EFFECTS OF NICOTINE AND STREPTOZOTOCIN ON THE CARDIOVASCULAR SYSTEM

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By

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ABSTRACT

Our study investigated the potential for a combination of diabetes and nicotine treatment to affect blood pressure in the rat. We used streptozotocin injection and oral nicotine feeding as models of type-1 diabetes and smoking respectively. Blood pressure was assessed using the indirect tail-cuff technique. In an attempt to further characterize our experimental model, we also observed body weight, plasma glucose and the contractility of aortic segments in various treatment groups. Our data was expressed as mean ± SEM, and significance was regarded as $P < 0.05$. We found that a combination of streptozotocin and nicotine treatment resulted in a significant elevation of systolic blood pressure compared with either treatment alone, or control. Furthermore, assessment of aortic contractility showed alteration of reactivity to both phenylephrine and sodium nitroprusside as a result of the combination treatment. We also observed a trend for our combination treatment to exacerbate the elevation of plasma glucose level seen in streptozotocin induced diabetic rat models. This study serves as an experimental basis to underline the importance of cessation of tobacco use for individuals with diabetes mellitus.

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The animal use protocol for this project was approved by the University of Saskatchewan Standing Committee on Animal Care and Supply.

Dedication

For my family (Lynn, Kerry, Kate, Julia and Bill),

And,

For Duncan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACh - acetylcholine
ADP - adenosine diphosphate
AGE - advanced glycation end-product
AHA - american heart association
ANOVA - analysis of variance
BP - blood pressure
CAD - coronary artery disease
CO - carbon monoxide
CVD - cardiovascular disease
DCCT - Diabetes Complications and Control Trial
EC_{50} - effective concentration 50%
E_{max} - maximum effect
ET-1 - endothelin-1
HR - heart rate
KCl - potassium chloride
MAP - mean arterial pressure
MRFIT - Multiple Risk Factor Intervention Trial
NADPH - nicotinamide adeninedinucleotide phosphate (reduced)
NIBP - non-invasive blood pressure
NO - nitric oxide
NOS - nitric oxide synthase
PHE - phenylephrine
PK-C - protein kinase-C
ROS - reactive oxygen species
SD - Sprague Dawley
SNK - Student-Newman-Keuls Test
SNP - sodium nitroprusside
STZ - streptozotocin
Tukey's-HSD - Tukey's Honestly Significant Difference Test
UKPDS - United Kingdom Prospective Diabetes Study
WKY - Wistar Kyoto

CHAPTER 1

INTRODUCTION

This study set out to consider whether a combination of diabetes and tobacco use would cause a change in blood pressure. The first part of this introduction will deal with the relevance of our research question to the general population. Our discussion of relevance will not only include the prevalence of cardiovascular disease, hypertension, and associated risk factors; but also statistical and experimental evidence for linkages between them. Next, we will consider what the literature has to say about the effect diabetes and tobacco use independently has on blood pressure, and why we implemented hyperglycaemia and nicotine treatment as a model of diabetes and tobacco use respectively. We will consider an experimental basis for the roles of glucose and nicotine in cardiovascular pathology, and how cardiovascular disease and hypertension are intimately related to one another. We will then discuss why studying the combination of diabetes and tobacco use is a worthwhile avenue of investigation.

1.1 Relevance of the Research Question

1.1.1 Prevalence of cardiovascular disease and associated selected risk factors

The American Heart Association (AHA) reported cardiovascular Disease (CVD) as the leading cause of death of people in the United States of America (USA) in the year 2000 (AHA 2002). Almost every heart awareness organization in the western world identifies CVD as a major cause of morbidity and mortality in their respective populations. Furthermore, these associations recognize both diabetes mellitus, and smoking as major risk factors for the development of cardiovascular disease (AHA 2002, Canadian Hearth

and Stroke Foundation 1999). However, not much has been done to investigate the potential interaction of these two risk factors in the development of cardiovascular disease. Through previous literature, one can establish the epidemiological link between these two risk factors, the concurrent development of hypertension, and the onset of cardiovascular disease. Hypertension is also recognized as a risk factor leading to certain types of heart disease (AHA 2002).

1.1.1.1 Tobacco Use

The American Lung Association (ALA 2003) reports that smoking has been identified as the primary source of morbidity and mortality worldwide due to a modifiable health risk factor. In the year 2001, it was estimated that there were 46.2 million smokers in the USA, or 22.6% of the overall population aged 18 and over (ALA 2003). Furthermore, Statistics Canada estimated that approximately 21.5% of all Canadians aged 12 and over between the years 2000-2001 were smokers. Based on observations made by the USA Center for Disease Control (CDC 2002), coronary heart disease was the number two cause of death due to smoking.

Hammond and Horn (1958) published the first major epidemiological study demonstrating a statistical link for smoking as a CVD risk factor. They reported that the death rate was approximately 57% higher in the smoking population studied as compared with the age matched non-smoking population, and that coronary heart disease was the causative mortal factor in 52.1 % of the excess mortality rate of this smoker group. More recently, the AHA reports that through 1995-1999, 33.5 percent of all people who died from a tobacco-related illness did so from a cardiovascular-related event (AHA 2003). Another meta-analysis of 10 studies conducted on the effects of second hand tobacco

smoke indicated that individuals subjected to passive smoke had a 30% increased risk of coronary heart disease (Glantz and Parmley 1991). These clinical studies, and many more like them, have served to attribute tobacco usage as a probable causative (risk) factor for the development of CVD (Kilaru et al 2001).

1.1.1.2 Diabetes mellitus

The struggle to control the complications of both major types of diabetes continues in order to decrease the morbidity and mortality outcomes of this disease. Diabetes mellitus is a serious problem in North America, and is reaching epidemic proportions in certain demographics of that population. According to the Canadian Diabetes Association, there are currently over 2 million persons with diabetes in Canada (CDA 2003). In a more local context, the prevalence of diabetes in the Saskatchewan population was around 4% for the general population, and about 12% in the province's aboriginal population (Saskatchewan Advisory Committee on Diabetes 2003). The AHA (2002) reports that in the year 2000, 75% of persons in the United States with diabetes mellitus died of some sort of heart or blood vessel disease. A review of the epidemiology of diabetes in the French population stated that 85-90% of persons with type-2 diabetes mellitus had some sort of cardiovascular complication (Eschwege and Guillanneuf 2001). The most prevalent complications noted in the French study were ischaemic heart disease and other non-specific myocardial events, resulting finally in cardiomyopathy.

1.1.1.3 Hypertension

The statistical link between hypertension and the development of cardiovascular pathologies is much easier to find. One such link is a meta-analysis published by MacMahon *et al* (1990) in The Lancet, which successfully correlated higher diastolic

pressure with an increased risk of coronary heart disease. The World Health Organization in their 2002 World Health Report suggests that '62% of strokes and 49% of heart attacks are caused by high blood pressure' (WHO 2002). Similar to the other risk factors, the experimental basis of the inter-relation between hypertension and heart disease will be discussed in a later section.

1.1.2 Statistical and experimental linkages between cardiovascular pathologies and associated risk factors

1.1.2.1 Cardiovascular complications associated with diabetes mellitus

Cardiovascular complications are a common occurrence, and cause of mortality, particularly in the progressive stages of diabetes mellitus (International Textbook of Diabetes Mellitus 1997). Although the profile of cardiovascular complications is somewhat different between type-1 and type-2 diabetes, most of these complications have been shown to occur in both types.

Vascular dysfunction. Impaired relaxation response of vascular tissues is a phenomenon common to both type-1 and type-2 diabetes (Creager et al 2003). In diabetes, vascular dysfunction is thought to occur due to factors influencing the function of both endothelial and vascular smooth muscle cells. Vascular dysfunction has been linked to the development of further cardiovascular complications such as atherosclerosis and hypertension (Creager et al 2003).

Clinical and experimental evidence suggest that at least part of this impaired vasorelaxation response is due to impaired endothelium-dependent vasorelaxation. Clinical observations in both type-1 (Johnstone et al 1993) and type-2 diabetes (McVeigh et al 1992) have shown impaired endothelium-dependent vasorelaxation in these patients.

