglycemia in the presence of insulin resistance. It has been hypothesized that the compensatory increase in plasma insulin concentration is not a benign phenomenon but that hyperinsulinemia may contribute towards the development of hypertension by a variety of different mechanisms.

(IV) INSULIN AND HYPERTENSION: THE POSSIBLE LINKS

This area has been the focus of intensive investigation, which has resulted in a lot of discussion and debate. The following section deals with the interactions of insulin with other organ systems that could potentially result in an increase in BP.

(i) Insulin-induced antinatriuresis

DeFronzo et al. were the first to demonstrate a direct sodium-retaining effect of insulin in healthy humans. They performed euglycemic insulin clamps in young subjects and found that urinary sodium excretion decreased within 30-60 minutes of a physiological increment in plasma insulin concentration and gradually reached a minimum, which was 50% lower than the basal rate (DeFronzo *et al.,* 1975). This observation has been subsequently confirmed in humans (Gans *et al.,* 1991), dogs (DeFronzo *et al.,* 1976) and rats (Kirchner, 1988). The hypothesis that hyperinsulinemia leads to renal sodium and fluid retention is based on the assumption that the kidneys of hypertensive patients maintain normal sensitivity to the antinatriuretic effect of insulin, in contrast to the peripheral tissues which are resistant to insulin's glucoregulatory effects. This premise has been directly confirmed in essential hypertensive patients, where it was demonstrated that although insulin-mediated glucose uptake was markedly lower in hypertensives, insulin-induced sodium retention was maintained when

compared to normotensive controls (Shimamoto *et al.,* 1994). In addition, it was recently reported that in hypertensive patients, insulin directly increased sodium reabsorption in the proximal and distal tubules (Endre *et al.,* 1994; Kageyama *et al.,* 1994).

These reports raise the possibility that insulin may cause sodium and volume overload in hypertensive patients, which could lead to hypertension. A recent study demonstrated for the first time that although the sodium retaining effect of insulin was maintained in hypertensive patients, they were resistant to the natriuretic effects of atrial natriuretic peptide (Abouchacra *et al.,* 1994). This novel finding raises the possibility that resistance to the natriuretic effects of atrial natriuretic peptide may be one of the mechanisms underlying the insulin-induced increase in BP. However, young subjects with essential hypertension do not have an increased body sodium content or an increased plasma volume or a reduced plasma renin concentration (Beretta-Piccoli *et al.,* 1982), indicating that these acute effects may not be sustained or may be compensated for over a longer period of time.

(ii) Insulin and the sympathetic nervous system

An increase in sympathetic activity secondary to an increase in plasma insulin concentration has been demonstrated in humans. Rowe et al. reported that elevations in plasma insulin levels caused a dose-dependent increase in plasma catecholamine levels and a concurrent increase in pulse and BP. However, they observed this effect only at pharmacological concentrations of insulin, which casts doubt with regard to the physiological relevance of their findings (Rowe *et al.,* 1981). Other studies have documented that fasting decreased catecholamine levels whereas feeding led to an increase in plasma

norepinephrine levels (DeHaven *et al.,* 1980; Landsberg and Drieger, 1989; O'Dea *et al.,* 1982). This increase in sympathetic activity would be expected to cause an increase in cardiac output, peripheral vasoconstriction and a consequent increase in BP. However, studies have demonstrated that although a physiological increase in insulin concentration causes an increase in muscle sympathetic activity and nerve firing rate, it results in a decrease in vascular resistance and either no change or a paradoxical decrease in BP (Anderson *et al.,* 1991; Berne *et al.,* 1992). In a series of elegant experiments, Baron et al. demonstrated that insulin caused a rightward shift in the norepinephrine dose response curve and that this effect was more pronounced in lean as compared to obese subjects (Baron *et al.,* 1994). They also found that insulin caused a 25% increase in the metabolic clearance of norepinephrine and that this effect was also blunted in obese, insulin-resistant subjects. Finally, they reported that obese subjects were more susceptible to the pressor effects of insulin than lean insulin-sensitive humans. The reason why insulin does not increase BP, despite stimulation of the sympathetic nervous system, is that insulin causes preferential vasodilation in the skeletal muscle vasculature and thereby leads to a redistribution of cardiac output to skeletal muscle (Baron 1993). Thus the vasodilatory effects of insulin offset the increase in cardiac output, an issue that will be addressed in detail in one of the subsequent sections.

(iii) Trophic effects of insulin

It has been reported that insulin, via its action on insulin-like growth factor receptors, causes an increase in vascular smooth muscle cell growth *in vitro* (Banskota *et al.,* 1989; King *et al.,* 1985). Furthermore, it has been demonstrated that insulin stimulates DNA synthesis in fibroblasts and vascular smooth muscle

cells (Capron *et al.,* 1986; Rechler *et al.,* 1974). Cruz et al. reported that chronic insulin infusion into one femoral artery in the dog caused vascular hypertrophy only on the ipsilateral side (Cruz *et al.,* 1961). Therefore, it is possible that chronic hyperinsulinemia may cause vascular hypertrophy and lead to narrowing of the lumen of resistance vessels, consequently raising vascular resistance and BP. Although this hypothesis has not been validated, it is important to mention that the hypertrophic effects of insulin have been observed only at supraphysiological concentrations and that the possibility of insulin exerting trophic effects at physiological concentrations remains to be determined.

(iv) Hemodynamic effects of insulin

The hemodynamic effects of insulin have been intensively investigated over the last 3 years and results indicate that not only does insulin exert a variety of metabolic effects but that it is also a powerful vasoactive hormone (Baron 1993). Although some early reports had suggested that insulin exerted cardiovascular effects, most of the effects were observed at pharmacological concentrations (Rowe *et al.,* 1981). This led to the notion that insulin did not modulate vascular smooth muscle activity under normal physiological conditions, a notion that has been proven incorrect in recent years. The first study that examined this issue in detail was that of Laakso et al. who reported that insulin, when infused intravenously, caused a dose-dependent increase in leg blood flow in humans and that this effect was independent of plasma glucose concentration (Laakso *et al.,* 1990). Subsequently, several investigators have confirmed this observation and it has been demonstrated that in lean, insulin-sensitive subjects, acute insulin infusion causes a rise in peripheral blood flow with an EC_{50} of about 40 μU/ml (Laakso *et al.,* 1992). This indicates that insulin exerts potent

vasodilator effects at physiological concentrations. Furthermore, it was demonstrated that insulin also caused significant increases in cardiac output with an EC_{50} of about 70 μU/ml (Baron and Brechtel, 1993). Studies revealed that although insulin caused both systemic and peripheral vasodilation, the increase in skeletal muscle blood flow far exceeded the increment at the systemic level (Baron and Brechtel, 1993). Thus insulin, by preferentially increasing skeletal muscle blood flow, redistributes the cardiac output to skeletal muscle (the major site of glucose utilization). This also explains why the insulin-induced increase in cardiac output and sympathetic activity do not cause a resultant increase in BP.

Even more fascinating are findings indicating that these vasodilator effects of insulin are markedly impaired in insulin-resistant states such as obesity and diabetes mellitus (for a review, please refer to Baron 1993). In obese subjects, the EC_{50} of the dose response curve to insulin's vasodilator actions was about threefold higher than that in lean controls. In NIDDM patients, the EC_{50} was about 17 fold higher, indicating a marked resistance to the vasodilator effects of insulin (Laakso et al. 1992). The compelling question that then comes to mind is whether resistance to insulin's vasodilator effects also occurs in hypertensive patients. Indeed, it was recently demonstrated that insulin-induced vasodilation in pre-constricted dorsal hand veins was impaired in essential hypertensive patients (Feldman and Bierbrier, 1993). The mechanism/s underlying insulin-mediated vasodilation remain to be determined and may include both systemic and local effects (Anderson and Mark, 1993). Several recent reports suggest that insulin may alter vascular tone via direct effects on intracellular calcium concentration in vascular smooth muscle cells (Sowers *et al.,* 1994; Standley *et al.,* 1993). Insulin has also been shown to attenuate the contractile responses of vascular smooth muscle to vasoactive amines, probably by causing changes in intracellular

calcium (Touyz and Schiffrin, 1994; Yagi *et al.,* 1988). Other reports strongly suggest a role for endothelium-derived nitric oxide in the insulin-induced vasodilation (Steinberg *et al.,* 1994), since infusion of a nitric oxide synthase inhibitor completely prevented the increase in insulin-mediated leg blood flow. Furthermore, methylene blue, a guanylate cyclase inhibitor, also abolished insulin-mediated venodilation, suggesting that insulin's effects are cGMP dependent (Grover *et al.,* 1995).

If the insulin-mediated decrease in vascular tone were impaired in insulin-resistant states, it could cause a resultant increase in the pressor response to various neurohumoral factors, thus increasing vascular resistance and BP (Baron 1993). Lending support to this hypothesis are findings that obese, insulin-resistant subjects have a greater sensitivity to the pressor effects of norepinephrine when compared with lean, insulin-sensitive controls (Baron *et al.,* 1994). Insulin resistance at the vascular level could, therefore, tip the balance in favor of the pressor forces and therefore predispose an individual towards hypertension. Whether such an effect occurs in essential hypertension remains to be elucidated, but current evidence strongly suggests such a possibility.

(v) <u>Insulin and the intracellular cation transport systems</u>

It was initially postulated that insulin may regulate the activity of Na^+-K^+-ATPase, an important cellular enzyme that extrudes sodium in exchange for potassium and is responsible for maintaining the normal resting potential in cells. This hypothesis stemmed from observations that the activity of this pump (which is insulin regulated) was reduced in essential hypertensive subjects as well as in experimental models of hypertension (Boon *et al.,* 1985; Canessa *et al.,* 1984; Postnov and Orlov, 1985). Such a reduction in Na^+-K^+-ATPase activity could

lead to increased intracellular sodium levels, which could sensitize the arteriolar smooth muscle cells to the pressor effects of catecholamines and angiotensin II. Although this is an attractive hypothesis, evidence suggests that such an abnormality is unlikely to be the cause underlying the increase in BP in hypertensive subjects. For example, it has been demonstrated that insulin stimulated potassium uptake (a reflection of Na^+-K^+-ATPase activity) is unrelated to insulin's stimulatory effect on glucose metabolism (Ferrannini *et al.,* 1988). Furthermore, insulin can promote cellular potassium uptake normally in hypertensive patients (DeFronzo and Ferrannini, 1991). Therefore, it does not seem that resistance to insulin's glucoregulatory effects also extends to its effects on the Na^+-K^+-ATPase enzyme.

Another cation pump that has been examined is the Na^+-H^+-antiporter, which is responsible for maintaining intracellular sodium concentration as well as intracellular pH. Increased activity of this pump in response to increased levels of insulin has been observed in various cell types in hypertensive subjects (Adragna *et al.,* 1982; Canessa *et al.,* 1987; Weder, 1985). Overactivity of this pump could result in increased sodium levels inside the cell, which would sensitize vascular smooth muscle cells to the effects of various pressor amines. In addition, increased sodium levels could result in an indirect increase in intracellular calcium concentration, which would also cause an increase in vascular tone. Finally, an increase in the activity of this proton pump would lead to intracellular alkalinization, which is a stimulus for vascular smooth muscle growth (Lever 1986).

Probably the most important observation that may explain the direct vasodilatory effects of insulin is that insulin has marked effects on intracellular calcium concentration (Sowers *et al.,* 1994). It has been reported that insulin

attenuates vascular smooth muscle calcium influx through both receptor and voltage-operated calcium channels. In addition, insulin also modulates the activity of Ca^{+2}-ATPase, which is responsible for the extrusion of calcium from cells (Standley *et al.,* 1993). Resistance to these effects of insulin would cause an increase in intracellular calcium levels and a consequent enhancement of vascular tone and BP. Thus, insulin has the ability to directly modulate several intracellular ionic pumps and thereby alter vascular tone and BP.

(V) SPECIFIC RESEARCH PROBLEM AND RESEARCH STRATEGY

(i) The research problem

A major limitation of studies linking insulin resistance and hyperinsulinemia to hypertension is that they do not establish causality. Although associations and correlations strongly favor such a link, this issue requires further experimental evaluation. In addition, these metabolic defects are not always associated with essential hypertension, neither are all insulin-resistant subjects hypertensive. Another confounding issue is whether the insulin resistance associated with hypertension is a cause or an effect of increased BP. It has been documented that epinephrine, acting primarily through the beta adrenergic receptor, markedly impairs hepatic as well as peripheral tissue sensitivity to increments in plasma insulin concentration (Diebert and DeFronzo, 1980). It could, therefore, be argued that a primary increase in sympathetic activity (increase in plasma catecholamines) may antagonize insulin action and lead to secondary insulin resistance. However, decreasing blood pressure with most antihypertensive drugs does not improve insulin sensitivity or decrease plasma insulin levels, which suggests that these metabolic defects are not secondary to hypertension. Resolution of this issue requires more direct and specific experimental

interventions, which were attempted in the present study. We examined the hypothesis that insulin resistance and hyperinsulinemia are causally related to hypertension.

(ii) Rationale

We addressed this issue from a pharmacological perspective and reasoned that if insulin resistance and/or hyperinsulinemia were responsible for the development of high BP, then a specific improvement in insulin action should result in a fall in BP. However, if these metabolic defects were not causally related to hypertension, or were secondary to it, then such metabolic improvements should not cause any resultant change in BP. Recent evidence suggests that the insulin resistance of hypertension is tissue and pathway specific, with the major defect residing in the glycogen synthesis pathway in the muscle (Ferrannini *et al.,* 1987). Therefore, drug interventions that specifically improve muscle glycogen synthesis and enhance insulin action should also prevent the increase in BP.

(iii) Experimental approach

To broaden the nature of our enquiry, we used both a genetic and an acquired model of experimental hypertension, which were: (a) the spontaneously hypertensive rat, which is thought to closely resemble human essential hypertension and (b) the fructose-hypertensive rat, where hypertension is induced in normotensive rats by feeding them a high fructose diet. We attempted to directly improve insulin sensitivity by employing three drug interventions, all of which have been shown to enhance insulin action. These drugs were: (a) vanadyl sulfate, the (+IV) form of the trace element vanadium (b) bis(maltolato)oxovanadium(IV), an organic vanadium complex that is more potent

and is associated with fewer gastro-intestinal side effects than vanadyl sulfate and (c) pioglitazone, a recently synthesized thiazolidinedione derivative that enhances insulin action, probably by sensitizing peripheral tissues to the effects of insulin. Insulin sensitivity was quantified by using the euglycemic, hyperinsulinemic clamp in conscious rats, which is considered to be one of the most precise methods available for assessing *in vivo* insulin action. Multiple, direct and specific drug interventions were employed in an effort to strengthen our experimental approach and the anticipated results.

(iv) Experimental models

(a) Spontaneous hypertension: Spontaneously hypertensive rats (SHR) have a genetic predisposition towards hypertension and have been shown to be insulin-resistant and hyperinsulinemic when compared to their genetic controls, the Wistar Kyoto (WKY) strain (Hulman *et al.,* 1991; Mondon and Reaven, 1988). Insulin stimulated glucose uptake was found to be lower in adipocytes isolated from SHR as compared to the WKY rats (Reaven *et al.,* 1989a). This decrease in insulin stimulated glucose transport was observed despite normal insulin receptor number, affinity and tyrosine kinase activity, suggesting that the defect in insulin action resided distal to the insulin receptor (Reaven *et al.,* 1989a). Furthermore, the defect in insulin action in adipocytes preceded the development of hypertension in the SHR (Reaven and Chang, 1991). Subsequent studies demonstrated that insulin clearance was decreased in the SHR, which was due to decreased removal of insulin by the kidneys and skeletal muscle rather than a defect in hepatic insulin extraction (Mondon *et al.,* 1989). Such a reduction in insulin clearance could also result in higher circulating insulin levels in the SHR. Reaven et al. studied the effect of a high fructose diet in the SHR and found that

although the fructose diet caused an increase in plasma insulin levels and BP in both the SHR and WKY rats, the increase was more pronounced in the SHR as compared with their WKY controls (Reaven *et al.*, 1990).

Although euglycemic clamp studies conducted in anesthetized SHR demonstrate that they are insulin-resistant, recent studies done in conscious SHR have challenged this notion (Buchanan *et al.,* 1992a; Buchanan *et al.,* 1992b; Frontoni *et al.,* 1992). SHR have been shown to be more responsive to stress (anesthesia or restraint) as compared to the WKY (McMurty and Wexler, 1981; Shah *et al.,* 1977). Consequently, there could be a greater release of endogenous catecholamines, which could, in turn, antagonize insulin action and lead to secondary insulin resistance in the anesthetized SHR. In agreement with this concept are results from clamp studies in conscious, minimally restrained rats that demonstrate no difference in insulin sensitivity in the SHR as compared to the WKY (Buchanan *et al.,* 1992a; Frontoni *et al.,* 1992). However, the presence of hyperinsulinemia in the SHR was confirmed even in the latter studies and it was proposed that increased insulin levels may contribute to the development of high BP. This issue could be evaluated by decreasing plasma insulin levels in the SHR and studying the resultant change (if any) in blood pressure. Such an attempt was made in the present investigation.

(b) Fructose-induced hypertension: The fructose-hypertensive model represents an acquired form of systolic hypertension, where the rise in BP is not genetically determined but is diet-induced (Hwang *et al.,* 1987). The finding that hypertension can be produced in normotensive rats by an experimental manipulation known to result in insulin resistance and hyperinsulinemia supports the contention that these metabolic defects are very closely linked to hypertension. Furthermore, exercise training (which caused an improvement in

insulin sensitivity) and somatostatin administration (which decreased hyperinsulinemia) to the fructose-fed rats attenuated the fructose-induced increase in BP (Reaven *et al.,* 1988; Reaven *et al.,* 1989b). Addition of clonidine to the drinking water of fructose-hypertensive rats caused a decrease in BP but did not improve insulin sensitivity or lower plasma insulin levels, suggesting that the metabolic abnormalities were primary and were not simply a consequence of high BP (Hwang *et al.,* 1987). Interestingly, high fructose feeding also elicits insulin resistance, hyperinsulinemia and hypertension in dogs and the metabolic defects antedate the increase in BP (Martinez *et al.,* 1994). Given the fact that we still do not know the relative contribution of genetic and acquired factors towards the insulin resistance observed in hypertension, it was important to examine the validity of the hypothesis under study in this model. Would the hypothesis hold true in a model where genetic susceptibility towards hypertensinogenic mechanisms is absent? Having discussed the experimental models that were employed in the study, we will next consider the drugs that were used as the experimental interventions.

(v) Drug interventions

(a) Vanadyl sulfate: Vanadium (atomic weight 50.94) is a ubiquitous group V transition element that exists in several valence forms (-1 to +5). Its essentiality in man has not been established as yet although it is estimated that in humans, the total vanadium pool ranges between 100-200 µg (Byrne *et al.,* 1978). Over the past decade, several laboratories have reported on the insulin-mimetic properties of vanadium *in vitro* (Clark *et al.,* 1985; Shechter and Karlish, 1980; Tolman *et al.,* 1979). Heyliger et al. in our laboratory were the first to demonstrate that oral sodium orthovanadate treatment corrected both the hyperglycemia and

myocardial abnormalities in diabetic rats (Heyliger *et al.,* 1985). During our initial studies, we observed that oral treatment with the vanadate (+5) form of vanadium lead to gastrointestinal side effects. Furthermore, since the LD_{50} of the vanadyl (+4) form of vanadium has been reported to be 2 times greater than the vanadate form, we decided to focus on the effects of oral vanadyl sulfate in streptozotocin (STZ)-diabetic rats (Ramanadham *et al.,* 1989; Ramanadham *et al.,* 1990a,b). Work from our laboratory has established the effectiveness of oral vanadyl sulfate in correcting various abnormalities in the heart and adipose tissue in STZ-diabetic rats and in enhancing the effects of insulin *in vivo* (Ramanadham *et al.,* 1989; Ramanadham *et al.,* 1990a). Another observation that surfaced from our studies was that vanadyl treatment not only decreased plasma glucose levels in STZ-diabetic rats (without an increase in plasma insulin) but that it also reduced insulin levels in control, non-diabetic rats (Heyliger *et al.,* 1985; Ramanadham *et al.,* 1990a,b). This suggested that vanadyl either potentiated or replaced the effects of endogenous insulin, resulting in a decreased requirement of insulin in non-diabetic rats. Vanadium has also been shown to enhance glycogen synthesis in the liver and muscle from control and diabetic rats (for a review, please refer to Shechter, 1990). Vanadyl, by improving glycogen synthesis in the muscle, could, therefore improve insulin sensitivity and ameliorate hypertension (if our hypothesis were valid).