This observation has been compared with similar results in experimental models of type-2 diabetes (Meraji et al 1987), as well as hyperglycemia (Tesfamariam et al 1990), which show similar impairment of endothelium-dependent vasorelaxation responses. The molecular basis for these events will be discussed in a subsequent section.

Atherosclerosis. Atherosclerosis is a chronic inflammatory process known to occur in both type-1 and type-2 diabetes (Steiner 1996). Therefore, one is not surprised to know that the major pathological manifestations of atherosclerosis in diabetic individuals are coronary artery disease (CAD), cerebrovascular disease, and peripheral artery disease (Lüscher et al 2003).

A number of disturbances in the metabolic pathways of vascular tissues have been proposed as the mechanism for development of atherosclerosis in the diabetic individual. In particular, a decreased bioavailability of nitric oxide (NO) leads to increases in vascular tone, vascular permeability, thrombolytic activity, and cellular proliferation (Creager et al 2003). A molecular basis for decreased NO bioavailability will be discussed in a later section. Also, dyslipidemia is a phenomenon seen in both type-1 and type-2 diabetes, and is an independent risk factor for CVD (Marks and Raskin 2000). Changes in these vascular parameters, as well as lipid metabolism, are thought to underlie the onset of atherosclerotic processes (Lopes-Virella 2001).

Heart failure. Statistical evidence as to the occurrence of heart failure in diabetes mellitus was first described in the Framingham Study (Kannel and McGee 1979). The exact cause of heart failure in diabetes is still a 'hotly contended' issue (Young et al 2001). Predisposing factors such as hypertension and coronary artery disease are thought to contribute to the development of heart failure. There is also evidence that suggests a

direct mechanism of diabetes-induced cardiomyopathy. Rubler et al (1972) first postulated that cardiomyopathy was associated with diabetes. Cardiomyopathy and heart failure both seem to be present in type-1 and type-2 diabetes (Zarich and Nesto 1989). However, the cause-effect relationships between cardiomyopathy, heart failure, and other predisposing factors are still not clear (Young et al 2001). Whatever the relative contributions of each predisposing factor, we might suppose that heart failure is at least in part generated due to the pathological cardiovascular processes of atherosclerosis and hypertension.

1.1.2.2 Cardiovascular disease associated with tobacco use and nicotine

Although it is easy to categorize the cardiovascular complications of diabetes mellitus due to the overt nature of that disease, the categorization of cardiovascular complications of tobacco and/or nicotine use is somewhat more complicated. The reason for this is the complicated toxicological profile of major forms of tobacco use, and controversy as to contribution of nicotine, and other chemical constituents, to the toxic properties of tobacco products. Cigarette smoking has been shown to induce many forms of cardiovascular disease including peripheral vascular disease, coronary heart disease and cerebrovascular disease (Burns 2003). Burns (2003) also points out that cigarette smoking contributed to an increase in the levels of other cardiovascular risk factors.

Much study is still concerned with precisely, which constituent(s) of tobacco smoke is/are involved in the development of cardiovascular disease. Most research points to nicotine, carbon monoxide and the oxidant gases of tobacco smoke as causative agents of CVD (Benowitz 2003). The acute pharmacological effects of nicotine are well described (Benowitz 1986), characteristically resulting in a transient increase in heart rate and

blood pressure. However, the extent to which chronic nicotine exposure contributes to cardiovascular disease processes is still not known. Current theory surrounding nicotine as a primary causative agent in cardiovascular pathology will be discussed in a later section.

1.1.2.3 Cardiovascular disease and hypertension

Although hypertension is closely related to CVD, it is not necessarily regarded as part of the same discrete disease. Major clinical/epidemiological studies have established a very clear link between hypertension and an increased risk of CVD (Kannel 2000, Neaton and Wentworth 1992). To complicate this matter further, certain forms of CVD, particularly those that lead to increased peripheral vascular resistance, may subsequently lead to the development of hypertension (Christlieb 1982). We seem to be left with a situation where the two, hypertension and CVD, are interdependent; the onset of one leads to the other, and likely results in a continuous spiral of negative aetiology. At the very least, this relationship is certainly a reaffirmation of the paradigm of integration, which is perhaps the most important fundamental characteristic of human physiology.

For the purposes of this discussion, it is likely most useful to focus on a discussion of hypertension as a risk factor for the development of CVD. To this point, a significant part of this discussion has included an epidemiological relevance for studying hypertension in the context of cardiovascular disease. Although it is not the intention of this thesis to observe end-points directly associated with CVD, it is our intention to briefly establish the importance of blood pressure, ergo hypertension, as an aspect of cardiovascular pathology. Considering the link between hypertension and the development of CVD is important if we wish to suggest that hyperglycaemia and nicotine

lead to an increase in blood pressure, and a subsequent increase in CVD risk. In fact, we already know from previous discussion that conditions that are associated with the introduction of hyperglycaemia and nicotine into a mammalian system will result in an increased risk for CVD. Further sections of this thesis will discuss an experimental basis for the idea that diabetes and smoking, or related experimental models, confer CVD risk through an effect on blood pressure.

Ever since the early-mid 1900's, physicians have strongly suspected that the occurrence of hypertension had a role in the development of CVD. Not until the past two decades have we begun to amass a broad body of statistical evidence that essentially supports this hypothesis. It is hard to say if any particular prospective or interventional study is a gold standard in illustrating the associations between present day risk factors and their alleged cardiovascular outcomes. In North America we would likely point to studies like The Framingham Heart Study, or The Multiple Risk Factor Intervention Trial (MRFIT) as the basis for much of our assumptions about cardiovascular disease origins (Kannel 2000, Neaton and Wentworth 1992). Although limited in the sense of discovering the underlying mechanisms, we can derive much important information from these studies on factors that relate to the development of hypertension and CVD. At the very least, these major studies have confirmed our initial suspicions that the occurrence of hypertension is a definite risk for CVD.

1.2 The Chronic Effect of Diabetes and Tobacco Use on Blood Pressure

If we suppose that combining a type-1 diabetic rat model with a nicotine treated rat model will lead to chronically elevated blood pressure, then there must be some basis for a change in blood pressure by either treatment alone. Much of the literature regarding the

sole action of either treatment on blood pressure is somewhat conflicting. However, when we begin to analyze the conditions of these separate observations, we are able to find a basis for our experimental hypothesis. We believe that the current controversy surrounding the effect of streptozotocin and nicotine treatment on blood pressure makes this a worthwhile area of investigation.

1.2.1 Diabetes, hyperglycaemia and blood pressure

Diabetic patients with hypertension have been shown to have an increased risk for further cardiovascular complications (Zarich and Nesto 1989). Hypertension can occur in both type-1 and type-2 diabetic patients (Epstein and Sowers 1992). However, the occurrence of hypertension in type-2 diabetes is not necessarily associated with nephropathy, as in type-1. Increased vascular resistance is thought to be a common factor in hypertension in both of these diabetic types, either due to atherosclerosis or vascular dysfunction (Sowers 2001).

Currently, the literature regarding the chronic blood pressure effects of streptozotocin (STZ) treatment in the rat is controversial. Yamamoto (1988) reported that higher doses (50-60 mg/kg) of STZ lead to more severe diabetes in 2-4 week old Wistar Kyoto (WKY) rats, and subsequently a modest but significant increase in blood pressure of approximately 20 mmHg. This increase in blood pressure reported by Yamamoto (1988) is sustained over a period totaling up to 20 weeks, detected by using an indirect tail-cuff method of blood pressure measurement. As will be discussed later, Yamamoto (1988) used an ambient temperature of 40 degrees Celsius for their indirect tail-cuff method that we believe has a major impact on their result. In a 2001 study from our lab we concluded that there was no significant increase in BP, as measured by direct cannulation of the

femoral artery, in Sprague Dawley rats treated with 60 mg/kg STZ (Wang et al 2001). Although these literature reports seem to disagree on whether STZ can cause blood pressure change, it is possible that change might occur in the presence of other confounding variables.