(b) Bis(maltolato)oxovanadium(IV) (BMOV): Inorganic vanadyl administration was associated with two problems: poor gastrointestinal absorption and some gastrointestinal side effects (Conklin *et al.* 1982; Underwood, 1977). We speculated that an organic compound would be more lipophilic than its inorganic counterpart and may, therefore, be better absorbed from the gastrointestinal tract. Subsequently, we synthesized BMOV by complexing one molecule of vanadyl with

2 molecules of the common food additive maltol (McNeill *et al.,* 1992). Chronic oral administration of BMOV in STZ-diabetic rats normalized plasma glucose levels without a concomitant increase in circulating insulin (Yuen *et al.,* 1993). Furthermore, BMOV was found to be 1.5 times more potent than vanadyl sulfate in lowering plasma glucose. The increased potency of BMOV as compared to vanadyl sulfate was also demonstrated in an acute study, where we examined the glucose-lowering effects of the two compounds after oral gavage in STZ-diabetic rats (Yuen *et al.,* 1995). We have also reported that the ED_{50} for intraperitoneally administered BMOV is 0.08 mmol/kg as compared to 0.22 mmol/kg for vanadyl sulfate, indicating that BMOV is approximately 3 times more potent than vanadyl in lowering plasma glucose (Yuen *et al.,* 1993). This suggests that the increased potency of BMOV is not a result of increased absorption but that it either has an increased bioavailability or that the complex permeates through the cell wall more readily. We observed 21% mortality at the highest administered dose of vanadyl but no mortality was noted with any dose of BMOV (Yuen *et al.*, 1993). A six month assessment of the possible toxicological effects of BMOV indicated that BMOV prevented the pathological changes seen in untreated diabetic rats without producing significant toxicity in control or diabetic treated animals (Dai *et al.,* 1994). Of the vanadium compounds tested to date, BMOV is the most potent and the best tolerated.

(c) Pioglitazone: Pioglitazone, a recently synthesized thiazolidinedione derivative, is another compound that exhibits insulin enhancing effects (Hofman *et al.,* 1991; Ikeda *et al.,* 1990; Sugiyama *et al.,* 1990a). Pioglitazone has been shown to improve insulin sensitivity and to attenuate hyperinsulinemia in insulin-resistant animal models (Kemnitz *et al.,* 1994; Sugiyama *et al.,* 1990b). This drug has also been shown to increase the rate of glycogen synthesis in the isolated

muscle of the insulin-resistant, hyperinsulinemic Wistar Fatty rat (Sugiyama *et al.,* 1990a). Furthermore, pioglitazone potentiated the insulin-mimetic effects of vanadate on glucose metabolism in isolated adipocytes from Wistar Fatty rats (Sugiyama *et al.,* 1990b). Implicit in the point of view outlined above is the assumption that none of these drugs possess any other antihypertensive effects and that they selectively improve insulin action. Although these compounds have been studied for their insulin enhancing effects in diabetes mellitus, their effects in hypertension have not been evaluated. Since it is now known that hypertension, like obesity and type II diabetes mellitus, is an insulin-resistant state in most instances, it is only logical to study their effects on high BP.

(VI) Working hypotheses

1) Insulin resistance and hyperinsulinemia play a role in the development and regulation of high blood pressure.
2) Vanadyl sulfate, BMOV and pioglitazone improve insulin sensitivity in insulin-resistant states. These three drugs will, therefore, enhance insulin sensitivity, decrease plasma insulin levels and prevent the development of high BP in the SHR if started before the SHR become hypertensive.
3) By enhancing the action of insulin, vanadyl sulfate will also reverse hypertension in the SHR (if given after hypertension becomes fully manifest in the SHR).
4) Vanadyl sulfate will improve insulin sensitivity and prevent hypertension in the insulin-resistant, hyperinsulinemic fructose-hypertensive rats.

MATERIALS AND METHODS

(I) STUDIES IN THE SPONTANEOUSLY HYPERTENSIVE RAT

(A) RESEARCH DESIGN AND EXPERIMENTAL PROTOCOLS

(i) Studies with vanadyl sulfate

(a) Prevention Study: 23 SHR and 18 WKY rats, all male, were procured at 4 weeks of age from Charles River, Montreal, Canada and were randomly assigned to four experimental groups: S (SHR-untreated, n=15), SV (SHR-vanadyl treated, n=8), W (WKY-untreated, n=12) and WV (WKY-vanadyl treated, n=6). Systolic BP in all groups was measured before starting vanadyl treatment. Subsequently, chronic vanadyl sulfate treatment ($VOSO_4$: nH_2O, Fisher Scientific, NJ, U.S.A.) was commenced on 6 week old SV and WV rats. Vanadyl sulfate was administered at a concentration of 0.75 mg/ml ad libitum in the drinking water. Starting at week 8, weekly measurements of systolic BP, plasma glucose and plasma insulin were done on all the groups. Food intake, fluid intake and body weight were measured once a week. In addition, fluid intake of rats on vanadyl was measured 5 times a week for calculation of the dose of vanadyl consumed.

(b) Reversal Study: At the start of week 11 (weeks denote the age of the rats), the untreated SHR and WKY from the prevention study were further grouped as follows: S (SHR-untreated, n=9), SV_1 (SHR-vanadyl treated, n=6), W (WKY-untreated, n=6) and WV_1 (WKY-vanadyl treated, n=6). The treated groups were started on vanadyl sulfate (0.75 mg/ml) in the drinking water at the beginning of week 11. Weekly measurements of BP, plasma glucose and plasma insulin were continued for the next 3 weeks. At 15 weeks of age

(termination), direct systolic BP measurements were done to validate the indirect BP readings obtained in the preceding weeks.

(c) Pair-Feeding Study: Since vanadyl sulfate decreased food/fluid consumption and body weight in the treated rats (see results below), a separate study was initiated in which one group of rats (SHR as well as WKY) was pair-fed with the corresponding vanadyl-treated group, but was not given vanadyl. This was done to observe if a decrease in food and fluid intake per se contributed towards the attenuation of hypertension in the vanadyl-treated rats. 23 SHR and 24 WKY (all male) were procured at 5 weeks of age from Charles River, Montreal, Canada and were grouped as follows: S (SHR-untreated, n=8), SV (SHR-vanadyl treated since the start of week 6, n=8), SF (SHR pair-fed with treated rats for food and fluid consumed, n=7); W (WKY-untreated, n=8), WV (WKY-vanadyl treated since the start of week 6, n=8) and WF (WKY pair-fed with treated rats, n=8). At weeks 5, 9, 10, 12, 15 and 16, systolic BP was measured by the indirect tail-cuff method, which had already been validated by direct arterial cannulation in the previous experiment. During the weeks mentioned above, five-hour fasted plasma samples were also collected via the tail vein and were later analyzed for glucose and insulin. At 15 weeks of age (after 10 weeks of vanadyl treatment) the rats were fasted overnight, plasma was collected and was later analyzed for urea-nitrogen, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and vanadium levels.

(d) Insulin Implant Study: Vanadyl sulfate caused a decrease in plasma insulin levels and BP in the SHR (see results below). In order to examine this issue further, a separate study was initiated in which 9 week old SHR and WKY (n=12

in each group) were treated with vanadyl as before. Three weeks after starting vanadyl treatment, six vanadyl-treated rats in each group were administered exogenous insulin (14000 pmol/kg/day as a subcutaneous insulin implant, Linshin Inc., Canada) while the other six were continued on vanadyl as before. This was done to restore plasma insulin levels in the vanadyl-treated rats to those that existed before vanadyl treatment and to then observe the resultant change in BP.

During the post-implant period, plasma insulin, plasma glucose and BP were measured one and three weeks after placement of the insulin implants. In addition, the rats were sampled for plasma catecholamines and glucagon before and three weeks after placement of the insulin implants. Plasma catecholamine samples were collected in an identical fashion in all the rats. The rats were handled frequently for a few days before sampling. The samples were collected within 2 minutes of touching the individual rat in an undisturbed environment and the same procedure was followed for every animal. Thus the catecholamine concentrations do not represent the absolute stress free, basal values. The main objective was to look for major changes in catecholamine concentrations that could occur secondary to hypoglycemia (in rats with insulin implants) and therefore, the rats were not chronically cannulated for catecholamine sampling.

(ii) <u>Studies with bis(maltolato)oxovanadium(IV)</u>

The present study was initiated with the following objectives: (a) to assess insulin sensitivity in the SHR and WKY rats by performing euglycemic hyperinsulinemic clamps in conscious, minimally restrained rats and (b) to

administer BMOV to SHR and WKY rats and to examine the effects of the drug on insulin sensitivity, plasma insulin levels and systolic BP.

Protocol: Five week old male SHR and WKY rats were procured from Charles River, Montreal Canada and were randomly assigned to 4 experimental groups: S (SHR-untreated, n=9), SO (SHR-BMOV treated, n=11), W (WKY-untreated, n=11) and WO (WKY-BMOV treated, n=9). Chronic BMOV treatment (0.75 mg/ml ad libitum in the drinking water) was initiated on 6 week old rats. A concentration of 0.75 mg/ml was chosen since previous results indicated that, at this concentration, BMOV lowered plasma insulin levels in non-diabetic rats while allowing them to gain weight at rates that were comparable to untreated controls (McNeill *et al.*, 1992). Once at week 5 and again starting at week 8, systolic BP, plasma insulin (5-hour fasted) and plasma glucose were measured weekly for the next 4 weeks. At termination (15-19 weeks of age), the rats were fasted overnight and hyperinsulinemic clamps were performed in conscious rats.

(iii) Studies with pioglitazone

Protocol: Five week old male SHR and WKY were procured from Charles River, Montreal Canada and were randomly assigned to 4 experimental groups: S (SHR-untreated, n=8), SP (SHR-pioglitazone treated, n=16), W (WKY-untreated, n=8) and WP (WKY-pioglitazone treated, n=15). Pioglitazone was a generous gift from the Upjohn Company, Kalamazoo, Michigan, U.S.A. Chronic pioglitazone treatment (0.1 mg/g in the rat chow) was initiated in 6 week old SP and WP rats, whereas the S and W groups received control rat chow of similar composition to that given to the treated groups, apart from the exclusion of the drug. Once at week 5 and again starting at week 8, systolic BP, 5-hour fasted

plasma insulin and plasma glucose were measured every week. Since pioglitazone caused a decrease in plasma insulin levels and BP in the SHR (see results below), seven pioglitazone treated rats (in each of the SP and WP groups) were administered exogenous insulin after week 11 (14000 pmol/kg/day as a subcutaneous insulin implant, Linshin Inc., Canada) while the other 8 were continued on pioglitazone as before. This was done in an attempt to restore plasma insulin levels in the treated rats to those that existed before pioglitazone treatment and to then observe the resultant change in BP. During the post-implant period, plasma insulin, plasma glucose and BP were measured for 2 weeks and subsequently, the animals were fasted for 20 hours and high dose insulin clamps were performed in the S and W groups as well as the rats without the insulin implants in the SP and WP groups. In a separate study, we also conducted low dose insulin clamps in SHR and WKY (11-13 weeks of age) after treating them with pioglitazone at the same dose as described above for the first study.

(B) METHODOLOGY

(i) Blood pressure measurement

(a) Studies with vanadyl sulfate: Indirect BP measurements (systolic) were conducted by using the tail-cuff method. Rats were removed from the animal room and taken to a quiet room at 0800; they were allowed free access to food and water. The rats were prewarmed for 10 minutes in a rat holder placed on a hot plate with a surface temperature of 32 degrees Celsius. Since the rats had been preconditioned to the BP measurement procedure, they became sedate within two minutes of being restrained in the rat holder. The cuff used was 35 mm in length and was placed at the base of the tail. A pneumatic pulse sensor

was taped to the tail and was connected to a pneumatic pulse transducer (Narco Bio Systems, Houston, Texas, U.S.A.). A programmed electro-sphygmomanometer PE-300 (Narco Bio Systems) was employed to keep the various parameters such as inflation/deflation rates and cycling interval constant. The reappearance of pulsations (on gradual deflation) signified the systolic BP. In each rat, 3 consecutive readings were taken and averaged to obtain the individual BP.

Direct BP was measured at the termination of the study. The rats were anesthetized with a short acting barbiturate, sodium methohexital (Brietal sodium, Eli Lilly, Toronto, Canada), which was given at a dose of 60 mg/kg body weight intraperitoneally. Anesthesia was maintained with nitrous oxide and a catheter (1 meter of polyethylene tubing PE 50 joined to 7 cm of PE 10) was introduced via the caudal artery and placed into the abdominal aorta of each rat (Schenk *et al.*, 1992). Previous studies (in which this method was employed) had demonstrated that the cardiovascular status and baroreflex sensitivities of rats were similar 5 and 72 hours after anesthesia and surgery (Bennett and Gardiner 1986; Hebden *et al.*, 1987). Therefore, five hours after surgery, BP was recorded continuously by connecting the catheter via a Gould p23db pressure transducer to a polygraph (Gould TA 2000; Gould, Cleveland, OH). At the time of recording BP, the rats were fully conscious, freely moving and had recovered from the effects of anesthesia.

(b) Studies with BMOV and pioglitazone: For these and all subsequent studies, indirect systolic BP was measured in conscious rats by using the indirect tail-cuff method *without* external preheating (Bunag and Butterfield 1982). The animals were preconditioned to the experimental procedure before conducting

actual measurements. The equipment used includes a BP sensor/cuff, a BP amplifier and an analog/digital recorder and printer (Model 179 semi-automatic BP analyzer, IITC INC., Woodland Hills, CA, U.S.A.). The various parameters such as cycling interval and inflation/deflation rates are kept constant by the semi-automatic apparatus. In this method, the reappearance of pulsations (on gradual deflation of the BP cuff) are detected by a photoelectric sensor and are amplified and recorded digitally as the systolic BP. An average of 3 such readings was taken as the individual systolic BP.

The major advantage of this method is that the recordings are carried out at a temperature of 26-27 degrees Celsius, thus eliminating the heat stress typical of other methods. Heat stress (occurs at temperatures greater than 30 degrees Celsius) has been shown to modify the BP of rodents profoundly and is best avoided during BP measurements. In a preliminary study, we validated readings obtained by this method by comparison with those recorded by direct intra-arterial cannulation. Briefly, six male Wistar rats were catheterized (carotid artery) and were allowed to recover for five hours. The catheter was exteriorized and simultaneous direct and indirect measurements were recorded. The means of twenty such readings were: indirect tail-cuff method 136±3 versus direct cannulation 135±3 mmHg, $P>0.05$. Thus indirect tail cuff recorded pressures were similar (within 5 mmHg) to those obtained by direct cannulation; similar results have also been reported by other laboratories (Hwang *et al.*, 1989; Reaven *et al.*, 1988).

(ii) Euglycemic hyperinsulinemic clamp technique

(a) Principle underlying the method: This method is considered to be one of the most precise indicators of *in vivo* insulin action (DeFronzo *et al.*, 1979) and

involves raising the plasma insulin concentration acutely to a physiological level and maintaining it at that level for 120-180 minutes. The plasma glucose concentration is held constant at basal level by a variable glucose infusion. Under steady state conditions of euglycemia, the glucose infusion rate equals the glucose uptake by all the tissues in the body (assuming that there is no endogenous hepatic glucose production). By infusing labeled glucose, hepatic glucose production can also be quantified and computed in the final results (DeFronzo *et al.*, 1979). This method, therefore, quantifies the ability of insulin to dispose of an infused glucose load (insulin sensitivity) during a period of physiological hyperinsulinemia.

In all our studies, we applied this technique in conscious, minimally stressed rats. Studies document that the SHR may be more responsive to stressful stimuli (such as anesthesia or physical restraint) than the WKY (McMurty and Wexler, 1981; Shah *et al.*, 1977). Increased stress can cause an increase in plasma catecholamines, which may antagonize insulin action. Therefore, it is desirable to use a method that causes the least amount of stress to the animals. Clamp studies done in conscious rats have been shown to be much less stressful than the application of anesthesia/physical restraint during the procedure (Buchanan *et al.*, 1992a).

BMOV study: Rats were conditioned to tail restraint by a modification of the approach of Buchanan et al. (Buchanan *et al.*, 1992a). In brief, the tail was passed through a hole (approximately 1.5 cm in diameter) in the cage, after which it was immobilized at a point halfway along its length by passing it through a soft cork and taping it distally. The rats had free access to food and water and were conditioned for increasing periods of time (30 minutes to two

hours, thrice a day) over 3 days prior to the clamp study. The rats were weighed daily and weight gain was comparable to that observed during the pre-conditioning period. The rats were fasted overnight (20 hours) before the clamp studies.

Four hours before the start of the insulin infusions, each animal was placed in a specially designed foam rubber jacket, which allowed free movement of all four limbs and forward vision. Subsequently, the rat was placed on a board with a belt positioning system which allowed it to be immobilized in the left or right lateral and supine positions. Lidocaine 1% was then infiltrated into the tissue on the ventral aspect of the tail. A 0.5 cm incision was made in the tail and the tail artery was cannulated with fine bore polyethylene tubing (PE 10) and flushed with heparin 50 U/ml of 0.9% saline. The tail vein was also cannulated percutaneously with a 24G Intracath (Jelco, Tampa, Florida, U.S.A.) attached to PE 50 tubing. The animal was returned to the cage and was allowed to recover with free access to water. 50 µL of blood was withdrawn immediately after surgery and at 15, 30, 60, and 120 minutes after surgery (but before the start of the insulin infusion) into tubes containing 9 mM EGTA and 8 mM glutathione (in final dilution) for determination of catecholamine levels. During the first 15 minutes of the clamp, baseline plasma glucose measurements were obtained. Thereafter, the following were infused:

(a) Insulin, 14 pmol/kg/min from 0-120 minutes (preceded by a loading dose of 3X, 2X and 1X for 1 minute each).

(b) Somatostatin, 920 pmol/kg/min from 0-120 minutes. Somatostatin was given to suppress endogenous insulin secretion completely. Since rat and human insulin share considerable immunoreactivity, it is necessary to suppress

endogenous rat insulin in order to precisely quantify circulating human insulin levels (since human insulin is infused during the clamps).

(c) D-[3-^{3}H]-glucose at a rate of 0.10 μCi/minute from -60 to 120 minutes, after an initial square-wave bolus over 1 minute. The tracer infusion is started prior to the initiation of the insulin infusion in order to label the glucose pool in the body. This allows the isotopic measurement of glucose turnover and hepatic glucose production as described by Steele (Steele, 1959). This measurement is necessary because the infused insulin may not suppress hepatic glucose production, especially if there is any hepatic insulin resistance present. Furthermore, the effect of insulin on hepatic glucose production may also be different in the SHR as compared to the WKY.

(d) During the clamps, 20% D-glucose was infused as needed to maintain plasma glucose at the preinfusion level.

30 μl of arterial blood was sampled at 5 minute intervals for determination of plasma glucose. At 100, 110 and 120 minutes, 200 μl of blood was withdrawn for measurement of steady state plasma insulin levels and tracer dilution and the animals were then sacrificed by an intravenous injection of pentobarbital (250 mg/kg).

(b) Calculation of insulin sensitivity: During the euglycemic clamp, two factors determine insulin sensitivity : (i) the ability of insulin to enhance glucose utilization and (ii) the ability of insulin to suppress hepatic glucose production. In an attempt to assess the individual contribution of these two factors, isotopically labeled tracer is used during the clamp procedure. Labeled glucose is infused prior to and during the clamp as a constant infusion and plasma specific activity is determined. The overall rate of glucose appearance (R_a) is

then determined by using the modified equation of Steele (Steele 1959). This method is based on the principle that the rates of appearance and utilization of an unlabeled metabolite in the plasma can be determined by the rate of infusion of a labeled metabolite and its specific activity in the plasma.

During the euglycemic clamp, the glucose appearance rate (R_a) in the plasma equals the sum of the hepatic glucose output (HGO) and the glucose infusion rate (GINF). Therefore, HGO can be calculated by subtracting GINF from the R_a. During conditions of steady state glucose and insulin concentrations, the rate of glucose utilization ($R_{d)}$ is equal to R_a. For each animal, a steady state value for plasma glucose, insulin, glucose production and glucose clearance was obtained by averaging the data recorded during the final 30 minute period; glucose clearance was calculated by dividing the R_d by the steady state glucose concentration. Insulin sensitivity was then expressed as the ratio of the steady state glucose clearance to the steady state plasma insulin value (Mitrakou *et al.*, 1992).

(c) Underestimation of hepatic glucose output: As outlined in the previous paragraph, the total rate of glucose appearance during steady state equals the sum of the exogenous glucose infusion and the endogenous glucose production by the liver, such that

$$R_a = GINF + HGO$$

Since the rate of production and the rate of utilization are similar at steady state,

$$R_a = R_d = GINF + HGO$$

Since HGO can be either positive or zero, R_d must always be greater than or equal to GINF. However, when the tracer dilution method is used, calculated

total glucose uptake can be less than GINF such that artifactually negative values for HGO are obtained (see results of clamp studies).

Thus, during steady state, HGO may actually be underestimated and this is an inherent error in the glucose clamp technique. The reason for this error is that the "glucose system" was modeled as a single compartment in which the infused glucose immediately and completely mixed throughout the pool. However, it was subsequently realized that the compartment consisted of at least two components: a rapidly mixing component (plasma) and a slowly mixing component (interstitial space i.e. where insulin actually acts) (Bergman *et al.*, 1989; Finegood *et al.*, 1987). During the glucose clamp, the entire glucose pool of the body is labeled by infusing tritiated glucose prior to the start of the clamp. However, when the clamp is initiated, the constant glucose infusion (20% D-glucose) increases the addition of glucose to the system by a factor of 3 to 4. This leads to a sharp drop in the specific activity of the plasma glucose space and causes dilution of the isotopically labeled tracer and an underestimation of HGO. Thus it is apparent that the underestimation of HGO is due to the rate of change of specific activity in the space from which glucose is infused and sampled, i.e. plasma.