1.2.2 Tobacco use, nicotine and blood pressure

A few important questions arise when one theorizes that smoking can lead to, or participate in, the development of hypertension under certain disease conditions. The reason that it is important to address these questions is due to the previous mentioned controversy over the attribution of nicotine to tobacco related disease processes. The first question is, 'Is there any evidence that smoking can lead to sustained clinical or experimental hypertension under any circumstances?' Secondly, 'What toxic agent, related to smoking, would be most likely involved in such a process?' Lastly, 'Is there any evidence at all that this particular agent is related to the development of hypertension, or a change in BP, in an experimental model?' If we can satisfy these three questions essentially with 'yes, nicotine, yes', then we have the basis for an argument that smoking, ergo nicotine consumption, leads to the development of hypertension, and subsequently, cardiovascular disease.

Smoking is one factor of many which has the demonstrated ability to transiently increase blood pressure. Smoking one cigarette usually results in a transient increase in both heart rate and blood pressure of humans (Benowitz 1986), and rats (Houdi et al 1995). This transient effect of smoking has been long attributed to the actions of nicotine on the sympathetic nervous system (Gebber 1969). Although the transient effect of nicotine is

to lead to an increase in blood pressure, it may not be correct to assume that this is also the chronic effect of nicotine on blood pressure levels.

The idea that smoking may lead to chronically elevated blood pressure is somewhat controversial. Some reports actually suggest that smoking may result in lower blood pressure under certain circumstances (Mann et al 1991). Contrary to this, a recent health survey from England suggests that perhaps small, but significant, elevations in systolic blood pressure occur as a result of cigarette smoking (Primatesta 2001). However, the differences seen in Primatesta's (2001) study, although statistically significant, are only actual differences of 1-2 mmHg. The 'small, but significant' elevations in systolic blood pressure in Primatesta's (2001) study hardly seem clinically relevant. A study by Mann *et al* (1991) demonstrated that hypertensive smokers over the age of 50 had significantly higher daytime ambulatory blood pressures compared with their non-smoking counterparts. Even within Mann's study the results seem somewhat paradoxical. Mann (1991) reports that younger smokers have lower daytime ambulatory blood pressure than their non-smoking counterparts. This apparent contradiction might suggest that the effect of cigarette smoking on blood pressure is bi-phasic. That is to say, smoking will initially lead to moderate decreases in a person's blood pressure level. Subsequently, over the longer course of smoking, this phenomenon might be reversed due to the continuous progression of disease and metabolic disturbance. This idea could certainly be consistent with the idea that nicotine treatment is a model for aging in the cardiovascular system (Thorin et al 1990).

The obvious limitations of the observational studies of smoking and blood pressure would be their inability to give us the direct culprit responsible for a change in blood

pressure. Nicotine has been suggested as one of the leading players in the development of CVD and related pathologies associated with cigarette smoking (Benowitz 2003). Nicotine is not the only postulated player in cardiovascular modification, as the list also includes vaso-active gases like carbon monoxide (CO), and other oxidant gases (Benowitz 2003). Subsequent sections will attempt to expand on this case for nicotine in chronic cardiovascular pathology.

One particular study suggests that chronic nicotine treatment will cause hypertension in rats. Swislocki et al (1997) have reported that, "smokeless nicotine exposure leads to sustained but reversible hypertension" in 8-week old Sprague Dawley rats". Contrary to Swislocki, Bui et al (1995) reported no significant difference of systolic blood pressure in 7-week old WKY rats that were implanted with similar nicotine tablets over a period of 6 weeks. Although both Bui et al (1995) and Swislocki et al (1997) used the same dose and method of delivery of nicotine, not to mention the same method of blood pressure measurement, it is possible that nicotine's effect on blood pressure is not consistent for different strains of rat. Therefore, we are still uncertain as to the potential for nicotine to chronically alter blood pressure levels in either the human or experimental animal.

1.3 Experimental Evidence for the Roles of Hyperglycaemia and Nicotine in Cardiovascular Pathology

As suggested previously, this section is a brief discussion regarding evidence of underlying mechanisms for hyperglycaemia and nicotine in the development of certain cardiovascular pathologies. Both of these agents are demonstrated to contribute to the initiation of atherosclerosis and further manifestations of cardiovascular disease (Lüscher et al 2003). It should be noted that this line of discussion only intends to serve as

rationale for the current set of experiments, or at least a suggestion that glucose and nicotine might be important elements of diabetes and smoking respectively. However, they may not necessarily be the sole pathological mechanism for the development of cardiovascular complications in diabetics or smokers.

1.3.1 Glucose as a cardiovascular pathological agent

Although it is likely that various metabolic factors contribute to diabetic cardiovascular complications, there is much evidence to suggest that glucose plays a central role in these processes. We can find partial evidence to support this theory in clinical studies that demonstrate the benefits of glycaemic control. We have already seen evidence in the previous section that suggests that conditions, which elevate blood glucose, can lead to functional changes in the cardiovascular system. Hyperglycaemic production of reactive oxygen species (ROS) is what is thought to underlie cellular metabolic changes, ultimately leading to functional changes in the cardiovascular system (Creager et al 2003). These functional changes in the cardiovascular system have the potential to directly affect blood pressure.

Treatment outcome studies are a major piece of evidence implicating glucose as the causative agent in the development of cardiovascular alterations. The Diabetes Control and Complications Trial (DCCT) studied the result of using an intensive treatment on the development of diabetic complications in type-1 diabetes (DCCT 1993). It was shown that the intensive treatment, as compared with a more conventional treatment regime, resulted in a daily average blood glucose level that was closer to normal, as well as reduced levels of glycosylated hemoglobin. The DCCT study also showed that the same subjects with better glycaemic control also had a reduced risk of developing

complications related to microvascular disease. There is also evidence which suggests that glycaemic control can improve macrovascular outcomes in patients with type-1 diabetes (Jensen-Urstad 1996). The United Kingdom Prospective Diabetes Study (UKPDS) investigated the effect of a more intensive treatment for NIDDM on its related complications (UKPDS 1998 a & b). Similar to the DCCT, the UKPDS found that a reduction in microvascular-associated pathologies in the intensive treatment group. It should be pointed out, however, that the intensive treatment in the UKPDS study only resulted in a very modest reduction in glycosylated hemoglobin levels. Significantly, the incidence of myocardial infarction, a macrovascular outcome, was 16% lower in the intensive therapy group of the UKPDS. Taken together, these two major studies suggest that blood glucose level plays a major role in the development of both micro and macrovascular complications of diabetes mellitus.

Previously it was described that vascular dysfunction is a complication associated with both type-1 and type-2 diabetes. The current theory surrounding the dysfunction of vascular tissues due to hyperglycaemia involves the production of oxygen derived free radicals, leading to further disturbances in the cell's metabolism (Creager et al 2003). Furthermore, it has been reported that treatment with an oxygen free radical scavenger attenuates the impaired vasorelaxation response (Kim et al 2002).

Increased levels of oxidative stress have been shown in both human diabetics (Oberley 1988), and a streptozotocin induced model of type-1 diabetes (Sano et al 1998). Hyperglycaemia is demonstrated to induce production of ROS in vascular endothelial cells (Giardino et al 1996). The production of ROS due to hyperglycemia occurs concurrently with increased protein kinase-C (PK-C) activity, advanced glycation end-

product (AGE) production, and flux through the polyol pathway (Koya and King 1998, Brownlee 1995, Lee et al 1995). However, the exact nature of the cause-effect relationship between these elements is still not clear. The mitochondrial electron transport chain is though to be the source of the excess generation of ROS due to hyperglycaemia. Nishikawa et al (2000) demonstrated that inhibiting mitochondrial production of ROS would inhibit hyperglycaemic-induced increases in PK-C activity, AGE production, and sorbitol accumulation. This would suggest that the production of ROS precedes the induction of PK-C, the formation of AGEs, and the accumulation of sorbitol. Unfortunately, the pathways do not seem to be that simple. Inoguchi et al (2000) suggests that the formation of ROS occurs due to hyperglycaemic activation of PK-C-dependent reduced nicotinamide adenine dinucleotidephosphate (NADPH) oxidase. Although it seems that these two references may contradict each other, it is entirely possible that both results are true. For instance, it seems that the production of AGEs can be initiated by ROS production, but formation of AGEs have also been demonstrated to result in the production of ROS (Wautier et al 2001, Tan et al 2002). Regardless of the precise mechanism of hyperglycaemia-induced ROS production, the phenomenon has been well demonstrated. Furthermore, we know that the over-production of ROS has definite downstream consequences on the function of cardiovascular tissues.

The involvement of ROS has been implicated in vascular dysfunction (Mayhan and Sharpe 1999a). One model in which ROS, specifically superoxide, has been proposed to lead to alter nitric oxide synthase (NOS) co-factor tetrahydrobiopterin (Milstien and Katusic 1999). Once uncoupled by deactivation of its co-factor, NOS will also begin to

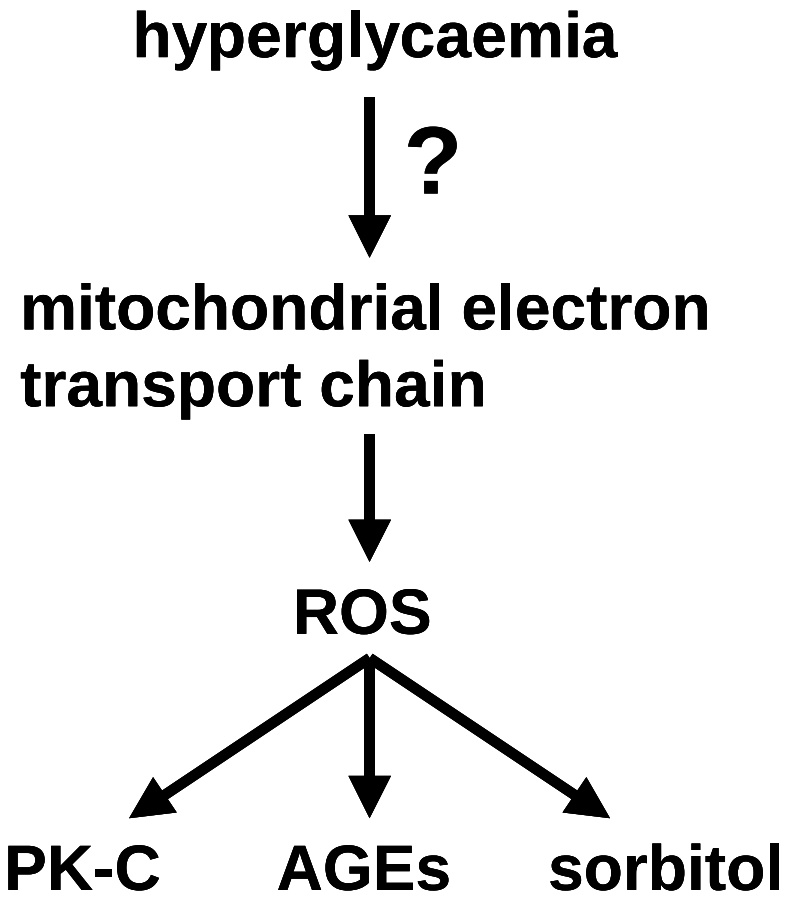

Figure 1-1: Proposed pathway for the generation of ROS due to hyperglycaemia.

produce oxygen-derived free radicals such as superoxide anion and hydrogen peroxide (Cosentino et al 1998). The end result of peroxynitrite is the decreased level of NO, and an increased production of oxygen derived free radicals. Subsequently, the end result of reduced NO level would be an impairment of the endothelium-dependent relaxation of vascular tissue.

ROS generated under these circumstances have also been shown to lead to the further alteration of metabolic pathways including activation of PK-C, production of AGEs, and induction of the polyol pathway (Nishikawa et al 2000). Although all of the three pathways have been identified to lead to diabetic complications, sorbitol accumulation via the polyol pathway is not implicated in the development of cardiovascular complications per se. The induction of PK-C will lead to increased contraction of vascular smooth muscle (Walsh et al 1996). Increased PK-C activity has also been associated with proliferation of vascular endothelium and smooth muscle, increased vascular permeability, and induction of expression of endothelin-1 (ET-1) (Koya and King 1998). The activation of AGEs has been shown to disrupt both intracellular and extracellular matrix proteins, generate free radicals via interaction with its receptor, interfere with NO levels, and stimulate the production of ET-1 (Brownlee 1995). The induction of PK-C and production of AGEs provide a further molecular basis for the dysfunction of cardiovascular tissues, and an alteration of blood pressure.

An attenuated level of NO production, induction of PK-C, and production of AGEs, provide, at least in part, a metabolic basis for altered reactivity in vascular tissues. Although we have focused this discussion on how these metabolic changes would likely affect vascular tissues, there is evidence that suggests that similar metabolic disturbances

occur in tissues not sensitive to insulin, such as cardiomyocytes (Koya et al 2000). This may suggest that vascular tissues are metabolically sensitive to extracellular glucose concentration in a way similar to non-insulin dependent type tissues.

1.3.2 Nicotine as a cardiovascular pathological agent

The molecular basis for nicotine as a cardiovascular pathological agent has many striking similarities to the previous evidence for glucose. There is experimental evidence in both humans and animals that demonstrates vascular dysfunction due to nicotine treatment (Mayhan and Patel 1997, Chalon et al 2000). Further evidence suggests that the mechanism of this nicotinic impairment is due to the generation of oxygen-derived free radicals, which leads to a decrease in NOS activity (Mayhan and Sharpe 1999b, Fang et al 2003). Perhaps most importantly, Mayhan and Sharpe (1996b) demonstrated that nicotine could decrease the amount of nitric oxide being released from a peripheral vascular bed in response to acetylcholine (ACh) or adenosine diphosphate (ADP). This observation is quite interesting in the light of the well-established pharmacological action of nicotine. Further to this, Mayhan and Sharpe (1996b) also reported that this decrease in NO release by nicotine could be attenuated with the concurrent application of the free radical scavenger superoxide dismutase. This evidence suggests that nicotine may cause vascular dysfunction in a manner similar to hyperglycaemia. In other words, nicotine and glucose could share a common mechanism in the development of vascular dysfunction; that is, a propensity to decrease the bioavailability of NO in vascular tissues. These alterations in the function of peripheral vascular reactivity could have major implications for the chronic expression of blood pressure levels.

1.4 Selection of a Type-1 Diabetes Experimental Model

As discussed in Section 1.1.2.1, the literature reports that the cardiovascular complications found in type-2 diabetes are also possible in type-1. The reason for the difference in the profile of cardiovascular complications of these two types of diabetes is not known. We chose to study a model of type-1 diabetes, because we suppose that certain conditions may lead to the same cardiovascular complications in type-1 diabetes, as in type-2 diabetes. Stimulating the onset of cardiovascular complications in type-1 diabetes with nicotine would have definite implications for treatment strategies in type-1 diabetic patients who smoke. It would emphasize an importance to educate type-1 diabetic patients as to why controlling a preventable risk factor, like smoking, makes a difference in their health outcome. Stimulating the onset of a cardiovascular complication in a type-1 diabetes model might also serve as a clue as to why a cardiovascular complications in type-2 diabetes occurs. As previously mentioned, the metabolic disturbances in type-1 and type-2 diabetes are quite different. However, the two conditions share the obvious similarity of poor glucose control. Therefore, even through the study of a type-1 diabetes like model, we might be able to gain clues as to the pathophysiological mechanisms which cause certain cardiovascular complications in type-2 diabetes.