In an attempt to keep the specific activity of the "plasma compartment" constant, many investigators tried to infuse a labeled infusion of 20% D-glucose, however, the results have been variable (for a review, please refer to Bergman *et al.*, 1989). Although infusion of labeled glucose does lead to a certain degree of correction in the estimation of HGO, it is perhaps not a very scientifically valid solution. This is because all it does is to reduce the dilution of the hot tracer and therefore guarantees a positive number for HGO, which is no more meaningful than a "true" negative one. Furthermore, although such a

correction has been attempted in dogs and humans, no published data exists regarding the use of spiked cold glucose infusate for correction of negative HGO in rats. One reason why this correction does not work well in rats is that very small blood samples are obtained as compared to dogs and humans and also because rats are more insulin-sensitive and have higher clamp glucose infusion rates (on a weight to weight basis) as compared to the bigger species. This limitation is widely accepted and it is ethical to say that HGO was fully suppressed during the clamp. For our studies, where cold glucose infusion rate exceeded the isotopically calculated R_a, the former figure was used to calculate total glucose disposal (R_d).

We could not attempt to spike cold glucose during the clamps because the maximum amount of tritium allowed in a rat carcass at UBC is 100 μCi/kg and our calculations indicated that we would exceed that limit. If we had attempted to spike the glucose, we would have administered an average of 130 μCi/rat/clamp while the permitted limit is only 31 μCi/rat/clamp. Therefore, in all the clamp studies, negative HGO values were obtained and HGO has been stated to be fully suppressed.

Pioglitazone study: Euglycemic clamps in conscious rats preconditioned to limited tail restraint were performed by a method identical to that described above for the BMOV study. However, in this study, we performed clamp studies at both high and low rates of insulin infusion, which allowed us to measure insulin sensitivity at both physiological and pharmacological steady state concentrations of insulin. It has been previously reported that the stress induced during this surgical procedure is minimal and that plasma catecholamine concentrations return to normal within 30 minutes of line

placement in the SHR, WKY (Bhanot *et al.*, 1994a) as well as Sprague Dawley rats (Bhanot *et al.*, 1994b).

During the first 15 minutes of the clamp, baseline plasma glucose and plasma glucagon measurements were obtained. Thereafter, the following were infused:

(a) Insulin, either 14 pmol/kg/min (for the low dose studies) or 70 pmol/kg/min (for the high dose studies) from 0-120 minutes (preceded by a loading dose of 3X, 2X and 1X for 1 minute each).

(b) Somatostatin, 920 pmol/kg/min from 0-120 minutes.

(c) D-[3-^{3}H]-glucose at a rate of 0.10 μCi/minute from -60 to 120 minutes, after an initial square-wave bolus over 1 minute. This was done for isotopic determination of glucose turnover as described above. Where cold glucose infusion rate exceeded the isotopically calculated R_a, the former figure was used to calculate total glucose disposal (R_d).

(d) During the clamps, 20% D-glucose was infused as needed to maintain plasma glucose at the preinfusion level.

30 μl of arterial blood was sampled at 5 minute intervals for determination of plasma glucose concentration. At 100, 110 and 120 minutes, 200 μl of blood was withdrawn for measurement of steady state plasma insulin levels and tracer dilution and the animals were then sacrificed by an intravenous injection of pentobarbital (250 mg/kg). For each animal, insulin sensitivity was expressed as the ratio of the steady state glucose clearance to the steady state plasma insulin value, as described above for the BMOV study.

(II) STUDIES IN THE FRUCTOSE-HYPERTENSIVE RAT

Male Sprague Dawley rats were procured locally (body weight 180-200 grams, 6 weeks of age). The animals were randomly assigned to 4 experimental groups: C (control-untreated, n=8), V (control-vanadyl treated, n=12), F (fructose-untreated, n=9) and FV (fructose-vanadyl treated, n=15). At week 6 (weeks denote the age of the animals) BP, plasma glucose and plasma insulin (5-hour fasted) were measured in all groups. Subsequently, chronic vanadyl sulfate treatment ($VOSO_4$: nH_2O, Fisher Scientific, NJ, U.S.A.) was initiated in the V and FV groups. Rats received vanadyl at a concentration of 0.75 mg/ml ad libitum in the drinking water. This concentration was chosen because previous studies had indicated that it decreased insulin levels in non-diabetic rats without altering plasma glucose levels (Pederson *et al.*, 1989).

One week after initiation of vanadyl treatment, the animals in the F and FV groups were started on a 66% fructose diet (Teklad Labs, Madison, WI, U.S.A.). The sodium content of the fructose diet was similar to that of the standard rat chow (standard chow: sodium 4 g/kg; fructose diet: sodium 4.2 g/kg). Systolic BP, plasma insulin (5-hour fasted) and plasma glucose were measured each week for the next 4 weeks. In addition, food intake, fluid intake and body weight of the animals were recorded every week. At termination, insulin sensitivity was assessed in conscious rats by the euglycemic, hyperinsulinemic clamp technique. As observed in the previous studies, vanadyl caused a decrease in plasma insulin concentration and systolic BP in the FV rats. To further examine this issue, V and FV rats (n=5 in each group) were administered exogenous insulin (daily subcutaneous ultralente insulin 14000 pmol/kg/day, Eli Lilly, Toronto, Canada) for 3 weeks. This was done in

an attempt to restore the plasma insulin levels in the FV rats to those seen in the untreated F group and to then observe the resultant change in BP.

Indirect systolic BP was measured in conscious rats using the indirect tail-cuff method without external preheating, as described earlier. Euglycemic hyperinsulinemic clamps were performed in a manner identical to that described in the BMOV study.

(C) BIOCHEMICAL ANALYSES

For all the studies, biochemical measurements were performed in an identical fashion, as described below:

(a) Plasma glucose was determined by the glucose oxidase method using kits purchased from Boehringer Mannheim, Laval, Quebec, Canada.

(b) Plasma insulin was determined by using a double antibody radioimmunoassay against porcine insulin standards (15-1200 pmol/L) with kits procured from Immunocorp, Montreal, Canada. The antiserum was generated in guinea pigs and was titered to bind 35-50% of insulin ^{125}I (freshly iodinated) in the absence of nonradioactive insulin. The sensitivity of the assay, defined as the least amount of insulin that could be distinguished from zero, was 15 pmol/L. This value was obtained from the 95% confidence limit of the intra-assay variation of 40 zero standards. Samples from all the weeks in each study were analyzed together at the end of the study to avoid inter-assay variations. Plasma glucagon was determined by using a double antibody radioimmunoassay against porcine standards (25-2000 ng/L). The antiserum was generated in rabbits by using porcine glucagon-HSA and was titered to

bind 20-40% of the glucagon-^{125}I in the absence of nonradioactive glucagon (ICN Biomedicals Inc., Costa Mesa, U.S.A.).

(c) In the pair-feeding study with vanadyl sulfate, GOT, GPT and urea nitrogen levels were assayed by colorimetric determinations with kits obtained from Sigma Diagnostics, St. Louis, U.S.A..

(d) Plasma vanadium analysis was done by electrochemical atomic absorption spectrophotometery according to the method employed by Mongold et al. (Mongold *et al.*, 1990) with minor modifications.

(e) During the clamp studies, plasma glucose was measured by the glucose oxidase method in a YSI 23A glucose analyzer (YSI Inc., Yellow Springs, OH, U.S.A.). Plasma insulin was assayed by a double antibody radioimmunoassay technique using porcine insulin standards (ICN Biomedicals Inc., Costa Mesa, U.S.A.).

(f) For determination of D-[3-^{3}H]-glucose concentrations, serum was diluted 1:4 with water and then added to an equal volume of perchloric acid, final concentration 2.5%. Proteins were precipitated by centrifugation at 2000xg for 10 minutes. Aliquots of supernatant were dehydrated for 6 hours at 55 degrees Celsius and counted in a ß-scintillation counter.

(g) Plasma catecholamines were measured by a radioenzymatic method (Amersham Inc., U.S.A.). The assay system utilizes the enzyme catechol-O-methyltransferase to catalyze the transfer of a [^{3}H]-methyl group from *S*-adenosyl-L-[methyl-^{3}H] methionine to norepinephrine and epinephrine. The

resulting products, [^{3}H] normetanephrine and [^{3}H] metanephrine are converted by periodate oxidation to [^{3}H] vanillin and extracted.

The catecholamine values reported in the various studies represent the mean of values obtained 60 and 120 minutes after surgery (unless otherwise specified), since the levels in all groups fell within the first 30 minutes and remained unchanged thereafter.

(D) STATISTICAL ANALYSES

(i) Sample size

We initially applied a two tailed power analysis (by using the standard deviation obtained from our preliminary study with vanadyl sulfate) for each of the dependent variables (insulin, glucose and BP) under study. The analysis indicated that for a probability (alpha) level of 0.05, the minimum sample size required for each experimental group was 6-8 rats; therefore a minimum of 6 rats/group were used in all the studies.

(ii) Statistics

All data are presented as means±SE. Data analysis for each study was performed by using analytical tests as described below. The independent variable in each study was the drug intervention (treated versus control). Since there were several dependent variables (glucose, insulin, BP, body weight), the differences among various group means were studied by using a multivariate analysis of variance (MANOVA), using the Number Cruncher Statistical Program (NCSS). MANOVA is the most powerful statistical procedure available for this type of analysis.

In MANOVA, it is assumed that individual group variance-covariance matrices are equal (a preliminary test is provided to determine the viability of this assumption). The test is used to determine if the mean vector (made up of the individual variate means) between various groups is different. The statistic used is called Wilk's Lambda, which is the multivariate extension of R-squared (from multiple regression). Significance tests are made by using an F-approximation to Wilk's Lambda.

For all the studies, a probability of $P<0.05$ was taken to indicate a significant difference between means. Once the MANOVA detected a significant difference in the mean vector, the individual variables were analyzed by employing the Newman-Keuls test for multiple comparisons. Changes within each group over time were analyzed by an analysis of variance (ANOVA) followed by a Newman Keuls test. Differences in the various parameters before and after administration of exogenous insulin (in rats given insulin implants) were compared by using the paired t-test procedure on the NCSS statistical package. For the vanadyl sulfate studies in the SHR, the systolic BP and plasma insulin values reported in the text represent the mean values from weeks 10 and 12 in the prevention study and weeks 11-13 in the reversal study. For all other studies in the SHR, the systolic BP and insulin values represent the mean values from weeks 9-11.

RESULTS

(I) STUDIES IN THE SPONTANEOUSLY HYPERTENSIVE RAT

(i) Studies with vanadyl sulfate

(a) Body weight

Representative results from the pair-feeding study are discussed below; similar results were obtained in the prevention and reversal studies. In agreement with earlier studies (Mondon and Reaven 1988), the untreated W rats gained weight at a faster rate than the untreated S group (figure 1). Although vanadyl consumption resulted in reduced weight gain in the SV and WV rats, they continued to gain weight throughout the study period. Body weight of the pair-fed SF and WF rats remained similar to the corresponding vanadyl-treated groups throughout the study.

(b) Plasma insulin concentration

Prevention/Reversal Studies: The SHR were hyperinsulinemic as compared to the WKY; vanadyl lowered plasma insulin levels in the SHR in both the prevention (SV: 252±23 versus S: 336±13 pmol/L, $P<0.01$) and reversal studies (SV_1: 264±13 versus S: 342±7 pmol/L, $P<0.001$, figures 2A and 3A) to control WKY values (W: 264±23 pmol/L, $P>0.05$ versus SV). This decrease in plasma insulin concentration was maintained throughout the study periods. The average percentage decrease in the plasma insulin values (fed values) seen after vanadyl treatment was about 20% in the SHR and 4% in the WKY. The decrease in plasma insulin levels in the treated WV rats did not attain statistical significance except at one time point (week 10, prevention study).

Pair-Feeding Study: Plasma insulin values in the pair-feeding study represent five-hour fasted values as opposed to fed values in the other two studies. The untreated SHR were hyperinsulinemic as compared to their WKY controls even as early as 5 weeks of age (figure 4A). Vanadyl reduced the fasting plasma insulin values in the SHR by about 35% and this decrease persisted throughout the study. Plasma insulin levels in the pair-fed SHR were intermediate between those seen in the untreated and vanadyl-treated groups. No change in plasma insulin levels was observed between the control, treated or pair-fed WKY. However, as the WKY grew older and heavier, their plasma insulin levels increased, which probably signified the combined (negative) effect of increasing weight and age on insulin sensitivity in these rats (DeFronzo and Ferrannini, 1991).

(c) Blood pressure

Prevention/Reversal Studies: In the prevention study, chronic vanadyl sulfate treatment resulted in a marked and sustained decrease in systolic BP in the SHR, which was noticed at all time points starting at week 8 (SV: 158±2 versus S: 189±1 mmHg, $P<0.001$, figure 2B). BP remained unchanged in the normotensive, treated WKY (WV: 127±1 versus W: 135±1 mmHg, $P>0.05$). Direct systolic BP measurements conducted at the termination of the study confirmed the previous indirect readings, which was a reflection on the validity of both the techniques used (figure 2). There was a difference of about 8 mmHg between the direct and indirect systolic BP readings, which was consistent in all groups except the untreated SHR. During the indirect BP measurements, the pulsations in the caudal arteries in the untreated SHR appeared earlier than in the other groups, probably due to the increased pulse pressure and the hyperdynamic state associated with hypertension. Consequently, the untreated SHR had to be

restrained for a relatively shorter period of time (about 3 minutes after the initial 10 minute warming period, as opposed to 5-10 minutes in the other groups), which shortened the period of restraint/external heating in that group. This may be the reason why the modest increase in the indirect BP readings (as compared with the direct measurements) that occurred in the other three groups was not observed in the untreated SHR. When vanadyl was started after the SHR were fully hypertensive (reversal study), it again caused a marked decrease in BP in the SHR (SV_1: 161±1 versus S: 188±1 mmHg, $P<0.001$, figure 3B) but had no effect in the WKY.

Pair-Feeding Study: The decrease in BP in the vanadyl-treated SHR was similar in magnitude to that seen in the prevention and reversal studies (figure 4B). However, the pair-fed SHR remained as hypertensive as the control, untreated rats and their BP remained unchanged. Also, no change in BP was noticed between the normotensive control, treated or pair-fed Wistar Kyoto rats (figure 4B).

Insulin Implant Study: Restoration of plasma insulin concentration in the vanadyl-treated SHR (table 1) reversed the effects of vanadyl sulfate on BP and this reversal was observed as early as 1 week after placement of the insulin implants (SV with insulin implants 190±3 versus SV without insulin implants 152±3 mmHg, $P<0.001$). No change in BP was observed in the vanadyl-treated WKY treated with exogenous insulin. Catecholamine levels in the vanadyl-treated SHR remained unchanged as compared to those seen in the untreated SHR (SV: 2470±203 versus S: 2284±377 pg/ml, $P>0.05$), which suggests that the antihypertensive effects of vanadyl may be independent of changes in sympathetic activity.

FIGURE 1

PAIR-FEEDING STUDY: Body weights in the six groups: S (SHR-untreated, n=8), W (WKY-untreated, n=8), SV (SHR-vanadyl treated, n=8), WV (WKY-vanadyl treated, n=8), SF (SHR pair-fed with SV but not given vanadyl, n=7) and WF (WKY pair-fed with WF but not given vanadyl, n=7). Vanadyl treatment was initiated at the start of week 6. Data are shown as means±SE.

* $P<0.05$; S different from SV and SF.

# $P<0.05$; W different from WV and WF.

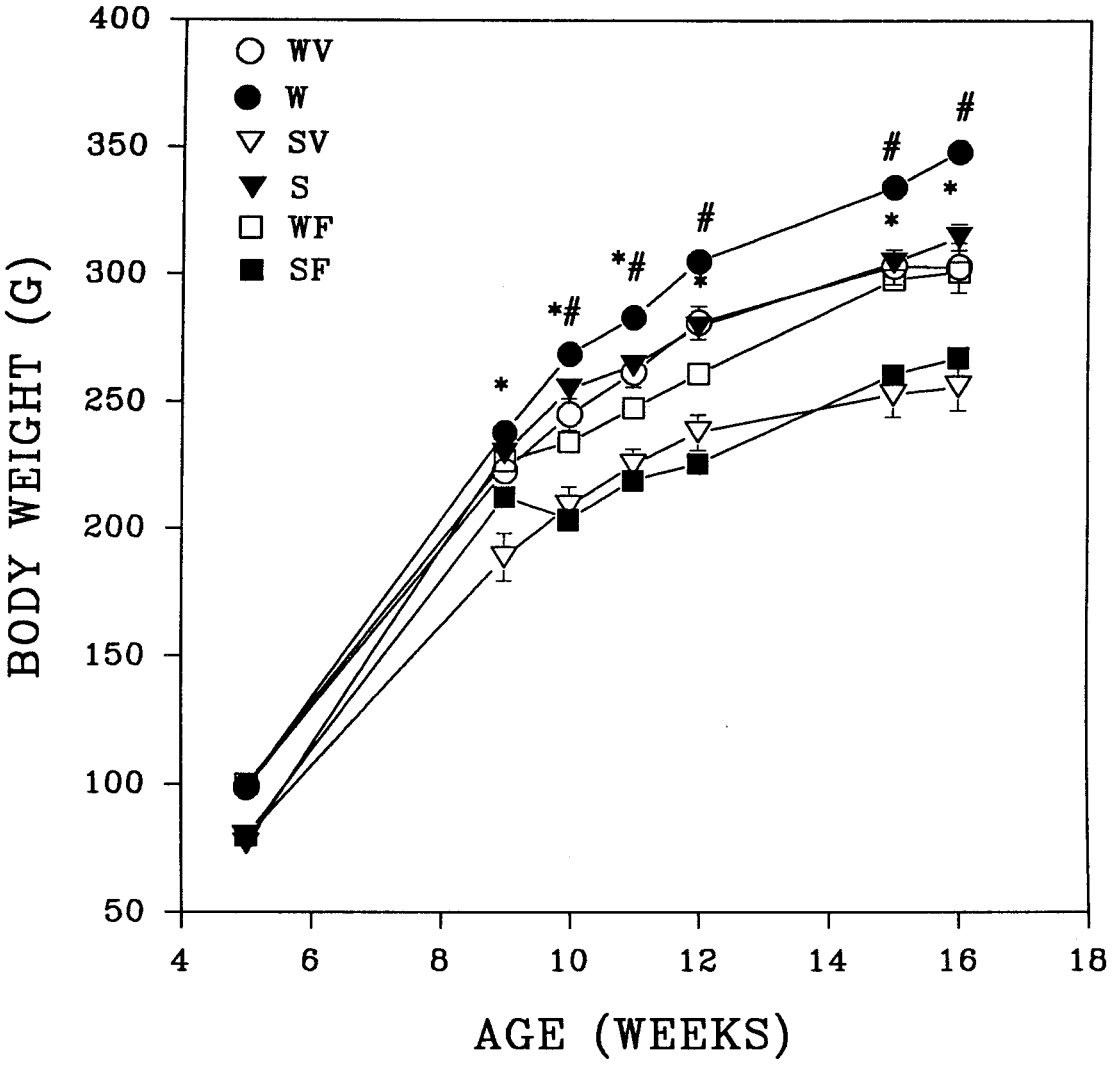

400
350
300
250
200
150
100
50
BODY WEIGHT (G)
WV
W
SV
S
WF
SF
4
6
8
10
12
14
16
18
AGE (WEEKS)

FIGURE 2

PREVENTION STUDY: (A) Plasma insulin levels and (B) Systolic blood pressure in the four groups (vanadyl treatment was started at the end of week 5): WV (WKY-vanadyl treated, n=6), W (WKY-untreated, n=12), SV (SHR-vanadyl treated, n=8) and S (SHR-untreated, n=15). Data are shown as means±SE.

* $P<0.001$ S different from SV at all time points after week 8. Systolic BP in both S and SV different from W and WV (the latter two not different from each other). The number of animals for direct BP measurements are shown in parentheses.

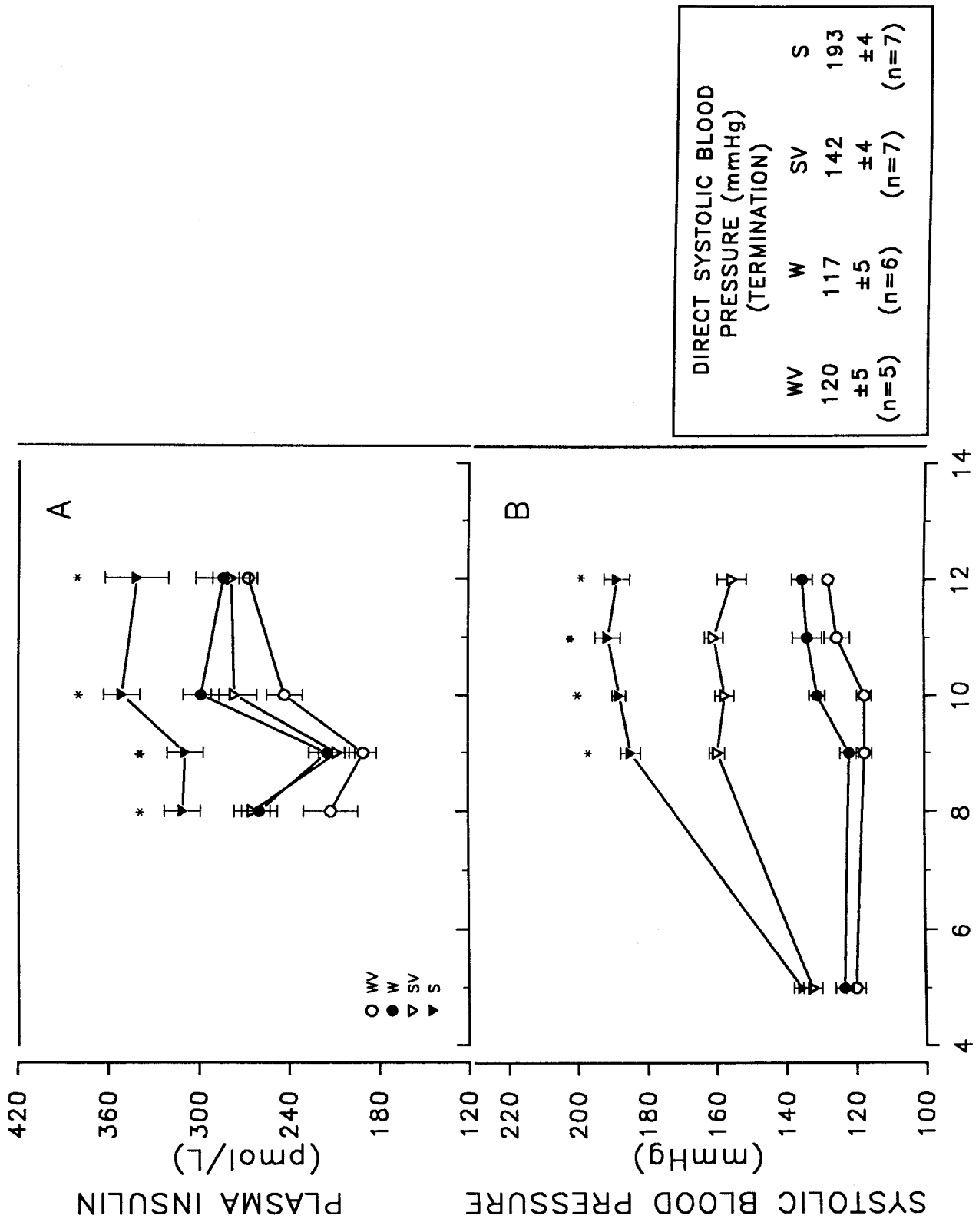

A
B
PLASMA INSULIN (pmol/L)
SYSTOLIC BLOOD PRESSURE (mmHg)
AGE (WEEKS)
WV
W
SV
S
DIRECT SYSTOLIC BLOOD PRESSURE (mmHg) (TERMINATION)
WV 120 ±5 (n=5)
W 117 ±5 (n=6)
SV 142 ±4 (n=7)
S 193 ±4 (n=7)

FIGURE 3

REVERSAL STUDY: (A) Plasma insulin levels and (B) Systolic blood pressure in the four groups (vanadyl treatment was started at week 11): WV_1 (WKY-vanadyl treated, n=6), W (WKY-untreated, n=6), SV_1 (SHR-vanadyl treated, n=6) and S (SHR-untreated, n=9). Data are shown as means±SE.

* $P<0.001$ S different from SV_1 after week 11. BP of both S and SV_1 different from W and WV_1 (the latter two not different from each other). The number of animals for direct BP measurements are shown in parentheses.

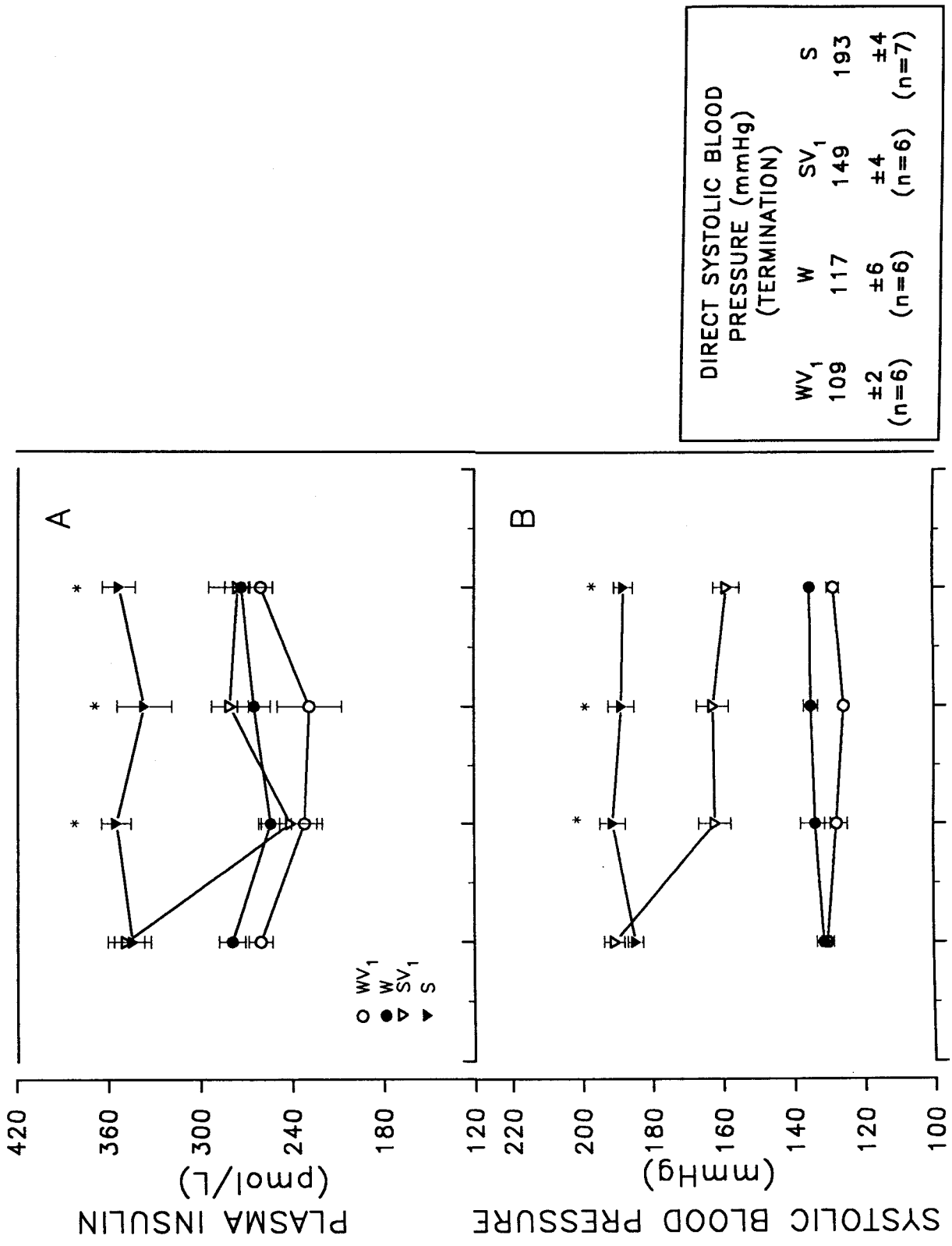
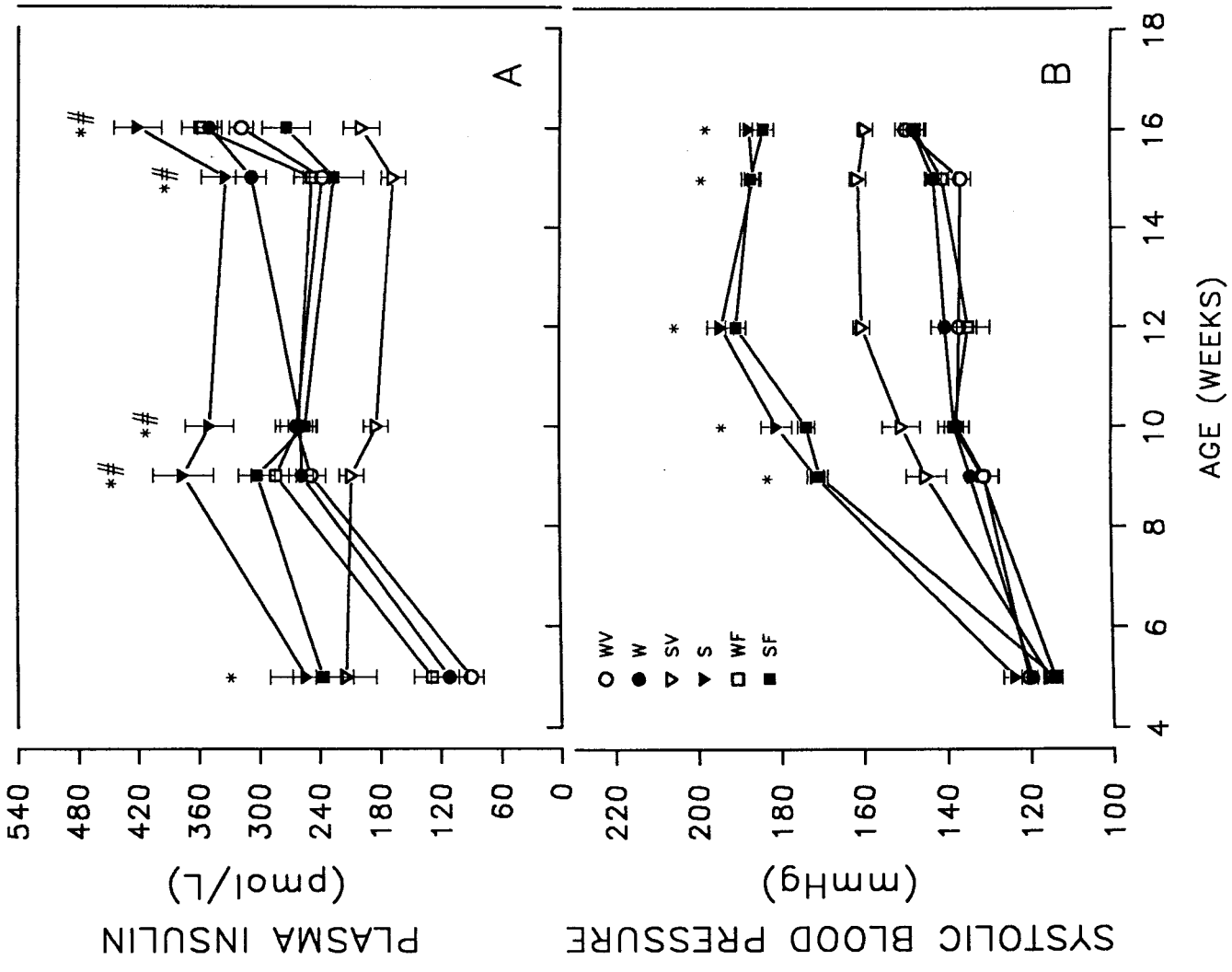

DIRECT SYSTOLIC BLOOD PRESSURE (mmHg) (TERMINATION)
WV1 109 ±2 (n=6)
W 117 ±6 (n=6)
SV1 149 ±4 (n=6)
S 193 ±4 (n=7)
A
B
WV1
W
SV1
S
PLASMA INSULIN (pmol/L)
SYSTOLIC BLOOD PRESSURE (mmHg)
AGE (WEEKS)

FIGURE 4

PAIR-FEEDING STUDY: (A) Plasma insulin levels and (B) Systolic blood pressure in the six groups: S (SHR-untreated, n=8), W (WKY-untreated, n=8), SV (SHR-vanadyl treated, n=8), WV (WKY-vanadyl treated, n=8), SF (SHR pair-fed with SV but not given vanadyl, n=7) and WF (WKY pair-fed with WF but not given vanadyl, n=7). Vanadyl treatment was initiated at the start of week 6. Data are shown as means±SE.

* $P<0.05$; S different from SV after week 9. Systolic BP in both S and SV different from W, WV and WF at all time points starting at week 9. # $P<0.05$; S different from SF.


A
B
PLASMA INSULIN
(pmol/L)
SYSTOLIC BLOOD PRESSURE
(mmHg)
AGE (WEEKS)
WV
W
SV
S
WF
SF

(d) Plasma glucose concentration

The average plasma glucose values in the various groups during the different study protocols ranged from 5.8-7.7 mmol/L (table 2). All the groups in the prevention, reversal and pair-feeding studies remained euglycemic (<8.5 mmol/L) throughout the respective study periods. No changes in plasma glucose levels were observed after vanadyl treatment in either the fed (prevention/reversal studies) or the 5-hour fasted (pair-feeding study) states. Furthermore, plasma glucose concentration remained unchanged in the vanadyl-treated SHR one week after placement of the insulin implants (6.0±0.2 versus pre-implant 6.0±0.1 mmol/L, $P>0.05$). However, a decrease in plasma glucose was observed 3 weeks post-implant (4.0±0.9 versus pre-implant 6.0±0.1 mmol/L, $P<0.05$), which was not accompanied by any change in plasma glucagon levels (table 1). Plasma catecholamines in the vanadyl-treated SHR given insulin implants showed an increase as compared to their pre-implant values (3 weeks post implant: 4940±662 versus pre-implant 2073±245 pg/ml), which may indicate either a compensatory response to a fall in plasma glucose or a direct effect of sustained hyperinsulinemia on the sympathetic nervous system.

(e) Food and fluid intake

Both food and fluid intake in the vanadyl-treated groups decreased as compared to the untreated groups. This was seen in all the treated groups to a similar extent and was noticed in the prevention, reversal and pair-feeding studies (tables 3A-C). The average decrease in food intake ranged from 10-15% and the decrease in fluid intake ranged from 30-40%. The pair-fed SHR and WKY were given food and fluid in amounts that were similar to the corresponding vanadyl-

treated groups, which was reflected as similar weight gain patterns between the pair-fed and the vanadyl-treated rats (figure 1).

(f) Dose of vanadyl consumed

Daily vanadyl dose calculated over the study periods showed an overall downtrend with time, due to the increasing body weight of the rats rather than a reduction in their fluid intake. The dose consumed ranged from 0.4-0.6 mmol/kg/day and was not different between the various treated groups.

(g) Hepatic function

Vanadyl treatment did not cause any change in plasma GPT in either the SHR or the WKY (S: 70±4, SV: 66±7 and SF: 70±4 u/l; W: 56±4, WV: 57±4 and WF: 60±3 u/l). Furthermore, vanadyl did not affect plasma GOT levels in either of the two rat strains (S: 18±1, SV: 15±1 and SF: 17±2 u/l; W: 17±2, WV: 16±2 and WF: 14±3 u/l).

(h) Renal function

Plasma urea-nitrogen values also remained unchanged in the treated SHR and WKY (SV: 10±1 and WV: 10±1 mmol/L) as compared with the respective untreated (S: 13±1 and W: 10±1 mmol/L) or pair-fed (SF: 11±1 and WF: 9±1 mmol/L) groups. Thus 10 weeks of vanadyl treatment did not cause any impairment of hepatic or renal function in either the SHR or WKY. In addition, none of the treated rats died or exhibited any signs of gastrointestinal disturbance throughout the experimental period.

TABLE 1

VARIOUS PARAMETERS FROM THE EXPERIMENTAL GROUPS IN THE EXOGENOUS INSULIN STUDY

RATS	BP (mmHg)			Insulin (pmol/L)			Glucagon (ng/L)	
	B	A1	A3	B	A1	A3	B	A3
S	205±3	191±5	197±1	330±12	402±12	396±54	398±35	310±23
SV	146±4†	137±6†	159±1†	210±30†	180±12†	144±24†	405±14	296±26
SI*	152±3†	190±3§†	189±4§‡	198±12†	360±48§‡	618±42†§‡	390±20	298±35
W	130±7†	136±4†	147±1†	228±24†	264±18†	294±24†	340±30	270±22
WV	135±2†	141±2†	147±2†	234±18†	276±12†	294±24†	287±16	316±25
WI*	135±4†	144±2†	148±3†	222±18†	324±12§	480±102§	307±33	258±30

Various parameters before (B), one week after (A1) and 3 weeks after (A3) placement of the insulin implants in the experimental groups: S (SHR-untreated, n=4); SV (SHR-vanadyl treated, n=6); SI (SHR-vanadyl treated with implants, n=6); W (WKY-untreated, n=4); WV (WKY-vanadyl treated, n=6) and WI (WKY-vanadyl treated with insulin implants, n=6). Data are shown as means±SE.

* Insulin dose from the insulin implants was 14000 pmol/kg/day and implants were given only in the SI and WI groups. BP denotes systolic blood pressure.
† $P<0.05$, different from S.
§ $P<0.05$, different from the respective pre-implant values.
‡ $P<0.05$, SI different from SV.

TABLE 2

PLASMA GLUCOSE (MMOL/L) IN THE EXPERIMENTAL GROUPS IN THE PREVENTION, REVERSAL AND PAIR-FEEDING STUDIES

PREVENTION STUDY:		Week 8	Week 9	Week 10	Week 12
W	(12)	6.7±0.1	6.6±0.2	6.7±0.2	6.5±0.1
WV	(6)	6.4±0.3	6.7±0.4	6.3±0.3	6.4±0.2
S	(15)	6.7±0.1	6.6±0.2	6.4±0.1	6.5±0.2
SV	(8)	6.8±0.2	6.6±0.1	6.0±0.1	6.7±0.3
REVERSAL STUDY:		Week 10 (no vanadyl)	Week 11	Week 12	Week 13
W	(6)	6.4±0.3	6.3±0.2	6.2±0.1	6.2±0.1
WV_1	(6)	6.2±0.1	6.4±0.1	6.4±0.4	6.3±0.2
S	(9)	6.5±0.2	6.2±0.2	6.4±0.2	6.2±0.2
SV_1	(6)	6.0±0.2	5.8±0.3	6.6±0.3	6.6±0.3
PAIR-FEEDING STUDY:		Week 5 (no vanadyl)	Week 9	Week 10	Week 12
W	(8)	6.9±0.2	8.2±0.2	7.1±0.2	7.0±0.1
WV	(8)	7.0±0.3	7.7±0.2	7.6±0.2	7.5±0.1
WF	(8)	7.0±0.1	7.3±0.1	7.6±0.2	7.4±0.2
S	(8)	6.9±0.1	8.1±0.2	7.1±0.2	6.9±0.1
SV	(8)	6.5±0.2	7.6±0.2	7.6±0.1	7.4±0.2
SF	(7)	6.2±0.1	8.0±0.3	7.6±0.2	7.0±0.3

Data are shown as means±SE. The numbers in parentheses indicate the number of animals in each group. S=untreated SHR, W=untreated WKY, SV and SV_1=vanadyl-treated SHR, WV and WV_1=vanadyl-treated WKY, SF=SHR pair-fed with SV but not given vanadyl and WF= WKY pair-fed with WV but not given vanadyl. Vanadyl treatment in the prevention and pair-feeding studies was started at the end of week 5/start of week 6 (weeks denote the age of the animals) and in the reversal study at week 11. In the pair-feeding study, plasma samples were collected after fasting the animals for 5 hours. However, values in the prevention and reversal studies are from fed animals.

TABLE 3A

FOOD AND FLUID INTAKE IN THE VARIOUS EXPERIMENTAL GROUPS IN THE PREVENTION STUDY

		Week 4 (before vanadyl)	Week 6	Week 8	Week 10	Week 12
Food intake: (g/day)						
W	(12)	21±1	21±2	20±1	18±1	19±2
WV	(6)	21±1	16±1†	18±1	17±2	17±1
S	(15)	22±1	21±1	21±1	18±1	19±1
SV	(8)	23±2	17±2*	19±1	19±2	18±1
Fluid intake: (ml/day)						
W	(12)	31±2	34±1	39±2	37±2	40±1
WV	(6)	33±1	27±1†	29±1†	29±1†	28±1†
S	(15)	36±1	35±2	37±2	42±1	44±2
SV	(8)	35±2	28±2*	27±1*	28±2*	30±1*

Data are shown as means±SE. The numbers in parentheses indicate the number of animals in each group. S=untreated SHR, W=untreated WKY, SV=vanadyl-treated SHR, WV=vanadyl-treated WKY.
Vanadyl treatment was started at the end of week 5/start of week 6 (weeks denote the age of the animals).
* $P<0.05$ SV different from S and W; † $P<0.05$ WV different from W and S.