1.5 The Combination of Diabetes and Tobacco Use

Clinical observations to date have reported that the vascular complications associated with diabetes are often accelerated in diabetic patients who smoke (Schwamberger and Flora 1975). Although we are starting to get a clearer picture on the causative agents involved with each of the risk factors for CVD, there are still great gaps in our

understanding of how we get from risk factor to disease. Figure 1-2 is a simplistic view of how the risk factors might stack together to effect the development of cardiovascular pathology. Panel A (Figure 1-2) depicts our classical view that each risk factor contributes to the development of CVD in an independent manner. Panel B (Figure 1-2) is a potential way in which the three risk factors in question would interact to affect the development of CVD.

Aside from the potential for hyperglycaemia and nicotine to act via a proposed common mechanism involving reactive oxygen species, there may be other ways in which the two may exacerbate each other's effects. Tsujimoto et al (1965) demonstrated that intravenous nicotine injection in dogs led to transient increases in both serum potassium and blood glucose levels. We now know that this effect of nicotine is likely due to its acute pharmacological effect to increase sympathetic activity (Benowitz 1986). Although there are many ways in which both substances lead to acute perturbations in metabolic pathways, the chronic effects of this combination has not been extensively studied.

From our review of the current literature, we know that hypertension is a phenomenon that is associated with diabetes and certain forms of tobacco use like smoking under certain circumstances. What we are still unclear on is precisely which circumstances lead to a hypertensive state in an individual with diabetes, or an individual who uses tobacco products. Although some studies have attempted to answer this question experimentally, there is nothing which conclusively demonstrates a positive chronic effect on blood pressure due to either STZ or nicotine treatment in an animal model. The major reason for this is that most studies do not follow their observations for a long enough period of time, or they are not properly designed to test whether either of the aforementioned

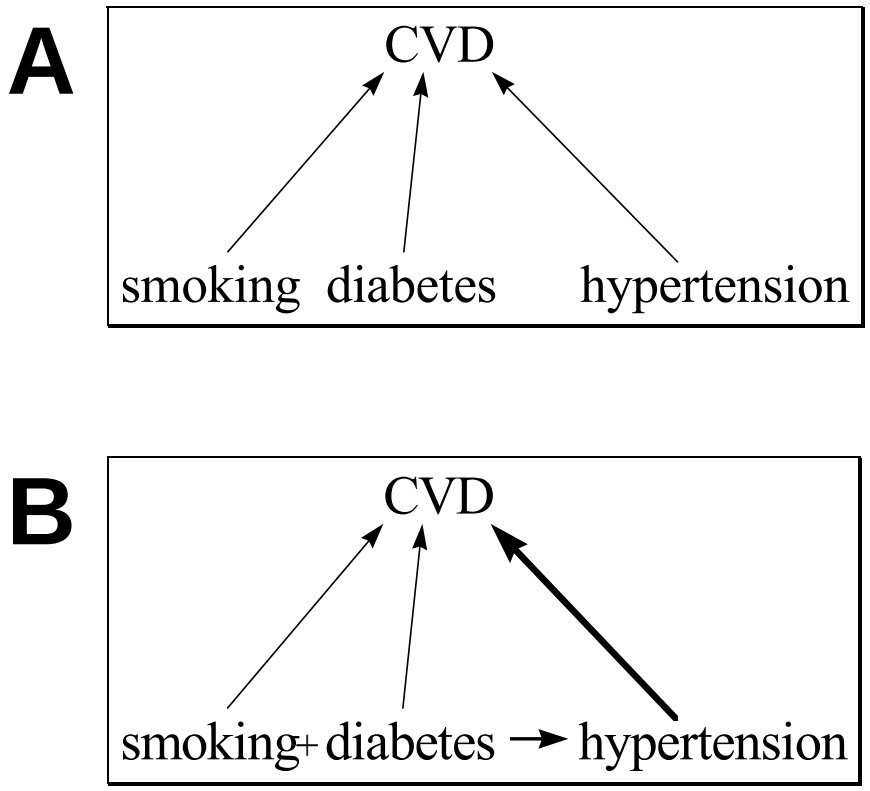

Figure 1-2: Potential cause-effect relationship between smoking, diabetes, hypertension and cardiovascular disease.

treatments affect blood pressure chronically. Therefore, we hope to clarify whether or not STZ and nicotine, either alone or in combination, have an effect on blood pressure that is characteristically chronic.

1.6 Hypothesis and Objectives

We hypothesize that combining nicotine uptake and hyperglycaemia will lead to an early onset and chronic elevation of blood pressure in rats. Hyperglycemia or nicotine treatment alone may induce hypertension if the treatment and observation period is long enough. Once combined, hyperglycemia and nicotine would elevate blood pressure much earlier than either treatment alone.

It is our objective to determine the effect that each treatment alone, STZ and nicotine, has on blood pressure. We would also like to determine if the combination of the two treatments, STZ and nicotine, lead to a change in blood pressure. Further to this, we will explore the potential mechanism of blood pressure change due to these treatments either alone or in combination.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal Care and Handling

All rats were housed at 21°C with 12-hour light/dark cycles, and fed standard lab chow and tap water *ad libitum*. The University of Saskatchewan Standing Committee on Animal Care and Supply has approved all animal protocols and animal handling procedures in this study.

2.2 Experimental Design

Male Sprague Dawley (SD) rats, 4-5 weeks of age, were received from the breeder (Charles River, Toronto, ON, Canada) and allowed 2-3 days to acclimatize to the local animal housing facilities. Then, blood pressure was measured in rats for 3 consecutive days to ensure that their pre-treatment baseline blood pressure was normal. Rats were then selected to either undergo nicotine pre-treatment for two months, or no treatment (control). The reason rats were pre-treated with nicotine, prior to STZ injection, is two fold. Firstly, this was done in an attempt to mimic an individual who uses tobacco products prior to diagnosis with diabetes. Secondly, rats were pre-treated with nicotine in an attempt to resolve the conflicting literature reports regarding the effect of chronic nicotine treatment on the blood pressure of rats (Swislocki et al 1997, Bui et al 1995). From previous experiments (Wang et al 2001), we had already established no effect of STZ treatment on blood pressure levels. Therefore, blood pressure, heart rate and body weight were followed for the period of two months pre-treatment with nicotine. After two months, both nicotine and control treatment groups were divided in half respectively.

Half of the rats from control group received streptozotocin injection. Likewise, half of the rats from nicotine group received streptozotocin injection. We then had four distinct treatment groups: control group, STZ group, nicotine group and STZ-N (STZ + nicotine) combination group. After the onset of streptozotocin-induced diabetes, blood pressure, heart rate and body weight were observed for one month. After the last blood pressure observation, rats were killed one at a time so that aortic contractility experiments could be done on fresh aortas from each rat. Furthermore, at the time of killing, blood samples were taken to measure plasma glucose, and various tissues taken for future analysis.

2.2.1 Type-I diabetes mellitus

A model of type-I diabetes mellitus was induced in rats using streptozotocin, and was used to treat rate in the STZ and STZ-N combination group. Streptozotocin was prepared in citrate buffer solution at a concentration appropriate to deliver a dose of 60 mg/kg in a bolus of approximately 1 ml via tail vein injection. Rats participating in non-STZ groups (control and nicotine) also received a 1 ml injection of citrate buffer solution, as placebo, without streptozotocin. Streptozotocin treatment was given 12 hours following the last gavage of rats in the STZ-N combination group. Onset of streptozotocin induced diabetic model was confirmed 2-3 days following injection by Chemstrip 5L urine strip test for glucose presence in the urine (Roche Diagnostics, Laval, QC, Canada).