TABLE 3B

FOOD AND FLUID INTAKE IN THE VARIOUS EXPERIMENTAL GROUPS IN THE REVERSAL STUDY

		Week 10 (before vanadyl)	Week 12	Week 14
Food intake: (g/day)				
W	(6)	18±1	19±1	18±1
WV_1	(6)	18±1	17±2	16±2
S	(9)	19±1	21±1	20±2
SV_1	(6)	19±2	15±1*	18±1
Fluid intake: (ml/day)				
W	(6)	36±2	38±2	38±2
WV_1	(6)	35±2	27±2†	29±2†
S	(9)	41±2	40±2	42±2
SV_1	(6)	40±2	29±2*	28±2*

Data are shown as means±SE. The numbers in parentheses indicate the number of animals in each group. S=untreated SHR, W=untreated WKY, SV_1=vanadyl-treated SHR, WV_1=vanadyl-treated WKY.
Vanadyl treatment was started at the start of week 11 (weeks denote the age of the animals).
* $P<0.05$ SV_1 different from S and W; † $P<0.05$ WV_1 different from W and S.

TABLE 3C

FOOD AND FLUID INTAKE IN THE VARIOUS EXPERIMENTAL GROUPS IN THE PAIR-FEEDING STUDY

		Week 5 (before vanadyl)	Week 6	Week 8	Week 10	Week 12
Food intake: (g/day)						
W	(8)	17±1	18±2	20±2	20±2	18±2
WV	(8)	17±2	16±2	21±2	18±2	19±1
WF	(8)	17±1		(PAIR-FED WITH WV)		
S	(8)	18±2	19±2	21±2	22±3	19±1
SV	(8)	17±1	14±2*	19±2	19±2	19±2
SF	(7)	18±2		(PAIR-FED WITH SV)		
Fluid intake: (ml/day)						
W	(8)	37±2	40±3	42±1	45±2	47±3
WV	(8)	38±2	21±2†	31±2†	37±3†	33±1†
WF	(8)	38±2		(PAIR-FED WITH WV)		
S	(8)	34±1	34±2	36±1	48±2	46±2
SV	(8)	35±2	20±1*	24±2*	28±2*	27±2*
SF	(7)	34±2		(PAIR-FED WITH SV)		

Data are shown as means±SE. The numbers in parentheses indicate the number of animals in each group. S=untreated SHR, W=untreated WKY, SV=vanadyl-treated SHR, WV=vanadyl-treated WKY, SF=SHR pair-fed with SV but not given vanadyl and WF=WKY pair-fed with WV but not given vanadyl.
Vanadyl treatment was started at the end of week 5/start of week 6 (weeks denote the age of the animals).
* $P<0.05$ SV different from S and W; † $P<0.05$ WV different from W and S.

(ii) Studies with bis(maltolato)oxovanadium(IV)

(a) Blood pressure, plasma insulin and plasma glucose concentration

Five-hour fasted SHR were hyperinsulinemic as compared to the WKY (table 4). BMOV also caused a sustained decrease in plasma insulin levels in the SHR (SO: 198±6 pmol/L, $P<0.0001$ versus S) without having any effect in the WKY (WO: 228±5 versus W: 223±4 pmol/L, $P>0.05$, table 4 and figure 6A). Interestingly, BMOV also caused a marked decrease in systolic BP in the SHR (SO: 149±3 versus S: 184±3 mmHg, $P<0.0001$, table 4 and figure 6B), but had no effect in the WKY. Five-hour fasted glucose in all groups remained normal (<8.5 mmol/L) and no change in plasma glucose was observed after BMOV treatment in either the SHR or WKY (table 4). Body weight in the BMOV-treated SHR and WKY was about 8-9% lower than their respective untreated groups (figure 5), but did not attain statistical significance ($P>0.1$ for mean of weeks 9-11 in the treated versus the respective untreated groups for both SHR and WKY, table 4).

(b) Euglycemic clamp study

During the 3 day conditioning period, weight gain in the rats was normal and similar to that seen during the previous weeks and none of the rats lost weight. Plasma catecholamine levels declined within the first 30 minutes of surgery in both the untreated SHR (plasma levels at 30 minutes: 866±50 versus those immediately after surgery 1333±100 pg/ml) and WKY (plasma levels at 30 minutes: 867±200 versus those immediately after surgery 1400±233 pg/ml). Steady state plasma glucose levels during clamps were similar in the four experimental groups (table 5) and were well matched to their corresponding basal concentrations.

FIGURE 5

BMOV STUDY: Body weights in the four groups: S (SHR-untreated, n=9), W (WKY-untreated, n=11), SO (SHR-BMOV treated, n=11), WO (WKY-BMOV treated, n=9). BMOV treatment was initiated at the start of week 6. Data are shown as means±SE.

* $P<0.05$; S and W different from SO and WO.

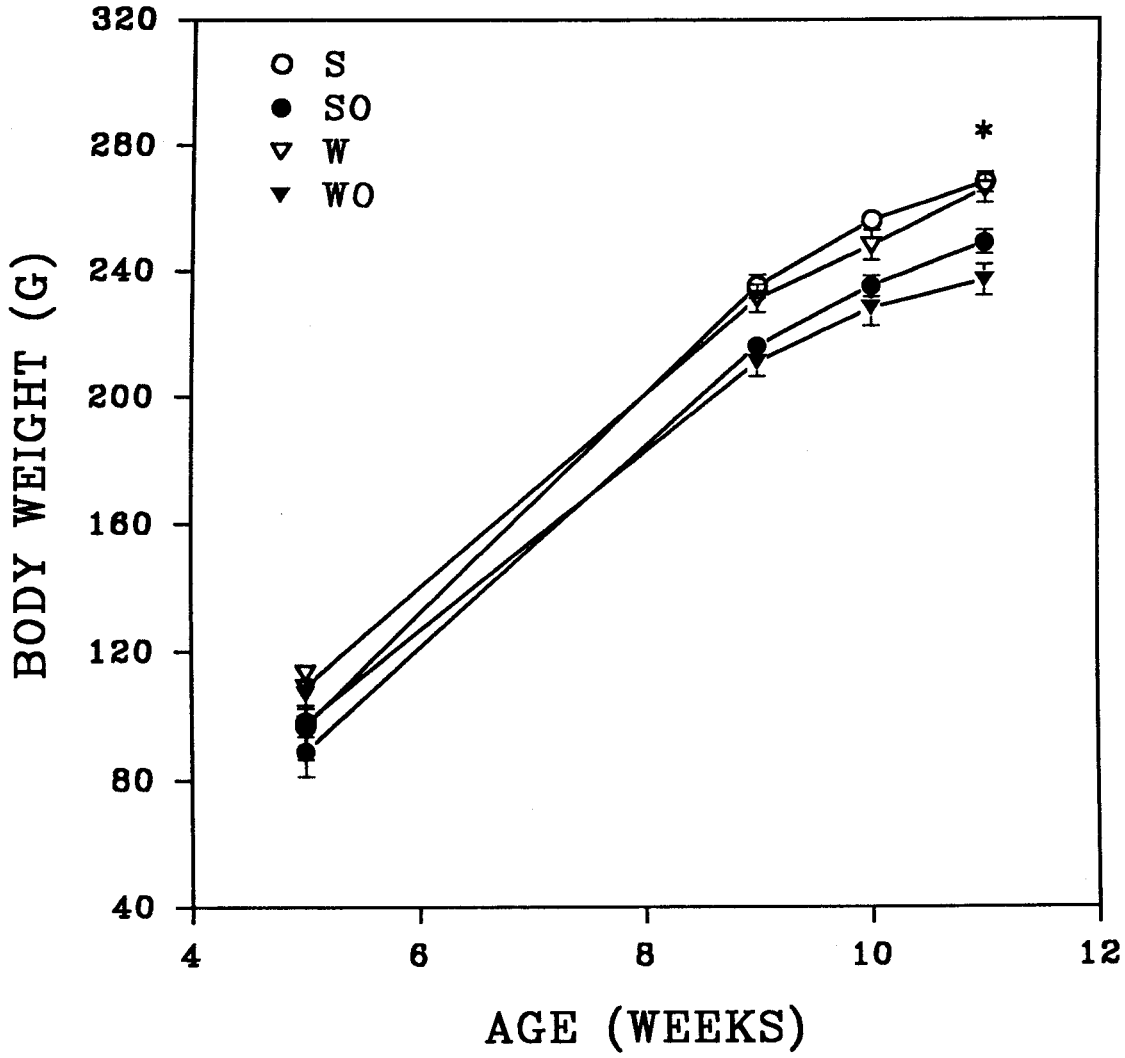

320
280
240
200
160
120
80
40
BODY WEIGHT (G)
S
SO
W
WO
*
4
6
8
10
12
AGE (WEEKS)

FIGURE 6

BMOV STUDY: (A) Plasma insulin levels and (B) Systolic blood pressure in the four groups: S (SHR-untreated, n=9), W (WKY-untreated, n=11), SO (SHR-BMOV treated, n=11), WO (WKY-BMOV treated, n=9). BMOV treatment was initiated at the start of week 6. Data are shown as means±SE.

* $P<0.0001$; S different from the other 3 groups. Systolic BP of SO was different from W and WO at weeks 9 and 11.

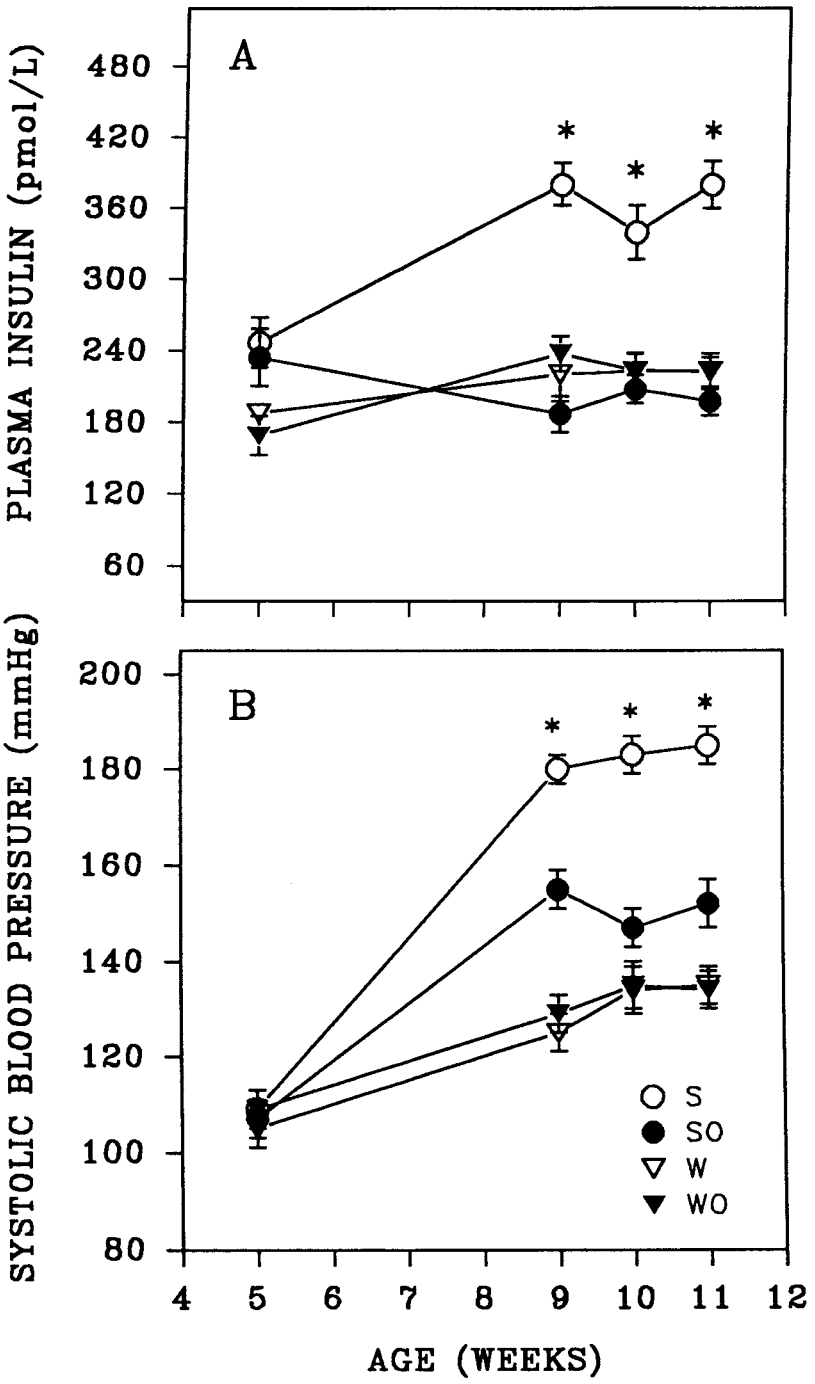

A
B
PLASMA INSULIN (pmol/L)
SYSTOLIC BLOOD PRESSURE (mmHg)
AGE (WEEKS)
480
420
360
300
240
180
120
60
200
180
160
140
120
100
80
4
5
6
7
8
9
10
11
12
*
S
SO
W
WO

TABLE 4

CHARACTERISTICS OF ANIMALS AT 9-11 WEEKS OF AGE IN THE BMOV STUDY

GROUP	S	SO	W	WO
Number	9	11	11	9
Weight (g)	253±10	233±10	248±8	225±8
Plasma glucose (5 hour fasted) (mmol/L)	6.2±0.1	6.0±0.1	6.3±0.1	5.9±0.1
Plasma insulin (5 hour fasted) (pmol/L)	366±13†	198±6*	223±4	228±5
Blood pressure (systolic) (mmHg)	184±3†	149±3*	134±5	134±3

Data are shown as means±SE. All data represent the average of values from weeks 9-11. S=untreated SHR, SO=BMOV-treated SHR, W=untreated WKY, WO=BMOV-treated WKY.

* $P<0.0001$, SO different from S. † $P<0.0001$, S different from the other 3 groups.

TABLE 5

RESULTS OF GLUCOSE CLAMPS IN THE BMOV STUDY

GROUP	S	SO	W	WO
Number	11	8	9	9
Basal glucose (20 hour fasted) (mmol/L)	3.2±0.1	3.3±0.1	3.2±0.1	2.8±0.1
Basal insulin (20 hour fasted) (pmol/L)	94±8	62±11	90±20	61±6
Clamp glucose (mmol/L)	3.3±0.1	3.0±0.2	3.4±0.1	3.0±0.2
Clamp insulin (pmol/L)	495±51	427±40	363±66	381±66
Clamp R_d (mmol/kg/h)	3.2±0.2	4.1±0.3	1.5±0.3	2.2±0.3
Clamp HGO (mmol/kg/h)	-1.4±0.2	-1.6±0.3	-0.4±0.2	-0.6±0.2
Clamp R_d / [G]/[I]† (ml/kg/h/pmol/L)	2.1±0.2‡	3.6±0.4*	1.2±0.1	2.4±0.5

Data are shown as means±SE. S=untreated SHR, SO=BMOV-treated SHR, W=untreated WKY, WO=BMOV-treated WKY. R_d=peripheral glucose disposal, HGO =hepatic glucose output.

† Clamp R_d / [G]/[I]= insulin sensitivity index (ISI) = steady state glucose clearance/steady state plasma insulin.

‡ $P<0.002$, S different from W.

* $P<0.01$, SO different from S.

HGO was completely suppressed in all groups. Negative values were obtained for HGO because cold glucose infusate was not "spiked" with D-[3-^{3}H]-glucose during these studies. When insulin sensitivity was expressed as the steady state glucose clearance per unit of steady state insulin, the untreated SHR were found to be more insulin-sensitive than the untreated WKY (table 5). BMOV treatment caused further enhancement in insulin sensitivity in the SO group ($P<0.01$ versus S).

(iii) Studies with pioglitazone

(a) Blood pressure, plasma insulin and plasma glucose concentration

Five-hour fasted SHR were hyperinsulinemic as compared to the WKY (S: 418±16 versus W: 234±10 pmol/L, $P<0.0001$). Pioglitazone caused a sustained decrease in plasma insulin levels in the SHR (SP: 200±7 pmol/L, $P<0.0001$ versus S) without having any effect in the WKY (WP: 226±4 pmol/L, $P>0.05$ versus W, figure 8A). Pioglitazone also caused a marked decrease in systolic BP in the SHR (SP: 150±3 versus S: 195±3, $P<0.0001$, figure 8B), but had no effect in the WKY (WP: 138±2 versus W: 140±2 mmHg, $P>0.05$). Five-hour fasted glucose levels in all groups remained normal (<8.5 mmol/L) and no change in plasma glucose was observed after pioglitazone treatment in either the SHR or WKY (table 6). Furthermore, the effects of pioglitazone on plasma insulin and BP were independent of any change in food intake, fluid intake or body weight (figure 7).

(b) Insulin implant study

Restoration of plasma insulin levels in the drug-treated SHR by using subcutaneous insulin implants (354±30 pmol/L, $P>0.05$ versus S and $P<0.0001$ versus SP without implants) reversed the effects of pioglitazone on BP and this reversal was observed as early as 1 week after placement of the insulin implants

(SP with insulin implants 183±3 mmHg versus SP without insulin implants 150±3 mmHg, P<0.001). No change in BP was observed in the treated WKY given exogenous insulin (WP with implants 139±2 mmHg, P>0.05 versus WP without implants). Catecholamine levels in the pioglitazone-treated SHR remained unchanged as compared to those seen in the untreated SHR (SP: 1219±141 versus S: 1124±101 pg/ml, P>0.05), which suggests that the antihypertensive effect of the drug may be independent of changes in sympathetic activity.

(c) Euglycemic clamp studies

During the conditioning period, weight gain in the rats was normal and similar to that observed during the previous weeks. Steady state plasma glucose levels in both the low and high dose clamp studies were similar between all four groups (tables 7A-B) and were well matched to their basal concentrations. Similarly, steady state insulin concentrations were similar in all the groups during both the high and low dose insulin clamp studies. Hepatic glucose production was fully suppressed during insulin infusion in all groups in both studies. During low dose insulin clamps, the untreated SHR were found to be more insulin-sensitive than the untreated WKY (table 7B). However, this difference was not observed during the high dose clamps, probably because the high steady state concentrations of insulin resulted in maximal stimulation of glucose utilization in all the groups. Surprisingly, neither the pioglitazone-treated SHR or WKY demonstrated any improvement in insulin sensitivity during either the low or high dose insulin clamp studies (tables 7A-B). Basal glucagon levels were significantly higher in the untreated SHR as compared with the untreated WKY and pioglitazone treatment had no effect on plasma glucagon concentrations in either the SP or WP rats (table 6).

FIGURE 7

PIOGLITAZONE STUDY: Body weights in the four groups: S (SHR-untreated, n=9), W (WKY-untreated, n=8), SP (SHR-pioglitazone treated, n=16), WP (WKY-pioglitazone treated, n=15). Pioglitazone treatment was initiated at the start of week 6. Data are shown as means±SE.

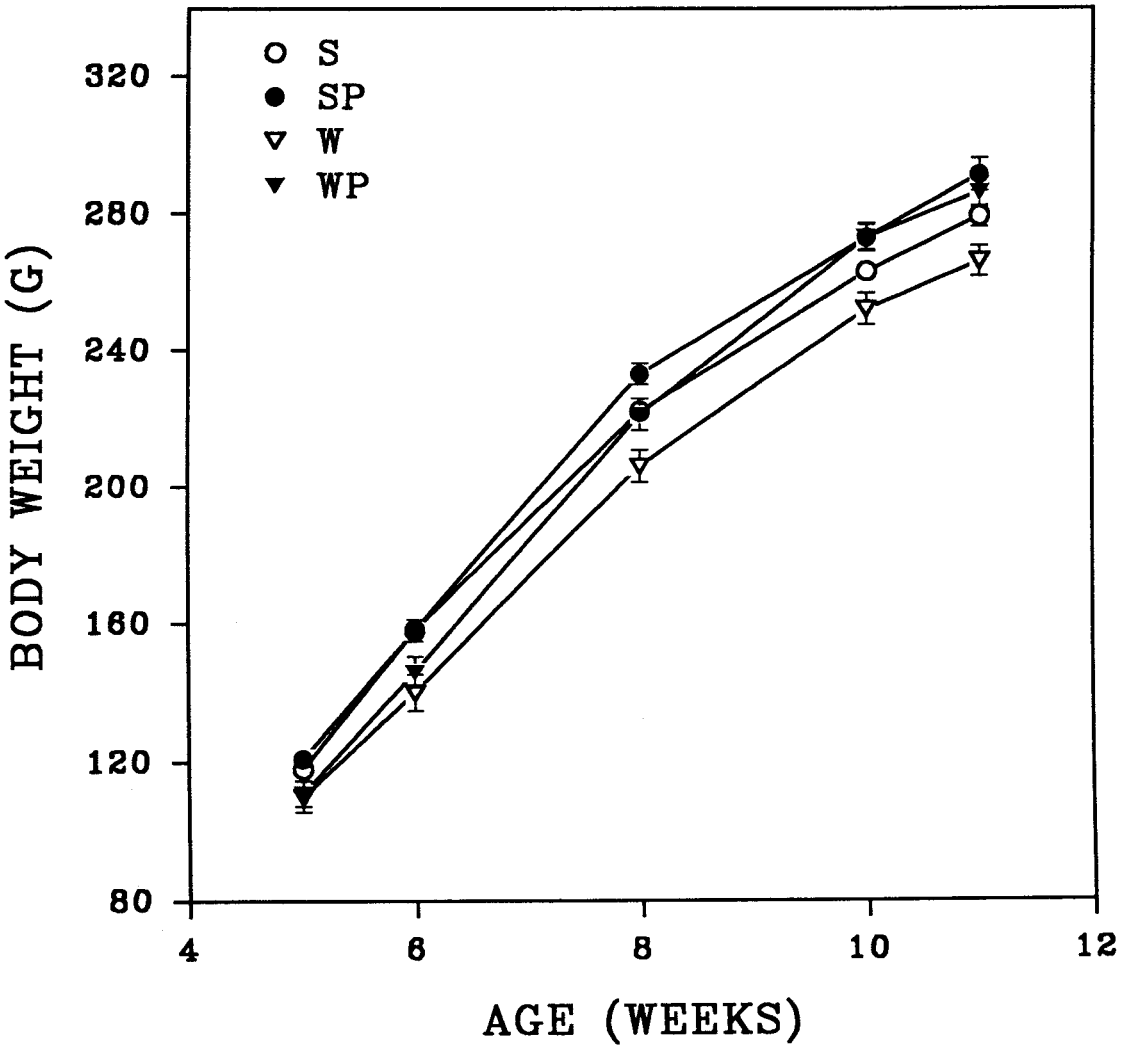

S
SP
W
WP
320
280
240
200
160
120
80
BODY WEIGHT (G)
4
6
8
10
12
AGE (WEEKS)

FIGURE 8

PIOGLITAZONE STUDY: (A) Plasma insulin levels and (B) Systolic blood pressure in the four groups: S (SHR-untreated, n=9), W (WKY-untreated, n=8), SP (SHR-pioglitazone treated, n=16), WP (WKY-pioglitazone treated, n=15). Pioglitazone treatment was initiated at the start of week 6. Data are shown as means±SE.

* $P<0.0001$; S different from the other 3 groups.

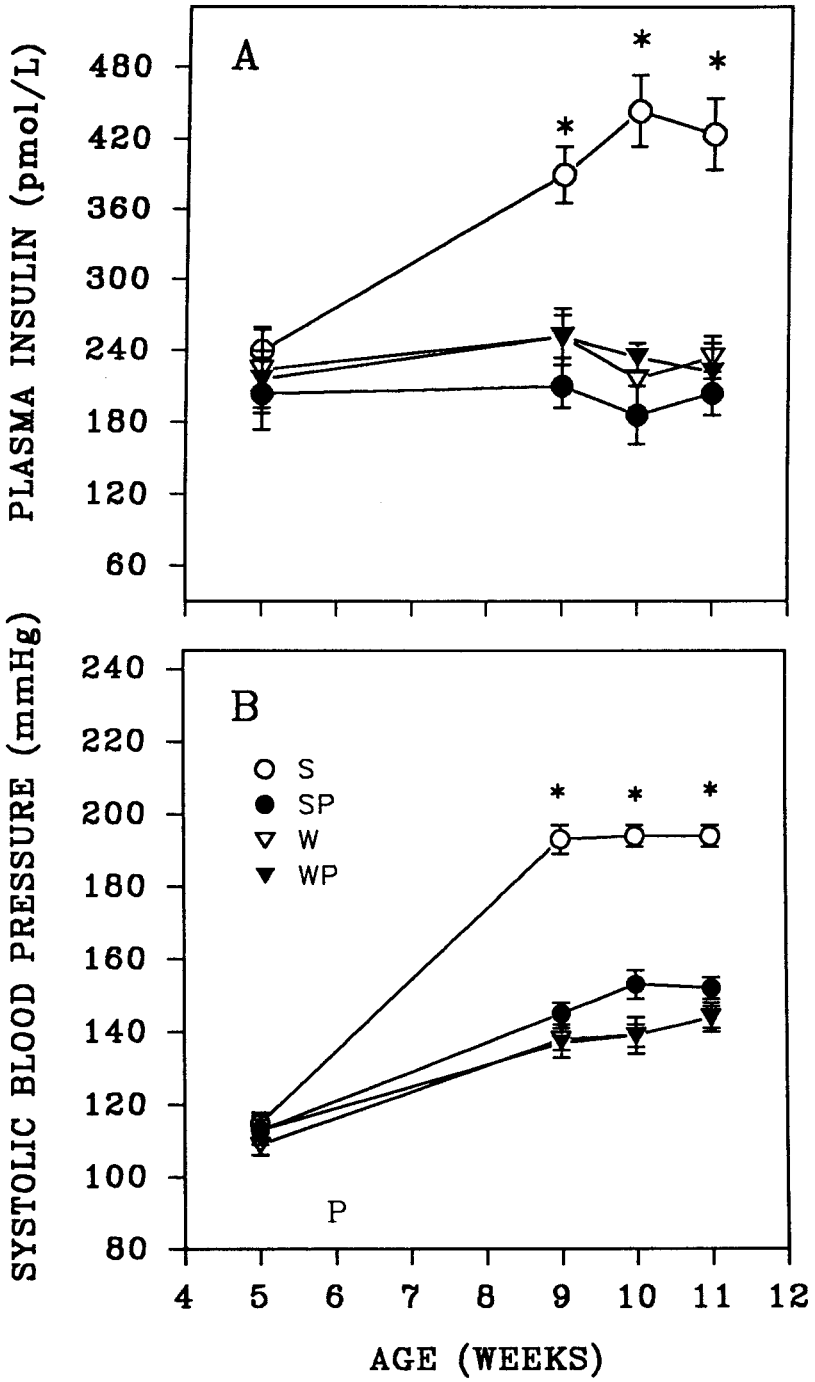

A
PLASMA INSULIN (pmol/L)
480
420
360
300
240
180
120
60
*
*
*
B
SYSTOLIC BLOOD PRESSURE (mmHg)
240
220
200
180
160
140
120
100
80
S
SP
W
WP
P
*
*
*
4
5
6
7
8
9
10
11
12
AGE (WEEKS)

TABLE 6

CHARACTERISTICS OF ANIMALS AT 9-11 WEEKS OF AGE IN THE PIOGLITAZONE STUDY

GROUP	S	SP	W	WP
Number	8	16	8	15
Weight (g)	262±10	270±8	250±10	270±12
Food intake (g/day)	23±1	23±1	22±1	22±1
Fluid intake (ml/day)	38±2	41±1	40±1	40±1
Plasma glucagon (20 hour fasted) (ng/L)	65±5	58±4	45±4*	45±4*
Plasma catecholamines (pg/ml)	1124±101	1219±141	896±118	1112±106
Plasma glucose (5 hour fasted) (mmol/L)	7.3±0.3	7.7±0.7	7.3±0.3	7.3±0.3

Data are shown as means±SE. S= untreated SHR, SP=pioglitazone-treated SHR, W=untreated WKY, WP=pioglitazone-treated WKY.

* $P<0.05$, W and WP different from S and SP.

TABLE 7A

RESULTS OF GLUCOSE CLAMPS AT A HIGH INSULIN INFUSION RATE (70 PMOL/KG/MIN) IN THE PIOGLITAZONE STUDY

GROUP	S	SP	W	WP
Number	8	6	8	8
Basal glucose (20 hour fasted) (mmol/L)	3.1±0.1	3.1±0.1	3.2±0.1	3.5±0.1
Basal insulin (20 hour fasted) (pmol/L)	81±19	68±11	95±13	112±17
Clamp glucose (mmol/L)	3.0±0.1	3.1±0.1	3.1±0.2	3.4±0.1
Clamp insulin (pmol/L)	2918±289	2789±216	2854±146	3423±312
Clamp R_d (mmol/kg/h)	8.8±0.3	9.7±0.4	8.4±0.2	10.3±0.3
Clamp HGO (mmol/kg/h)	-4.8±0.2	-5.5±0.4	-4.6±0.2	-5.6±0.3
Clamp R_d/[G]/[I] (ml/kg/h/pmol/L)	1.0±0.1	1.2±0.1	1.0±0.1	1.0±0.1

Data are shown as means±SE. R_d = peripheral glucose disposal, HGO=hepatic glucose output.
Clamp R_d/[G]/[I]=insulin sensitivity index (ISI)=steady-state glucose clearance/steady-state plasma insulin. S=untreated SHR rats, SP=pioglitazone-treated SHR rats, W=untreated WKY rats, WP=pioglitazone-treated WKY rats.

TABLE 7B

RESULTS OF GLUCOSE CLAMPS AT A LOW INSULIN INFUSION RATE (14 PMOL/KG/MIN) IN THE PIOGLITAZONE STUDY

GROUP	S	SP	W	WP
Number	5	6	6	6
Clamp weight (g)	308±7	294±6	284±10	308±9
Basal glucose (20 hour fasted) (mmol/L)	3.4±0.1	3.5±0.1	3.3±0.1	3.6±0.1
Basal insulin (20 hour fasted) (pmol/L)	66±4	91±1	111±9*	126±17*
Clamp glucose (mmol/L)	3.2±0.1	3.4±0.1	3.3±0.2	3.6±0.1
Clamp insulin (pmol/L)	466±11	491±18	418±36	484±14
Clamp R_d (mmol/kg/h)	11.7±1.1	9.9±0.3	4.0±0.9#	4.0±0.3#
Clamp HGO (mmol/kg/h)	-10.5±1.0	-8.8±0.3	-3.3±0.8#	-3.3±0.2#
Clamp R_d/[G]/[I] (ml/kg/h/pmol/L)	7.9±0.8	6.1±0.3	3.1±0.8#	2.2±0.1#

Data are shown as means±SE. R_d = peripheral glucose disposal, HGO=hepatic glucose output. Clamp R_d/[G]/[I]=insulin sensitivity index (ISI) = steady-state glucose clearance/steady-state plasma insulin. S=untreated SHR rats, SP=pioglitazone-treated SHR rats, W=untreated WKY rats, WP=pioglitazone-treated WKY rats.

* $P<0.05$ versus S; # $P<0.05$ versus S and SP.

(II) STUDIES IN THE FRUCTOSE-HYPERTENSIVE RAT

(a) Blood pressure, plasma insulin and plasma glucose concentration

Fructose feeding caused an increase in 5-hour fasted plasma insulin levels and this increase persisted throughout the study (mean baseline insulin: 230±20 versus mean at 9-11 weeks: 366±9 pmol/L, figure 9A). Vanadyl sulfate (0.4-0.6 mmol/kg/day) completely prevented the rise in plasma insulin concentration in the fructose-fed rats (mean baseline insulin: 253±14 versus mean at 9-11 weeks: 211±6 pmol/L, $P>0.05$). Vanadyl also caused a modest decrease in insulin levels in the control group (baseline: 241±15 versus mean at 9-11 weeks: 181±19 pmol/L, $P<0.05$). As illustrated in figure 9B, BP in the fructose-fed rats increased from 124±3 to 160±3 mmHg, $P<0.001$). This increase in systolic BP was evident 2 weeks after starting the fructose diet and persisted throughout the study. In contrast, BP did not rise in the fructose-fed rats treated with vanadyl sulfate (mean at baseline: 131±3 versus mean at weeks 9-11: 126 ±3, $P>0.05$). No changes in BP were seen in the control group (baseline: 125±5 versus mean at 9-11 weeks: 126±4 mmHg, $P>0.05$).

(b) Insulin implant study

Restoration of plasma insulin concentration in the fructose-fed-vanadyl treated rats (FV with insulin implants 340±20 versus FV without implants 214±35 pmol/L, $P<0.001$) reversed the effects of vanadyl sulfate and caused a corresponding increase in BP (FV with insulin implants 170±10 versus FV without implants 121±3 mmHg, $P<0.001$). This increase in BP was independent of changes in body weight (FV with implants 353±8 versus FV without implants 348±7 grams, $P>0.05$). However, no change in BP was seen in the control-treated rats (V with implants 122±5 versus V without implants 119±4 mmHg,

P>0.05). In addition, no change in 5-hour fasted plasma glucose levels was observed after the administration of exogenous insulin in either the control-vanadyl treated or fructose-vanadyl treated rats.

Fructose feeding did not cause any change in food intake, fluid intake or body weight when compared to the untreated controls. Vanadyl treatment resulted in a reduction in weight gain in both the V and FV rats (table 8). The average plasma glucose values in the various groups ranged from 6.3-7.8 mmol/L. Five-hour fasted glucose levels in all groups remained normal (<8.5 mmol/L) and no change in plasma glucose was observed after vanadyl treatment in either the V or FV groups (table 8).

(c) Euglycemic clamp study

During the 3 day conditioning period, weight gain in the rats was normal and similar to that seen during the previous weeks and none of the rats lost weight. Steady state plasma glucose levels were similar in the four experimental groups (table 9) and were well matched to their corresponding basal concentrations. Mean plasma insulin levels during the final 30 minutes of the clamp were also similar in all four groups. Hepatic glucose production was completely suppressed in all groups. Negative values were obtained for HGO because cold glucose infusate was not "spiked" with D-[3-^{3}H]-glucose during these studies. As is evident from table 9, the F group was severely insulin-resistant as compared to the normotensive C group. Vanadyl treatment caused a marked enhancement in insulin sensitivity in the FV rats and restored their insulin sensitivity to control levels. Plasma catecholamine levels were calculated by taking the mean of values obtained 60 and 120 minutes after surgery, since the levels in all groups fell within the first 30 minutes and remained unchanged

thereafter. Catecholamine levels in the FV rats did not decrease as compared to the F rats not given vanadyl (table 8), suggesting again that the antihypertensive effects of vanadyl are independent of any change in sympathetic activity. In addition, there was no difference in catecholamine levels between the C and V groups (C: 915±228 versus V: 1478±493 pg/ml, $P>0.05$) and the values among all 4 groups were similar at all time points after surgery.

FIGURE 9

FRUCTOSE STUDY: (A) Plasma insulin levels and (B) Systolic blood pressure in the four groups: C (control-untreated, n=8), V (control-vanadyl treated, n=12; vanadyl treatment started at week 6), F (fructose-untreated, n=9; fructose diet started at week 7) and FV (fructose-vanadyl treated, n=15; vanadyl started at week 6 and fructose diet at week 7). Data are shown as means±SE. V and F in the figure denote the start of the vanadyl and fructose treatments respectively.

* $P<0.001$, F different from the other 3 groups.

# $P<0.05$, V different from C.

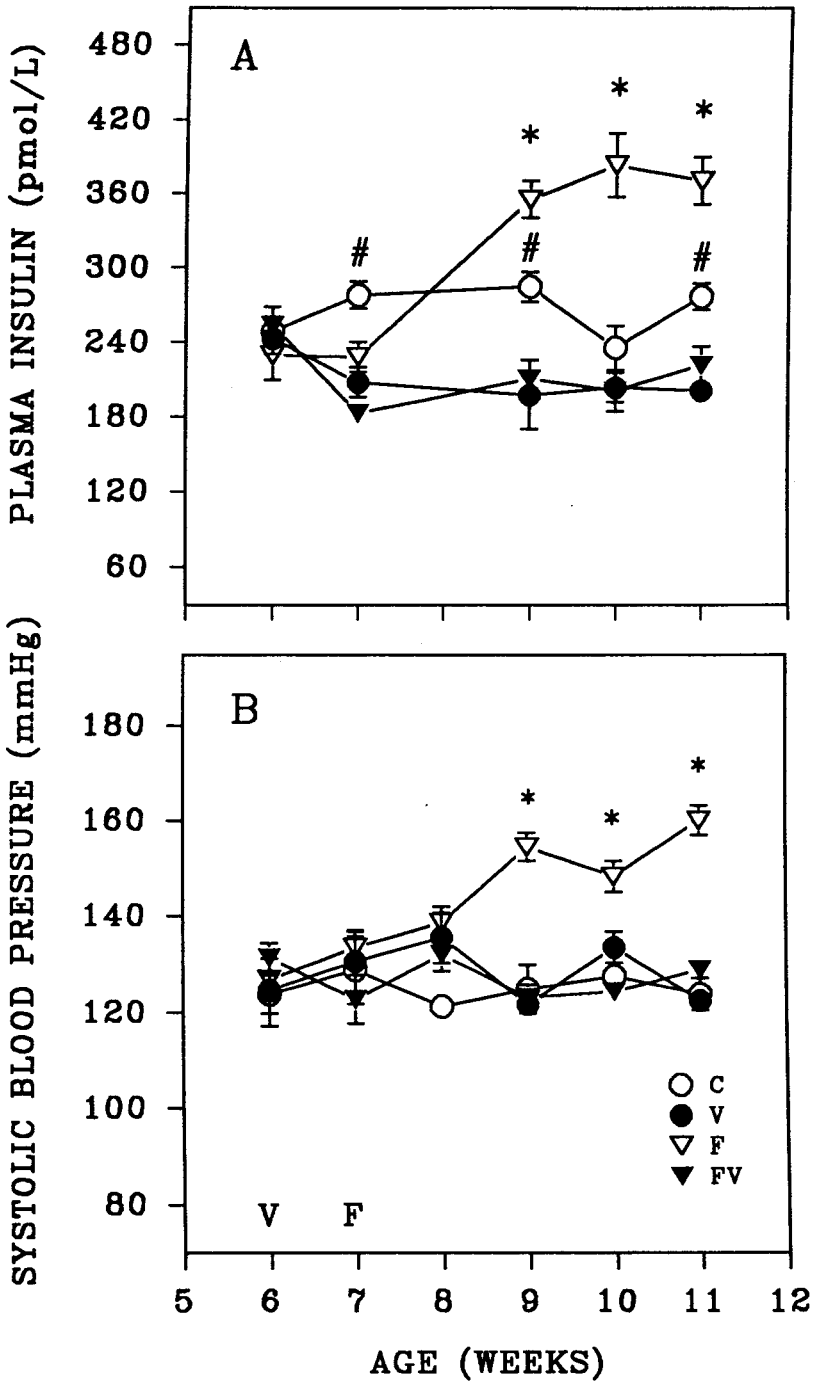

A
B
PLASMA INSULIN (pmol/L)
SYSTOLIC BLOOD PRESSURE (mmHg)
AGE (WEEKS)
480
420
360
300
240
180
120
60
180
160
140
120
100
80
5
6
7
8
9
10
11
12
C
V
F
FV
V
F

TABLE 8

CHARACTERISTICS OF ANIMALS AT WEEKS 6 (BASELINE) AND 12 IN THE FRUCTOSE STUDY

GROUP	C	F	V	FV
Number	8	9	12	15
$Weight_{W6}$ (g)	199±4	208±5	211±6	203±5
$Weight_{W12}$ (g)	387±8	389±6	334±10*†	327±7*†
Plasma catecholamines (pg/ml)	1978±493	1044±69#	915±45#	1743±109
$Plasma\ glucose_{W12}$ (5 hour fasted) (mmol/L)	6.9±0.3	7.1±0.3	7.1±0.3	7.2±0.4

Data are shown as means±SE. W6, W12=at week 6 and 12 respectively. C=control, F=fructose-treated, V=control-vanadyl treated, FV=fructose-vanadyl treated.

* $P<0.05$, different from C.
† $P<0.05$, different from F.
# $P<0.05$, different from C and FV.

TABLE 9

RESULTS OF GLUCOSE CLAMPS IN THE FRUCTOSE STUDY

GROUP	C	F	V	FV
Number	4	4	4	4
Basal glucose (20-hour fasted) (mmol/L)	3.6±0.0	4.2±0.1*	3.2±0.1	3.4±0.4
Basal insulin (20-hour fasted) (pmol/L)	189±58	289±96	76±44	178±29
Clamp glucose (mmol/L)	3.4±0.3	4.3±0.3	3.6±0.1	3.8±0.4
Clamp insulin (pmol/L)	475±53	498±104	351±72	389±42
Clamp R_d (mmol/kg/h)	1.4±0	0.8±0.01†	1.8±0.2	1.9±0.4
Clamp HGO (mmol/kg/h)	-1.1±0.4	0.3±0.2	-0.8±0.1	-0.1±0.6
Clamp R_d/[G]/[I] (ml/kg/h/pmol/L)	0.8±0.1	0.4±0.1†	1.4±0.3	1.3±0.3

Data are shown as means±SE. C=control, F=fructose-treated, V=control-vanadyl treated, FV=fructose-vanadyl treated. Clamp R_d/[G]/[I]=insulin sensitivity index (ISI)= steady-state glucose clearance/steady-state plasma insulin. R_d=peripheral glucose disposal. HGO=hepatic glucose output.

* $P<0.05$, different from C and V.

† $P<0.05$, different from the other 3 groups.