2.2.2 Nicotine treatment

Rats in the nicotine and STZ-N combination group received oral nicotine dosage. Dosing rats via oral gavage had the advantage of being able to standardize the dose of nicotine based on weight. This was particularly important to us due to the lengthy nature of our experimental time course, and expected weight gain of our experimental animals. Oral

nicotine dosage was given twice daily at 08:30 and 17:00. Oral gavage was done using a 2-inch, 8 gauge, gavage needle (Popper and Sons, New Hyde Park, NY, USA). (-)-Nicotine tatarate salt (Sigma, St. Louis, MO, USA) was dissolved in distilled water under low light conditions, placed in a light resistant container, and stored at 4°C prior to experiment. A dose of 1.5 mg/kg was given, for each gavage, in a bolus of ~2 ml, totaling a dosage of 3 mg/kg/day. The rationale for this dose is based upon the consideration that an average smoker consumes a package of cigarettes per day. From previous literature we know that an average cigarette delivers a dose of up to 8.4 mg of nicotine (Benowitz et al 1990). A package of Canadian cigarettes contains 25 pieces. We assume that the average person weighs 70 kg. Therefore, we estimate a relevant daily nicotine intake in mg/kg/day according to the following calculation:

- 8.4 mg nicotine/smoke × 25 smokes per pack = 210 mg nicotine/day
- 210 mg nicotine/day ÷ 70 kg = 3 mg nicotine/kg/day

However, when calculating the effect of nicotine concentration in the circulation, one has to be aware that the metabolism of nicotine might be quite different between human and rat. Animals in other groups not participating in nicotine treatment (control and STZ) received a 2 ml bolus of tap water, as placebo, via the same gavage method. Animals would not receive a gavage dose the morning prior to blood pressure measurement.

2.3 Experimental Parameters

2.3.1 Cardiovascular endpoints

2.3.1.1 Blood pressure and heart rate

Blood pressure (BP) and heart rate (HR) were measured using a Model-29 SSP non-invasive blood pressure (NIBP) tail cuff amplifier (IITC Inc. Woodland Hills, CA, USA).

A baseline value for blood pressure was taken on three consecutive days prior to treatment. Blood pressure was then measured once per week following the first day of treatment for the duration of the study. Rats were placed in a restrainer (IITC Inc.) and fitted with the tail cuff transducer in a temperature-controlled incubator at 28 ± 2 °C for optimum conditions. Chamber temperature was monitored using a thermistor probe (Omega Instruments, Stanford, CT, USA). Experimental data were acquired and logged using an MP100 data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA), and a personal computer (IBM, Armonk, NY, USA).

A sample original recording of blood pressure and heart rate data is depicted in Figure 2-1. The top channel indicates cuff pressure, the second channel indicates pulse deflections of the tail artery, and the third channel is the ambient temperature. Blood pressure was measured using the following procedure. The tail cuff was pumped to a pressure safely above the occlusion pressure of the tail artery. The cuff pressure was then bled off at a rate of about 10 mmHg/sec until no pressure remained. The cuff was not pumped up again for another 30 seconds in order to let the tail artery rest between trials. The inflation-deflation procedure was repeated until 3-4 clear results were obtained. Following the experiment, traces were analyzed for systolic and mean pressure as well as heart rate. Systolic pressure is taken as the point at which the deflections in the pulse channel (refer to Figure 2-1) resume, as the cuff pressure decreases below occlusion pressure. Mean blood pressure is taken at the point at which the deflections in the pulse channel reach maximum amplitude after the systolic pressure point. The diastolic pressure is derived using the equation normally used to calculate mean arterial pressure.

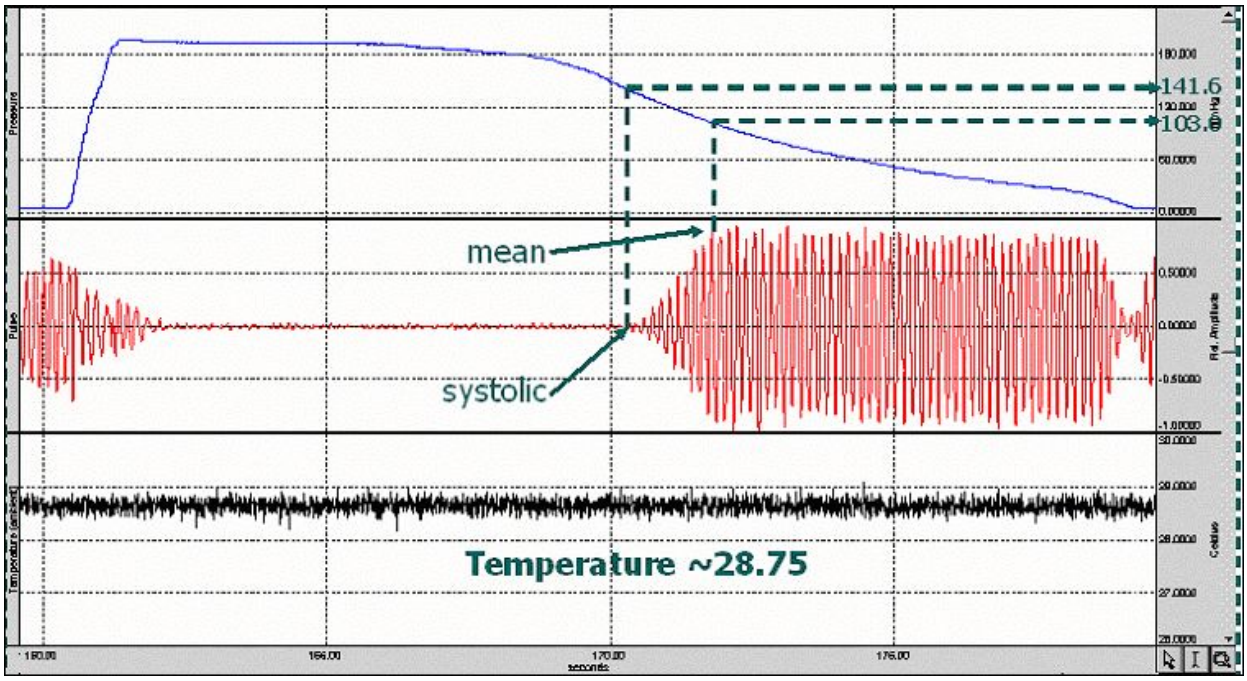

Figure 2-1: Sample tail cuff raw data recording from a Model-29 SSP NIBP tail cuff amplifier (IITC Life Sciences Inc).

The following expression is the standard equation for solving the mean pressure when systolic and diastolic pressure is known:

$$\text{MAP (mean arterial pressure)} = P_{diastolic} + 1/3\ (P_{systolic} - P_{diastolic})$$

The equation is re-written as follows:

$$P_{diastolic} = (3MAP - Psystolic) \div 2$$

The tail cuff interpretation of systolic and mean pressure has previously been verified by comparing the method to simultaneous cannulation measurement (Bunag & Butterfield, 1982). Heart rate is interpreted from the traces as the number of pulse deflections in one minute, taken from an area of the trace with a stable recording, prior to the commencement of blood pressure trials. Temperature of the chamber was logged on the third channel to verify the chamber temperature at the time of each measurement.

2.3.1.2 Aortic contractility

Thoracic aorta was excised, cleaned, and cut into 2 mm rings as previously described (Wang et al 1989). Care was taken not to damage the endothelial tissue layer of each aortic segment. Tissue was immediately hung in a 10 ml organ bath (Harvard Apparatus Inc., Holliston, MA, USA), and attached to a force displacement transducer (Grass Medical Instruments, Quincy, MA, USA). Data from the force-displacement transducers were acquired and logged using an MP100 unit, and personal computer. Aortic rings were stretched under 2 g passive tension, and bathed in Kreb's-Hanseleit saline solution that was bubbled with 95% oxygen (O_2)/5% carbon dioxide (CO_2) gas mixture (Praxair, Saskatoon, SK, Canada). Tissues were left to stabilize for one hour, while tension was continuously adjusted to 2 g passive force. Three consecutive potassium chloride (KCl) challenges (60 mM KCl) were delivered to contract tissues to verify and stabilize the

contractile response. PHE, ACh and SNP were purchased from Sigma, and 1 ml aliquots of 3 mM solution were prepared, protected from light, and frozen.