DISCUSSION

(I) Effects of vanadyl sulfate in spontaneously hypertensive rats

Results from this study confirmed previous observations that SHR are hyperinsulinemic as compared to their genetic WKY controls (Hulman *et al.*, 1991; Mondon and Reaven 1988). In this study, the vanadyl form of vanadium was used, since previous reports from our laboratory suggested that it was better tolerated than other forms of vanadium (Cros *et al.*, 1992). Vanadyl sulfate, in doses of 0.4-0.6 mmol/kg/day, lowered both plasma insulin levels as well as systolic BP in the SHR. The decrease in plasma insulin concentration observed in the prevention, reversal and pair-feeding studies was quite marked and was accompanied by concurrent decreases in systolic BP. Furthermore, the effects of vanadyl were independent of changes in plasma glucagon or catecholamines, suggesting that the effects were not mediated by a change in sympathetic activity. Although these findings do not prove that these events are causally related, they do provide indirect support for such a link. The observation that the pair-fed SHR remained as hypertensive as the untreated SHR indicates that the antihypertensive effect observed was specific to vanadyl and that the decrease in BP was independent of any change in food/fluid consumption or body weight. Vanadyl did not lower BP in the normotensive WKY, neither did it have any significant effect on their plasma insulin values.

Some of the well recognized insulin-like effects of vanadium include activation of both glucose transport and glycogen synthesis in rat adipocytes and skeletal muscle (Dubyak and Kleinzeller, 1980; Tolman *et al.*, 1979), inhibition of lipolysis (Duckworth *et al.*, 1988) and stimulation of lipogenesis (Fantus *et al.*, 1990). Fantus et al. reported that the vanadate form of vanadium caused a

marked increase in insulin-stimulated receptor kinase activity and prolonged insulin-stimulated lipogenesis in rat adipocytes (Fantus *et al.,* 1994). Other studies suggest that the glucoregulatory effect of vanadium is mediated either via an insulin-independent cascade or via its action at a site distal to the insulin receptor (Strout *et al.,* 1989). Whatever the precise mechanism/s of action of the drug may be, they get translated *in vivo* as an improvement in glucose utilization. Not only does vanadyl lower glucose levels in diabetic rats (without any increase in plasma insulin), it also causes a decrease in insulin levels in non-diabetic rats without any change in plasma glucose concentration (Ramanadham *et al.,* 1990a,b). Thus, it seems that either by replacing or potentiating the action/s of endogenous insulin, vanadyl causes a feedback inhibition of insulin release in non-diabetic rats.

Previous studies have demonstrated that in hyperinsulinemic rats, experimental interventions that decrease plasma insulin levels also attenuate increases in BP (Reaven *et al.,* 1988; Reaven *et al.,* 1989b). We recently reported that chronic metformin treatment in the SHR causes a decrease in insulin levels and BP that is very similar to that observed with vanadyl (Verma *et al.,* 1994). Furthermore, the decrease in BP in the metformin-treated rats was reversed when insulin levels in the metformin-treated SHR were restored to those that existed before treatment. In the present study, replacement of plasma insulin levels in the vanadyl-treated SHR to those that existed before treatment also caused a corresponding increase in BP. This effect was evident as early as 1 week after placement of the insulin implants, when post-implant plasma insulin and glucose values in the rats were similar to those seen in the untreated SHR. These findings suggest that hyperinsulinemia may increase BP in rats, a view that is further supported by studies documenting that hyperinsulinemia can elicit many

hypertensinogenic mechanisms such as activation of the sympathetic nervous system, increase in renal sodium and water reabsorption and proliferation of vascular smooth muscle tissue (for a review, please see DeFronzo and Ferrannini, 1991).

Implicit in the point of view outlined above is the assumption that vanadyl does not exhibit any other antihypertensive properties and that it selectively improves insulin action. Although the vanadate (+5) form of vanadium has been shown to affect the activities of various intracellular enzymes *in vitro* (mostly at pharmacological concentrations), vanadate is reduced intracellularly to the vanadyl (+4) state (Cros *et al.,* 1992; Sakurai *et al.,* 1990). Vanadyl, in turn, is a very poor inhibitor of cellular enzyme systems (Cros *et al.,* 1992; Shechter 1990). We are not aware of any study documenting direct antihypertensive effects of vanadyl at concentrations similar to those used in the present study. In addition, the observation that the antihypertensive effect of vanadyl could be reversed simply by raising plasma insulin levels in the vanadyl-treated rats to those seen in the untreated SHR indicates that hyperinsulinemia is closely related to an increase in BP in the SHR. Since it was not possible to alter the rate of insulin release with the type of implants used in this study, plasma insulin values in the vanadyl-treated SHR given insulin implants exceeded those seen in the untreated SHR three weeks after placement of the insulin implants (causing a decrease in plasma glucose and an increase in plasma catecholamines). However, what is perhaps important is the observation that reversal of vanadyl's antihypertensive effect was evident even one week post-implant, when plasma glucose and insulin levels in the rats with implants were similar to those in the untreated SHR. Although we cannot unequivocally explain why plasma insulin levels in the rats increased 3 weeks post-implant as compared to those that were observed one

week post-implant, the subcutaneous insulin implants were probably not fully functional by the end of the first week.

A few other interesting observations surfaced from the present study and are briefly mentioned below. Although vanadyl lowered plasma insulin concentration in the SHR to control WKY levels, BP in the SHR did not decline to normotensive values. This suggests that hyperinsulinemia may be only one of several factors causing or predisposing towards high BP in the SHR. Another observation that deserves mention is that although vanadyl sulfate decreased plasma insulin levels in the SHR, it had no effect on these metabolic parameters in the WKY. Recent studies in our laboratory indicate that although vanadium compounds lower plasma insulin levels in non-diabetic Wistar and Sprague Dawley rats (Bhanot *et al.,* 1993a; McNeill *et al.,* 1992), they do not affect insulin levels in the WKY (Bhanot *et al.,* 1993b). Furthermore, the WKY show remarkably different effects to other metabolic insults when compared to other rat strains. For example, the WKY are resistant to the effects of streptozotocin-induced diabetes (Iwase *et al.,* 1987) and show marked differences in heart rate, cardiac function and plasma triglycerides as compared to Wistar and Sprague Dawley diabetic rats (Ramanadham *et al.,* 1990a; Rodrigues and McNeill, 1986). Thus the WKY seem to exhibit certain metabolic differences when compared to other rat strains.

Although the plasma insulin levels in the prevention/reversal studies are from fed rats and those in the pair-feeding study are from 5-hour fasted rats, the fed values are not very different from the fasted ones, which would appear to be in conflict. However, rats consume most of their food at night and we have observed that they do not eat much during the first few hours in the day. Hence the 5-hour fasted values do not represent the "true" fasted state, since the animals would

normally not eat during that time. The reason for taking 5-hour fasted values was to decrease the intra-group variation in plasma insulin levels which could occur due to an occasional rat consuming small amounts of food during the day. Therefore the 5-hour fasted values actually represent postabsorptive insulin levels rather than the "true" fasted levels and are not very different from the fed values.

Another finding that needs to be addressed pertains to the plasma insulin concentration in the pair-fed SHR (figure 4). Although the magnitude of the increase in BP in the pair-fed rats was similar to that observed in the untreated SHR, the pair-fed rats had lower plasma insulin levels as compared to the untreated SHR. This would appear to be in conflict with the hypothesis that hyperinsulinemia is closely related to an increase in BP in the SHR. However, the lower plasma insulin concentration in the pair-fed SHR is a reflection of an increased fasting period in the rats (about 10 hours) as compared to that of the untreated and vanadyl-treated SHR (5 hours). This is because the pair-fed rats consumed all of their diet within the first 3-4 hours (from the time it was administered) as opposed to the untreated and vanadyl-treated groups who fed throughout the night. Therefore, when the animals were fasted for blood collection (from 8AM-1PM), the pair-fed rats were actually about 10-hour fasted as compared to the other groups, which were 5-hour fasted. Since hyperinsulinemia in the SHR is postabsorptive and is not seen in 10-hour fasted rats (discussed later), it is apparent that the pair-fed rats would not be hyperinsulinemic at the time of blood sampling. The observation that pair-fed rats consume most of their food within 3-4 hours of administration has been subsequently confirmed in other studies in our laboratory (unpublished observations), which reinforces the view

that very careful consideration should be given to the nutritional status of the SHR at the time of blood sampling for insulin measurements.

Some investigators have reported toxicity with vanadyl administration at levels much lower than those administered in the present study (Domingo *et al.*, 1991a,b), whereas others have not documented toxic effects even at higher doses (Mongold *et al.*, 1990). In studies reporting toxicity at lower vanadyl concentrations, the rats were made extremely diabetic (Domingo *et al.*, 1991a,b), no control-treated rats were included in the study and the effects of diabetes per se (as opposed to those of vanadium) were not excluded. In the present investigation, none of the rats died in any of the three studies conducted and no gastrointestinal, hepatic or renal toxicity was observed after 10 weeks of vanadyl treatment. Not only did the vanadyl-treated rats continue to gain weight throughout the experimental period, the pair-fed rats also gained weight at rates that were similar to the treated rats. This suggests that the reduced weight gain caused by vanadyl administration is due to the reduced food and fluid intake in the treated rats rather than any additional toxic effect of vanadyl. Plasma vanadium levels were not detectable in the untreated and pair-fed SHR and WKY. Plasma vanadium levels in the treated animals ranged from 0.48-1.07 μg/ml, which correspond with the levels at which vanadyl exhibits anti-diabetic effects (Mongold *et al.*, 1990).

In summary, this study confirmed the presence of hyperinsulinemia in the SHR as compared to their WKY controls. Vanadyl sulfate caused concurrent and sustained decreases in both plasma insulin levels and systolic BP in the SHR. Increasing plasma insulin levels in the vanadyl-treated SHR to those that existed before treatment reversed vanadyl's effects on BP. This suggests that either hyperinsulinemia may contribute to the development of high BP in the SHR or that

if hyperinsulinemia is not causally related to hypertension, then the underlying mechanism may be closely related to the expression of both disorders.

(II) Effects of bis(maltolato)oxovanadium(IV) in spontaneously hypertensive rats

In the previous study, we had not examined the effect of vanadyl sulfate on insulin sensitivity in the SHR. Although euglycemic clamp studies conducted in anesthetized SHR demonstrated that they were insulin-resistant, studies done in conscious SHR had challenged this notion. Studies done in conscious, minimally restrained rats showed no difference in insulin sensitivity in the SHR as compared to the WKY. However, the presence of hyperinsulinemia was confirmed even in those studies (Buchanan *et al.,* 1992a,b) and it was proposed that increased insulin levels may contribute towards the development of high BP in the SHR. Therefore, the precise nature of the relationship between these metabolic abnormalities and hypertension in the SHR remained elusive. Consequently, we decided to examine the effect of vanadium compounds on insulin sensitivity in the SHR. In earlier studies, we had observed that BMOV was more potent and better tolerated than vanadyl sulfate. In addition, it had a less negative effect on weight gain in rats as compared with vanadyl sulfate (McNeill *et al.,* 1992). Therefore, we chose BMOV over vanadyl sulfate for this study and examined the effect of BMOV on insulin sensitivity, hyperinsulinemia and systolic BP in the SHR.

In view of reported alterations in glucose metabolism induced by general anesthesia (Clark *et al.,* 1990; Lang *et al.,* 1987), glucose clamp studies were performed in conscious rats. During glucose clamps, the total R_d value at a given insulin concentration represents the sum of insulin-dependent and insulin-independent glucose disposal, both of which are influenced by plasma glucose levels. To exclude any underestimation of insulin action (due to minor differences in steady state plasma glucose levels between groups), we calculated insulin

sensitivity by dividing the steady state glucose clearance by the steady state plasma insulin. Results from our study demonstrate that the SHR are not insulin-resistant (but rather more insulin sensitive) as compared to their WKY controls. These results are in agreement with those from studies where clamps were performed in conscious rats (Buchanan *et al.,* 1992a; Frontoni *et al.,* 1992) but are in conflict with those obtained after clamping in anesthetized rats (Mondon and Reaven 1988; Hulman *et al.,* 1991).

This apparent conflict between results from studies conducted in conscious and anesthetized rats has been attributed to an exaggerated stress response to anesthesia in the SHR, which could result in secondary insulin resistance (Buchanan *et al.,* 1992a). Stress-induced increase in counter-regulatory hormones results in negative effects on glucose metabolism due to antagonism of insulin action as well as reduction in insulin secretion (Diebert and DeFronzo, 1980; Porte and Robertson, 1973). In another study, Rao recently reported that part of this discrepancy could be explained by the variability in insulin clearance that occurs during hyperinsulinemic clamp studies in rats (Rao, 1993). He suggested that such a variability in insulin clearance could result in an increased risk of a type (II) statistical error (especially when small sample sizes were used), which could mask the differences in insulin sensitivity between the SHR and WKY. However, in Rao's study, clamps were performed in anesthetized rats and catecholamine levels were not measured; therefore, the possibility of an increased stress response in the SHR could not be excluded.

Another obvious and important advantage of performing glucose clamp studies in conscious rats is that it circumvents the alterations in the physiological state of the animal that occur secondary to anesthesia. For example, pentobarbital anesthesia has been shown to antagonize insulin-induced

suppression of hepatic glucose output as well as insulin-induced peripheral glucose utilization (Clark *et al.,* 1990). Furthermore, anesthesia results in multiple physiological changes in BP, heart rate, body temperature and arterial pH (Baum *et al.,* 1985; Wilson *et al.,* 1987a,b). Due to these reasons, we chose to conduct clamp studies in conscious rats. Buchanan et al. reported that catecholamine concentrations returned to normal within four hours of cannulation (of the tail artery and vein) in animals preconditioned to partial restraint by the tail (Buchanan *et al.,* 1992a). In a modification of his method, it was recently reported that catecholamine concentrations returned to normal within 30 minutes of line placement in both Sprague Dawley rats and SHR (Cheung and Bryer-Ash, 1994). In the present study, we measured plasma catecholamine concentrations prior to starting the clamp infusions and found them to be similar in the untreated SHR and WKY groups.

As mentioned above, although the SHR do not appear to be insulin-resistant as compared to the WKY, they have been shown to exhibit postabsorptive hyperinsulinemia. The obvious question that then comes to mind is that if the SHR have normal insulin sensitivity, then why do their plasma glucose levels not decrease in the presence of hyperinsulinemia. In a recent study, it was reported that as compared to WKY rats, SHR have an exaggerated insulin response to an intravenously administered glucose load, an enhanced glucose tolerance and similar insulin-mediated glucose transport into skeletal muscle (Buchanan *et al.,* 1992b). Briefly, the authors studied both 4-hour and 12-hour fasted rats and observed that whereas 4-hour fasted rats were hyperinsulinemic as compared to the WKY, 12-hour fasted SHR had insulin levels that were similar to those seen in the WKY. Furthermore, in 4-hour fasted SHR, the decrease in plasma glucose did not attain statistical significance (although a

trend towards lower plasma glucose levels was observed), whereas in the 12-hour fasted SHR, plasma glucose values were lower than those seen in the WKY. Results from our study are similar to those of Buchanan et al. in that glucose levels after a 5-hour fast were similar between the SHR and WKY and are, therefore, reported as being normal. Although we did not measure plasma glucagon levels in the present study, we observed in one of our subsequent studies that there is increased glucagon secretion in the SHR, which could allow them to maintain normal glucose levels despite being more insulin sensitive than the WKY. Furthermore, the SHR exhibit an enhanced sympathetic response to feeding; therefore, a consequent increase in plasma catecholamine levels could also antagonize insulin action and prevent a fall in plasma glucose concentration in the fed state.

What is more important is the observation that the acute insulin responses to a glucose load (after both a 4-hour as well as a 12-hour fast) were 2-3 fold higher in the SHR as compared to the WKY rats, which was accompanied by an increased glucose disappearance rate in the SHR (Buchanan *et al.,* 1992b). Thus, the primary reason for postabsorptive hyperinsulinemia in the SHR seems to be hypersecretion of insulin in response to glucose. Furthermore, this hypersecretion of insulin does not seem to be related to insulin resistance, since the 3-O-methylglucose transport rates into the skeletal muscle isolated from the SHR and WKY rats were similar at physiological as well as pharmacological concentrations. Although a few studies done *in vitro* have demonstrated that there is a decrease in basal and insulin-stimulated glucose transport in isolated adipocytes from SHR as compared to WKY (Reaven and Chang, 1991; Reaven *et al.,* 1989a), results from two recent studies (Buchanan *et al.,* 1992b; Frontoni *et al.,* 1992) that directly examined glucose metabolism in skeletal muscle (which is

the primary site of glucose utilization) indicate that the SHR are not insulin-resistant as compared to the WKY. Therefore, results from studies conducted in isolated adipocytes do not contradict the observation that the SHR show normal insulin sensitivity, but rather suggest a differential regulation of carbohydrate metabolism in the muscle and adipose tissue in SHR.

If hyperinsulinemia contributed to an increase in BP in the SHR, then a decrease in plasma insulin levels should also attenuate the hypertension. Results of the present study support this hypothesis, since BMOV improved insulin sensitivity, decreased insulin levels and caused concurrent decreases in BP in the SHR. Interestingly, several recent reports indicate that compounds that enhance insulin sensitivity and thereby lower insulin levels also decrease BP in rats (Meehan *et al.,* 1993a; Morgan and Mark, 1993; Pershadsingh *et al.*, 1993). Taken together, these data suggest that either hyperinsulinemia contributes towards the development of high BP in rats or that the underlying mechanism is closely related to the expression of both these disorders. It may be argued that BMOV, an organic vanadyl complex, may also affect factors other than insulin i.e. it may decrease BP due to a direct vascular effect. Although such an effect cannot be excluded, we have considered several possibilities. Vanadyl is a very poor inhibitor of cellular enzyme systems (Cros *et al.,* 1992; Shechter 1990) and we are not aware of any study that indicates any direct antihypertensive effect of BMOV or vanadyl *in vivo* at concentrations employed in this study. Furthermore, if the antihypertensive effect of BMOV were due to a direct vascular effect, the drug should also have lowered BP in the WKY-BMOV treated rats (which was not observed), unless the SHR responded differently (as compared to the WKY) towards the direct effects of BMOV.

A few other observations from this study deserve mention. First, BMOV caused a 8-9% decrease in body weight in both the SO and WO groups and the contribution of such a decrease in body weight towards the observed improvement in insulin sensitivity cannot be excluded. BMOV does cause a decrease in body weight in SHR, WKY and other rat strains once the animals weigh 280-300 grams or more. However, we have consistently found that BMOV, at the concentration employed in this study, does not affect body weight gain in rats from 9-11 weeks of age (McNeill *et al.,* 1992; Yuen *et al.,* 1995). In fact, one of the primary reasons for using BMOV as the experimental intervention for this study was that it had a minimal effect on body weight gain as compared with other vanadium compounds. We had done a pilot study and were aware of the effects of BMOV before conducting this particular study. Our aim was to examine the effects of BMOV on insulin and BP before the appearance of any difference in body weight between the treated and untreated rats. Results of the study indicate that the antihypertensive effects of BMOV were present during weeks 9-11, when there was no difference in weight between the untreated and BMOV-treated SHR. However, to the extent that the modest decrease in body weight could have contributed towards the observed improvement in insulin sensitivity, our results do not allow us to exclude such a contribution.

Second, although the SHR were not insulin-resistant as compared to their WKY controls, BMOV further improved insulin sensitivity in the SHR. Such an improvement in insulin sensitivity could also affect BP in an independent manner that may not be related to hyperinsulinemia; results from our study, however, do not allow us to rule out such an effect.

Finally, there was a definite trend towards an improvement in insulin sensitivity in the treated WKY ($p=0.052$, such that the possibility of a type II

statistical error cannot be excluded) and the absolute increase in insulin sensitivity in the BMOV-treated WKY was comparable to that seen in the treated SHR. However, no difference in the 5-hour fasted plasma insulin levels was observed between the treated and untreated WKY. Interestingly, several studies in our laboratory indicate that although vanadium compounds lower plasma insulin levels in non-diabetic Wistar and Sprague Dawley rats, they do not affect insulin levels in the WKY (Bhanot *et al.,* 1993a,b; Bhanot *et al.,* 1994a,b). Furthermore, as discussed in the previous section, the WKY seem to exhibit certain metabolic differences when compared to other rat strains, which is an issue worthy of investigation.

In conclusion, the main finding of this study was that although the SHR are not insulin-resistant as compared to the WKY, they exhibit higher postabsorptive insulin levels. Furthermore, BMOV, a drug that improved insulin sensitivity and decreased insulin levels also caused a sustained decrease in BP in the SHR. Although these findings do not establish causality, they support the notion that hyperinsulinemia may be a contributing factor towards an increase in BP in this animal model of hypertension.

(III) Effects of pioglitazone in spontaneously hypertensive rats

In the BMOV study, we found that although the SHR were not insulin-resistant as compared to the WKY, BMOV further improved insulin sensitivity in the SHR (Bhanot *et al.,* 1994a). Such an improvement in insulin sensitivity could affect BP in an independent manner that may not be related to hyperinsulinemia. In addition, vanadium compounds are known to inhibit several cellular phosphatases including the Na^+-K^+-ATPase (Shechter 1990), which could have resulted in a decrease in BP independent of their effects on plasma insulin. Finally, treatment with BMOV caused a modest decrease in body weight, which

could have contributed towards the observed increase in insulin sensitivity with vanadium treatment (Bhanot *et al.,* 1994a). Therefore, in the present investigation, we studied the relationship between insulin action and hypertension in the SHR by employing pioglitazone, a drug that is pharmacologically distinct from vanadium but has similar effects on glucose metabolism.