Concentration-effect curves were then established using cumulative doses of vaso-active agents to investigate contraction and relaxation properties of the tissues. In the case of phenylephrine (PHE), the initial dose was given immediately following stabilization of the tissue after the 3rd KCl contraction. Time was given between each dose in order for any effect of PHE on the tissue to plateau prior to the next dose. After the final dose had been given, and time allowed for the peak response of that dose, ACh (1 μM) was given to ensure that the endothelial tissue was intact.

In the case of ACh and sodium nitroprusside (SNP), tissues were pre-contracted with PHE (0.1 mM) and allowed to reach a plateau before the initial dose. Also, time was given between each dose in order for the tissue response to each dose level to plateau before administration of the next dose.

A sample trace of one aortic contractility experiment is depicted in Figure 2-2. The primary observation from this trace is the differential in the tension of the tissue in grams (Δg) before and after the administration of a dose. Concentration-effect curves are thenconstructed, and used to calculate either the effective concentration at 50% response (EC_{50}), the amount of drug at which half of the maximal effect occurred; or maximum effect (E_{max}), the maximum response of a tissue to a given drug. EC_{50} values were derived from the sigmoidal equation from curves constructed where 100% response was simply the maximal response in that tissue. E_{max} values were derived from the sigmoidal equation from curves constructed where the maximal response of that tissue to a drug is expressed as a percentage of the maximal response elicited by a standard vasoconstrictor

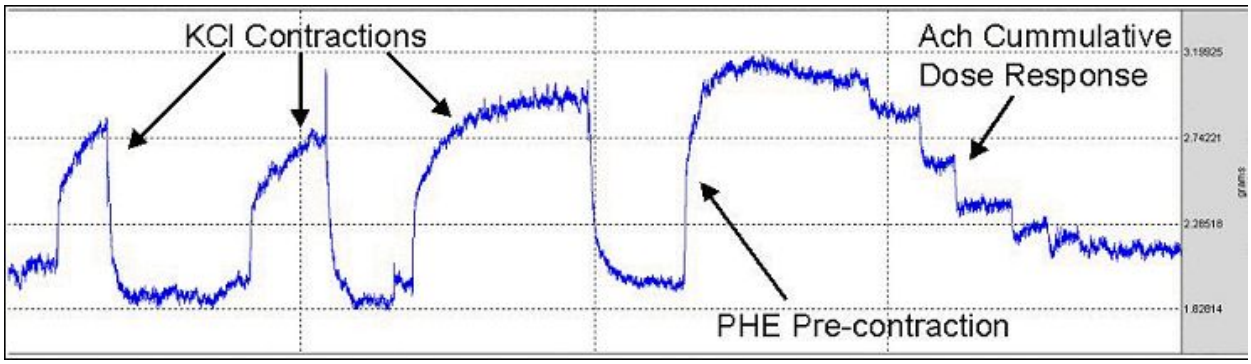

Figure 2-2: Sample recording from aortic contractility experiment.

agent. In the case of ACh and SNP, E_{max} is the maximum relaxant response of aortic tissue to either ACh or SNP, using the stable vascular tissue tension developed in the presence of PHE as 100% tension. In the case of PHE, E_{max} is the maximum response of aortic tissue to PHE, expressed as a percentage of the contractile magnitude of a 60 mM KCl-induced contraction of that tissue.

2.3.2 Metabolic Endpoints

2.3.2.1 Body Weight

Rats were weighed once per week during the time of blood pressure measurement. Each rat was taken from its cage, and placed in a small dish atop an Ohaus LS2000 portable balance (Ohaus Scale Corporation, Florham Park, NJ, USA). The weight observation was taken after the rat settled down, and the scale reading stabilized.

2.3.2.2 Plasma Glucose

Blood samples were taken at the end of the three-month study. Samples were collected by cardiac puncture, and bled into a heparinized collection tube (Beckton-Dickson, Franklin Lakes, NJ, USA). Plasma glucose levels were measured at The Royal University Hospital (Saskatoon, SK, Canada) using a Beckman LX20 automated assay system (Beckman Instruments, Fullerton, CA, USA).

2.4 Statistical Analysis

During pre-treatment screening, Grubb's test for detecting outliers was applied to rats that had 3-day systolic blood pressure that seemed abnormally high or low, and were excluded if they were determined to be outliers. Data from a single variable and treatment group was expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) was used to determine if statistical differences existed between the

mean values of the four experimental groups. Tukey's Honestly Significant Difference (Tukey's-HSD) and Student-Newman-Keuls (SNK) post-hoc statistical tests were used to do inter-group comparisons where ANOVA returned a significant difference between groups (Zar 1999). Means were considered significantly different at a P value less than 0.05. Statistical analysis was preformed using a personal computer running SPSS for Windows version 11.0.1 (SPSS Inc., Chicago, IL, USA).

In all results presented, n represents one observation corresponding to one rat, except in the case of aortic contractility. In the case of aortic contractility, n represents one observation corresponding to one aortic ring. Aortic rings were taken from 2-3 different rats for the purposes of one test.

The method of analysis in the two post-hoc techniques is, in theory, very similar. The way in which Tukey's and SNK measure the statistical significance of the mean differences between groups is comparable, aside from a slight difference in terms of the final ranking and grouping of the results suggested by the tests. It is this particular difference, which compelled us to process both methods.

The first technique, namely the Tukey's-HSD method of honestly significant differences, is known to be a versatile and easy to compute method that makes it possible to answer essentially any question about the means that may remain once the ANOVA is completed. Such questions would address the issue of the significance of the differences between intermediate means, or means that involve data (or groups) other than the one having the largest mean vs. the one having the smallest mean. In addition, the Tukey's method has proven to show great results when the interest lies only in the pairwise

differences between means, which is exactly the context of interest in this experiment (Zarr 1999).

Due to the vast array of advantages provided by the Tukey's test, one may wonder why a second method may be required. Although the two methods are nearly identical in theory with respect to pairwise comparisons, the SNK is used in this context to compliment Tukey's-HSD in situations where this test, due to the conservative nature of Tukey's-HSD, makes the results inconclusive. Such a situation arises when a difference in means in a pairwise comparison lies near the critical value at the selected significance level. In such a case, Tukey's may interpret the value as being both significant and insignificant, therefore placing the particular mean in two different subsets. It is clear that in practice a particular mean can only belong to a single group. Consequently, the P values for the test will be altered, and therefore, erroneous. Fortunately, the more flexible nature of SNK resolves this problem by associating such 'borderline' values to the correct group.

If SNK resolves the inconsistency problem with Tukey's-HSD, why not simply use SNK alone? The answer to this question is the fact that SNK doesn't ensure that family-wise errors are below the rejection level.

Following the above discussion, it is clear that the combination of both post-hoc tests will ensure the validity of the results provided in Chapter 3, and the subsequent conclusions in Chapter 4.

CHAPTER 3

RESULTS

As established in Chapter 2, the following experimental parameters were monitored in our experimental rats: blood pressure, heart rate, aortic contractility, body weight, and plasma glucose. Most importantly, changes were observed in the systolic but not the diastolic blood pressure of rats in the STZ-N combination group. Another interesting finding was that no significant changes in heart rate in any of our treatment groups were observed as compared with our control. Furthermore, significant changes in the response of aortic ring segments to both phenylephrine and sodium nitroprusside were noted, but not to acetylcholine. Finally, our observations pertaining to body weight and plasma glucose confirmed the already well-demonstrated characteristics of a streptozotocin-induced diabetic rat model.

3.1 Blood Pressure and Heart Rate

Observations relating to blood pressure and heart rate are reported in this section in two different ways. Firstly, the mean value of each one of the four treatment groups is compared for the final week of the experiment, which is referred to as the 'final week of observation'. The second manner in which observations are reported is by calculating an average of all blood pressure and heart rate mean values taken over the course of the STZ-N combination treatment. This second reporting method is referred to as the 'treatment duration average'.

3.1.1 Systolic blood pressure

Systolic blood pressure was evaluated on a weekly basis for each treatment group as previously described in Section 2.3.1.1. It was found that the mean systolic blood pressure in the two months prior to the onset of STZ-N combination was not significantly different in nicotine treated rats (112 ± 2.0 mmHg, n=16) from those of control (115 ± 3.1 mmHg, n=13).