Results from the present investigation confirm our previous observations that chemically diverse drugs that have the common property of attenuating hyperinsulinemia in the SHR also lower BP (Bhanot *et al.*, 1994a,b; Verma *et al.*, 1994). Pioglitazone caused marked and sustained reductions in plasma insulin levels and BP in the SHR. More importantly, the effects of pioglitazone were independent of changes in food intake, fluid intake or body weight, which clearly demonstrates that there remains a strong association between hyperinsulinemia and high BP in the SHR and that this link is independent of any change in food/fluid intake or body weight.

This study also confirmed our previous observation that the SHR are more insulin-sensitive than the WKY, when euglycemic clamp studies are performed in conscious rats. As has been discussed before, the apparent discrepancy in insulin sensitivity could be due to an exaggerated stress response to anesthesia in the SHR, which could result in secondary insulin resistance. In the present study, there was no difference in plasma catecholamine concentrations from 30 minutes to two hours after line placement in the rats, suggesting that all four groups had a similar response to the particular surgical technique employed. The finding that fasted plasma glucagon levels were much higher in the SHR as compared to the WKY supports the contention that the SHR are more insulin-sensitive than the WKY and therefore secrete more glucagon in an effort to maintain basal glycemia. Although the SHR are not insulin-resistant as compared

to the WKY, they exhibit postabsorptive hyperinsulinemia, which has been shown to be the result of a primary pancreatic beta cell hyper-responsiveness (to a glucose load) in the SHR (Buchanan *et al.,* 1992b). Results of the present study clearly indicate that SHR are hyperinsulinemic after a 5-hour fasting period but that their insulin levels are not higher than those of the WKY after a 20-hour fasting period.

The absolute values of the insulin sensitivity index for the untreated SHR and WKY rats during low dose insulin clamps in the pioglitazone study are about three times higher than those observed in the BMOV study (tables 5 and 7B), although the steady state plasma insulin concentration in the two studies is similar. This is because the rats in the pioglitazone study were about 4-5 weeks younger (and therefore also weighed less) than those in the BMOV study (pioglitazone study: 11-13 weeks of age; BMOV study: 15-19 weeks of age). Both an increase in age and body weight have a negative influence on insulin sensitivity, which is reflected in the results obtained from the two studies. More importantly, these results indicate that the relative differences in insulin sensitivity between the SHR and WKY are maintained even in older rats.

The primary finding of this study is that it is possible to decrease BP in the SHR without improving insulin sensitivity. Although pioglitazone has been demonstrated to improve insulin sensitivity and to correct GLUT4 transporter deficiency in obese, insulin-resistant rats, mice and monkeys (Kemnitz *et al.,* 1994; Sugiyama *et al.,* 1990a), it does not appear to improve insulin sensitivity in lean rats (Sugiyama *et al.,* 1990b). Our results support this observation and demonstrate that pioglitazone has no effect on insulin sensitivity in either the SHR or WKY at physiological or pharmacological concentrations. The obvious question that then needs consideration is as to how pioglitazone could attenuate

hyperinsulinemia in the SHR without improving insulin sensitivity. Although the occurrence of hyperinsulinemia is usually considered to be a compensatory response to overcome the existing insulin resistance, the SHR seem to be unique in that they secrete 2-3 times more insulin in response to a glucose load, which results in nutrient stimulated hyperinsulinemia. Thus hyperinsulinemia in the SHR is not secondary to insulin resistance and occurs due to hypersecretion of insulin by their pancreatic beta cells. Therefore, a drug that decreases insulin secretion from the pancreas could attenuate hyperinsulinemia in the SHR without affecting insulin sensitivity. Results of the present study, however, do not allow us to evaluate such an effect. Furthermore, pioglitazone was shown to decrease BP in the one kidney-one clip rat model of hypertension without altering insulin sensitivity (Zhang *et al.,* 1994). Thus it appears that in lean, hypertensive rats, the antihypertensive effects of pioglitazone are not invariably associated with an improvement in insulin-mediated glucose disposal.

Recent evidence indicates that insulin may alter vascular tone via direct effects on intracellular calcium concentration in vascular smooth muscle (VSM) cells (Touyz *et al.,* 1994). Insulin has also been shown to attenuate the contractile responses of VSM to vasoactive amines, probably by causing changes in intracellular calcium (Anderson and Mark 1993; Yagi *et al.,* 1988). More recently, it was demonstrated that physiological doses of insulin decreased VSM responses to angiotensin II, arginine vasopressin and norepinephrine in cultured cells from resistant rat arteries (Touyz *et al.,* 1994). Thus, it seems that insulin may have an inhibitory influence on the contractile responses to circulating amines and "resistance" to this effect in vascular smooth muscle would, therefore, manifest as an increase in peripheral vascular resistance and a resultant increase in BP. Such an effect would be complemented by the effects of hyperinsulinemia

on other organ systems such as the sympathetic nervous system. The observation that thiazolidinedione compounds also attenuate calcium-dependent VSM contractile responses to vasoactive amines (Pershadsingh *et al.,* 1993) raises the possibility that these drugs may lower BP not only by improving the response to the metabolic effects of insulin but also by altering cellular calcium responses to vasoactive agents. This notion was reinforced by results from a recent study where it was demonstrated that pioglitazone attenuated the voltage dependent calcium influx in a VSM cell line as well as in freshly dispersed, tail artery cells obtained from Sprague Dawley rats (Zhang *et al.,* 1994).

Other studies have demonstrated that pioglitazone inhibits DNA synthesis as well as growth of VSM cells, probably by affecting intracellular calcium concentration (Dubey *et al.,* 1993). Therefore, pioglitazone, by decreasing intracellular calcium concentration, could decrease VSM tone and thereby lower BP. In our study, pioglitazone completely prevented the increase in BP in the SHR as opposed to the partial reduction in BP observed with vanadium compounds. This also suggests that pioglitazone may have additional antihypertensive effects that may not be related to insulin resistance. Furthermore, it was recently demonstrated that pioglitazone also had direct effects on VSM that could contribute towards its antihypertensive actions (Buchanan *et al.*, 1995). Specifically, it was reported that pioglitazone blunted the contractile responses to norepinephrine, arginine vasopressin and potassium chloride in aortic rings obtained from normotensive Sprague Dawley rats, possibly by inhibiting agonist mediated calcium uptake in VSM. However, the current finding that reversal of the effects of pioglitazone on BP could be obtained simply by raising plasma insulin levels in the treated rats (to those that existed before treatment) strengthens the contention that the underlying pathogenetic

mechanism is closely related to the expression of hyperinsulinemia and hypertension in the SHR. This notion is further supported by the finding that the effects of pioglitazone on insulin and BP were independent of changes in plasma glucagon and catecholamine concentrations.

Another recent study demonstrated that although insulin attenuated the vascular response to norepinephrine in aortic rings obtained from WKY rats, it failed to do so in SHR (Lembo *et al.*, 1995). What was even more fascinating was the observation that the effect of insulin to reduce vascular reactivity was impaired even in pre-hypertensive 5-week old SHR. These findings are consistent with the notion that insulin may play an important modulatory role in VSM contraction and that resistance to insulin's inhibitory effects on VSM may be present in hypertensive animals/humans. For example, a decrease in the ability of insulin to attenuate vasoconstrictive responses to norepinephrine coupled with an exaggerated pressor response to hyperinsulinemia in the SHR (Brands *et al.*, 1994) could result in an increase in peripheral vascular resistance and a consequent rise in BP. Furthermore, pioglitazone, via its inhibitory effects on VSM calcium transients, could result in a decrease in VSM tone and could thereby lower BP in the SHR. Thus, resistance to insulin's glucoregulatory effects may not be "selective" but may extend to the effects of insulin on VSM, which may also help explain why hyperinsulinemia could contribute towards an increase in BP in both the insulin-resistant fructose-hypertensive rats as well as the insulin sensitive SHR. Whether insulin plays a permissive or a causal role in the pathogenesis of hypertension remains to be determined, but current evidence strongly suggests that this metabolic hormone also plays an important hemodynamic role via its interactions with other vasoactive peptides.

In summary, the SHR exhibit higher postabsorptive plasma insulin levels as compared to the WKY. Furthermore, pioglitazone, a drug that decreased insulin levels in the SHR also caused sustained decreases in BP without affecting insulin sensitivity. Although these findings do not establish causality, they support the contention that hyperinsulinemia is closely linked to an increase in BP in the SHR.

(IV) Effects of vanadyl sulfate in fructose-hypertensive rats

The fructose-hypertensive rat model represents an acquired form of systolic hypertension, where the rise in BP is not genetically determined but is diet-induced (Hwang *et al.,* 1987). Although the precise mechanism by which hypertension develops in fructose-fed rats has not been defined, it has been proposed that the rise in BP is secondary to the development of insulin resistance and hyperinsulinemia. The objective of the present study was to examine the relationship between insulin resistance, hyperinsulinemia and BP in fructose-hypertensive rats. To this aim, vanadyl sulfate was administered to fructose-fed rats and the effects of the drug on insulin sensitivity, plasma insulin levels and systolic BP were studied.

Results from this study confirm previous reports that feeding otherwise healthy rats a fructose diet results in insulin resistance, hyperinsulinemia and hypertension (Hwang *et al.,* 1987; Reaven *et al.,* 1988). The fructose diet (66% fructose, 12% fat and 22% protein) was specially prepared such that it had a sodium, protein and fat content very comparable to the standard rat chow. Therefore, the fructose-induced hypertension was not secondary to changes in dietary sodium intake. It has been reported that the fructose-induced increase in BP is not accompanied by any change in plasma renin activity or angiotensin levels (Hwang *et al.,* 1989), although the exact role of the renin-angiotensin system in this model of experimental hypertension is still unknown. Evidence

suggests that fructose feeding leads to insulin resistance and a compensatory hyperinsulinemic response which, in turn, may lead to volume overload and hypertension (Hwang *et al.,* 1989). This notion is supported by studies demonstrating that insulin promotes renal sodium absorption in a variety of species (Baum *et al.,* 1987; DeFronzo *et al.,* 1975; DeFronzo *et al.,* 1976).

If fructose-induced hypertension were secondary to an increase in plasma insulin, then a decrease in insulin levels should have prevented the rise in BP. Our results are consistent with this hypothesis, since vanadyl sulfate improved insulin sensitivity and attenuated both the increase in plasma insulin levels and BP. Vanadyl treatment did not alter plasma catecholamine levels, suggesting again that it lowered BP without any change in sympathetic activity. Furthermore, restoration of plasma insulin concentration in the vanadyl-treated, fructose-fed rats caused a corresponding increase in BP. Reversal of vanadyl's effects on BP after raising insulin levels to those observed in the untreated fructose group was independent of changes in body weight, which strengthens the contention that hyperinsulinemia contributes towards the genesis of fructose-induced hypertension. Additional support for this hypothesis comes from studies demonstrating that exercise training (which improved insulin sensitivity and decreased insulin levels) and somatostatin administration (which decreased hyperinsulinemia) attenuated the fructose-induced rise in BP (Reaven *et al.,* 1988; Reaven *et al.,* 1989b). Also, administration of clonidine to fructose-fed rats inhibited the increase in BP but did not improve the associated metabolic defects (Hwang *et al.,* 1987). This suggests that the defects in carbohydrate metabolism are not secondary to an increase in sympathetic activity.

Interestingly, a modest decrease in plasma insulin in the control vanadyl-treated rats did not cause a decrease in BP, nor did exogenous insulin treatment

increase BP. The question arises as to why hyperinsulinemia causes hypertension in insulin-resistant rats without doing so in insulin sensitive control animals. One possibility is that insulin sensitive tissues would increase glucose utilization (in response to the increase in insulin levels), which may initiate local autoregulatory vasodilator reflexes in order to increase local blood flow. By contrast, insulin resistance could prevent such vasodilator responses and thereby result in increased vascular resistance.

During the clamp studies, somatostatin was used to suppress endogenous insulin secretion since its effects on glucose clearance are less than those of other agents that have been used to suppress insulin release (Mondon and Reaven 1988). The use of somatostatin during clamp studies is based on the assumption that it has no direct effect on tissue glucose metabolism. Although it has been reported that somatostatin causes a small increase in glucose clearance in dogs, Baron et al. did not document such an effect during human clamp studies (Baron *et al.,* 1987). Furthermore, Buchanan et al. reported that during low dose insulin infusion in rats, there was no effect of somatostatin on glucose clearance after basal insulinemia was established (Buchanan *et al.,* 1992a). Although somatostatin has been shown to alter levels of plasma glucagon and growth hormone, these findings suggest that the effects of somatostatin on glucose clearance may occur only in the face of low insulin levels or that there may exist a difference in the effects of somatostatin on glucose clearance between different species. If glucagon and growth hormone play a different role in glucose metabolism in control, fructose and fructose-vanadyl treated groups, the use of somatostatin could have influenced our results. However, looking at the magnitude of the differences in insulin sensitivity between the groups studied, it is perhaps reasonable to conclude that the effects of

somatostatin cannot fully account for the observed differences in insulin sensitivity.

Vanadyl treatment resulted in a marked enhancement of insulin sensitivity in the fructose-fed rats and restored their insulin sensitivity to control values. However, this was accompanied by a decrease in body weight in the fructose-vanadyl group as compared to the untreated fructose group. It may, therefore, be argued that the improvement in insulin sensitivity in the fructose-vanadyl group may be secondary to their lower body weight rather than due to a direct effect of vanadyl itself. Although we do not have an unequivocal answer to this question, we have considered several possibilities. The control-vanadyl treated rats also showed a similar decrease in body weight (as compared to the untreated controls), yet their insulin sensitivity remained unchanged. If a decrease in body weight were the major factor causing an improvement in insulin sensitivity, then there should have been a corresponding increase in insulin sensitivity in the control-vanadyl group, which was not observed. Furthermore, we have expressed insulin sensitivity as the "insulin sensitivity index" (Bergman *et al.,* 1987), which accounts for changes in body weight that may otherwise confound the results. What is even more important is that the effects of vanadyl on plasma insulin and BP were also observed from weeks 9-11, when body weight in the fructose and fructose-vanadyl groups was similar. Finally, reversal of vanadyl's effects on BP after administration of exogenous insulin was also independent of change in body weight.

It has been reported that fructose-induced hypertension in Wistar rats is concentration and duration dependent (Dai and McNeill, 1995). Interestingly, the authors also reported that the increase in BP that occurred after fructose-feeding preceded the increase in plasma insulin concentration. However, in that study,

fructose was administered in the drinking water, which resulted in marked alterations in food and fluid intake in the fructose-fed rats. Specifically, the fructose-fed rats drank more and ate less than the control rats. A decrease in food intake can profoundly affect most metabolic parameters (including plasma insulin levels), which could have confounded the results of the particular study. Subsequent studies in our laboratory have demonstrated that when rats are administered fructose in the diet form (which does not alter food and fluid intake), the onset of hypertension is preceded by hyperinsulinemia (unpublished observations). Furthermore, in the study by Dai and McNeill, it was observed that the increase in BP occurred one week after initiation of the fructose solution, which is in contrary to the results obtained from studies in which fructose was given in the diet form (where the increase in BP was not evident for about 2 weeks after initiation of the high fructose diet). Thus, it seems that administering fructose in the drinking water as opposed to giving it in diet form can result in important differences in the metabolic and hemodynamic parameters in rats. It has also been observed that when fructose-hypertensive rats are rendered hypoinsulinemic and diabetic (with streptozotocin), they still become hypertensive (Dai and McNeill, 1992). However, hypertension in the fructose-hypertensive-diabetic rats cannot be compared with that of fructose-hypertensive rats, since the induction of the diabetic state can result in multiple changes in other organ systems (such as renal, autonomic, VSM). In addition, the hyperglycemia that occurs in the diabetic rats can lead to marked alterations in the cellular content of various ions such as magnesium, which may be important in the pathogenesis of fructose-induced hypertension, as discussed below.

Two recent reports on fructose-induced hypertension have documented interesting findings that deserve mention. The first one deals with the effects of

dietary fructose versus dietary magnesium deficiency in the etiology of the fructose-induced increase in BP. In that study, the authors hypothesized that it was the magnesium deficiency in the fructose diet rather than the fructose content of the diet that lead to fructose-induced insulin resistance (Balon *et al.,* 1994). The fructose diet is reasonably matched for sodium and potassium with control rat chow but is low in magnesium (magnesium content in the fructose diet is one third that of normal rat chow). The authors reported that when rats were fed a high fructose diet that had a magnesium content similar to that present in normal rat chow, the rats did not develop insulin insensitivity or an increase in BP. This was in contrast to the rats fed the conventional fructose diet, who did exhibit insulin resistance, hyperinsulinemia and hypertension. Although insulin sensitivity was assessed by the hindquarter perfusion method (which is not an accurate measure of *in vivo* insulin sensitivity), the results were interesting, especially since several recent reports suggest that magnesium deficiency may cause insulin resistance. In our experiments, the fructose diet was deficient in magnesium and since serum magnesium levels were not measured, we cannot rule out the possibility that dietary magnesium deficiency could be partly/fully responsible for causing insulin resistance in the fructose-fed rats. However, regardless of the mechanism causing insulin resistance in fructose-hypertensive rats, the consequent metabolic and hemodynamic abnormalities that occur are prevented by drugs that improve insulin sensitivity. Furthermore, results from our insulin implant study clearly indicate that hyperinsulinemia and hypertension are very closely related in these rats. Therefore, the findings from the study mentioned above do not contradict our hypothesis in any way but rather shed light on one of the possible mechanisms underlying the severe insulin resistance observed in fructose-fed rats.

In the second report by Brands *et al.,* it was demonstrated that a high fructose diet does not raise mean arterial pressure, when BP is assessed by chronically catheterizing the animals (Brands *et al.,* 1994). The authors suggested that the hyperinsulinemia and the increase in sympathetic activity that occur secondary to fructose feeding could increase BP lability in rats, which could lead to increased BP upon acute handling of the rats (as is done in the tail-cuff method). However, after chronic catheterization, they were unable to demonstrate any increase in BP. Although the study documents an important observation, there are several points that the authors seemed to have overlooked while discussing their results. First, the fructose diet that they used was matched for the vitamin and mineral content with normal rat chow and was, therefore, not different in magnesium content. As discussed above, if magnesium deficiency were the cause of insulin resistance, then no change in any parameter would be expected in their study, since the rats were not on a magnesium deficient diet. The finding that the authors did not observe hyperinsulinemia in the fructose-fed rats strongly suggests that indeed the rats were not insulin-resistant and hence would not be expected to be hypertensive (if the insulin-hypothesis is valid). Second, they only examined the short term effects of the diet (2 weeks), and may have missed the chronic effects of fructose feeding on BP. We and others have observed that the fructose-induced increase in BP can be measured only 14-18 days after initiation of the fructose diet and are not present up to 2 weeks of starting the diet. Therefore, although the study by Brands et al. suggests that fructose feeding may increase BP lability to stressful situations rather than mean arterial BP, they may have missed the actual long term effects of the diet (acknowledged briefly by the authors in the manuscript). Although we did not conduct direct BP measurements in our study, other studies in our laboratory

have demonstrated that the fructose-fed rats are hypertensive even when direct intra-arterial BP measurements are made (unpublished observations). Although the direct BP measurements are about 5-8 mmHg lower than the indirect values, the relative differences in the control and fructose-fed rats are maintained. Therefore, although the study by Brands et al. may seem to be in conflict with many other reports showing that fructose feeding causes hypertension, there are significant differences in their protocol that may explain their discrepant findings.

In summary, vanadyl sulfate prevented the fructose-induced increase in plasma insulin and BP. The effects of vanadyl on BP could be reversed by restoring plasma insulin levels in the vanadyl-treated rats to pre-treatment levels. This suggests that hyperinsulinemia may contribute towards the development of high BP in fructose-hypertensive rats.

CONCLUSIONS

(i) SHR are not insulin-resistant but rather are more insulin sensitive than the WKY.

(ii) SHR exhibit postabsorptive hyperinsulinemia and are hyperinsulinemic as compared to the WKY after a 5-hour fasting period.

(iii) Drug interventions (vanadyl sulfate, BMOV, pioglitazone) that decrease hyperinsulinemia also attenuate hypertension in the SHR. The effect of the drugs on BP can be reversed by restoring the plasma insulin levels in the drug-treated SHR to those observed in their untreated counterparts.

(iv) The antihypertensive effects of pioglitazone in the SHR are independent of its effects on insulin sensitivity, which suggests that hyperinsulinemia may be unrelated to insulin resistance in the SHR.

(v) Vanadyl sulfate completely prevents fructose-induced insulin resistance, hyperinsulinemia and high BP; the effect of the drug on BP can be reversed by restoring plasma insulin levels in the vanadyl-fructose-treated rats to those that are seen in their untreated fructose-fed counterparts.

These data indicate that either hyperinsulinemia may contribute to the development of high BP in both the SHR and the fructose-hypertensive rats or that the underlying mechanism is closely related to the expression of both these disorders.

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