Final week of observation. Following the nicotine pre-treatment period, our type I diabetic model was induced in selected rats and measurement of blood pressure was continued for the four distinct treatment groups described previously in Section 1.2. A summary of systolic blood pressure data is presented in Table 3-1. At the final week of treatment (Figure 3-1), systolic blood pressure in the control group was 113 ± 4.2 mmHg (n=4), in the STZ group was 122 ± 4.5 mmHg (n=5), in the nicotine group was 108 ± 5.1 mmHg (n=8), and in the STZ-N combination group was 151 ± 14.1 mmHg (n=4). ANOVA detected a significant difference between treatment groups ($P < 0.05$). Table 3-2 clearly indicates the statistical significance of pairwise comparisons among groups involving the STZ-N combination group. When compared with any of the other groups, namely control group, nicotine group, and STZ group, the systolic blood pressure in STZ-N combination rats is significantly higher than in the other groups. Conversely, other pairwise comparisons between groups other than the STZ-N combination group do not indicate a significant statistical difference. The table also indicates that diabetes alone or nicotine alone does not have a significant impact on the systolic blood pressure in the subject rats.

Table 3-1: Summary of systolic blood pressure.

	Treatment Group (mmHg)			
	Control (n=4)	STZ (n=8)	Nicotine (n=5)	STZ-N Combination (n=4)
Final Week of Observation	113 ± 4.2	122 ± 4.5	108 ± 5.1	151[*,A] ± 14.1
Treatment Duration Average	120 ± 1.7	128 ± 2.9	114 ± 4.9	142[*,B] ± 7.6

Values are expressed as mean ± standard error of the mean. [*]Indicates statistical difference at $P < 0.05$ compared with all other groups according to [A]Tukey's-HSD and [B]Student-Newman-Keuls post-hoc tests.

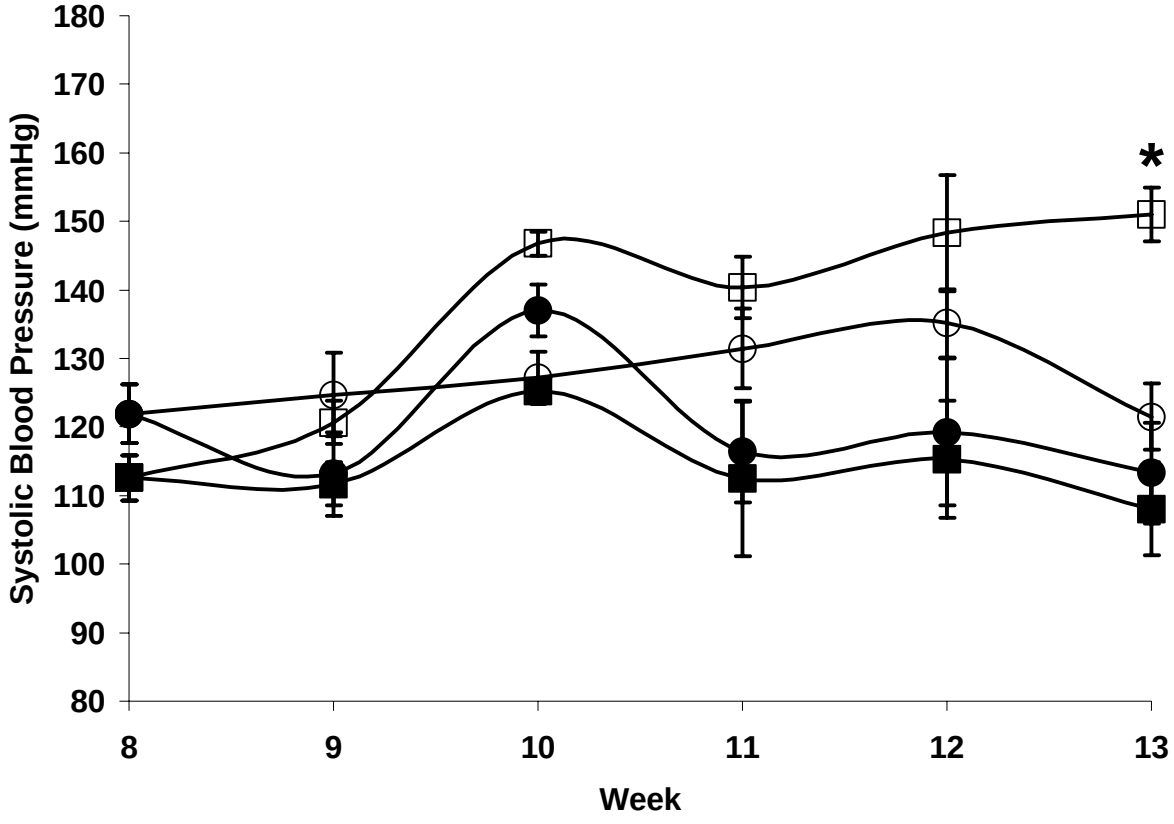

Figure 3-1: Systolic blood pressure of rats for the duration of STZ-N combination treatment (●control, ○STZ, ■nicotine, □STZ-N combination). *Indicates significance at $P < 0.05$ as compared with all other groups according to Tukey's-HSD.

Table 3-2: Proposed subsets following Tukey's-HSD[a,b] post-hoc statistical analysis on systolic blood pressure means during the final week of observation.

Treatment Group	N	Subset for alpha = .05	
		1	2
Nicotine	5	108.1	
Control	4	113.3	
STZ	8	121.5	
STZ-N Combination	4		151.0
Significance		0.557	1.000

Means for groups in homogeneous subsets are displayed. Means of one subset are considered significantly different at $P < 0.05$ from means of any other subset.
a-Uses Harmonic Mean Sample Size = 4.848.
b-The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Treatment duration average. Over the treatment duration (Figure 3-1), the systolic blood pressure of the control group was 120 ± 1.7 mmHg (n=4), the STZ group was128 ± 2.9 mmHg (n=8), the nicotine group was 114 ± 4.9 mmHg (n=8), and the STZ-N combination group was 142 ± 7.6 mmHg (n=4). The data in Table 3-3 shows that the systolic blood pressure of STZ-N combination rats was significantly higher when compared with any of the other three groups for the treatment duration.

Due to the nature of the post-hoc statistical tests used, one clarification about the data in Table 3-3 is required. As can be seen in the lower portion of Table 3-3, the Tukey's-HSD reports the mean of the diabetes group in both subsets. Due to the repetition of the diabetic mean value in Tukey's-HSD, the P-Values in the Tukey's-HSD results (lower portion of Table 3-3) have no significance.

One final observation from Table 3-3 we feel should be mentioned is that the SNK pairwise comparison between control group, nicotine group, and STZ group showed no significant difference in the systolic blood pressure over the treatment duration.

3.1.2 Diastolic blood pressure

Diastolic blood pressure was observed using methods previously described in Section 2.3.1.1, for each one of the treatment groups. Mean values for diastolic blood pressure at the last week of observation, as well as the treatment duration, are summarized in Table 3-4. A statistical difference in diastolic blood pressure ($P < 0.05$) was noted between the mean of nicotine group (68 ± 2.1 mmHg, n=16) and control group (77 ± 2.5 mmHg, n=13) during the 2-month pre-treatment period with nicotine (n number is much larger for these groups prior to the separation of the groups for STZ treatment). However, no statistical difference arose in diastolic pressure during the last week of observation

Table 3-3: Proposed subsets following Tukey's-HSD[a,b] and Student-Newman-Keuls[a,b] post-hoc statistical analysis on systolic blood pressure means during the treatment duration.

	Treatment Group	N	Subset for alpha = .05	
			1	2
Student-Newman-Keuls (a,b)	**Nicotine**	5	114.018	
	Control	4	120.223	
	STZ	8	128.072	
	STZ-N Combination	4		142.225
	Significance		.099	1.000
Tukey's-HSD (a,b)	**Nicotine**	5	114.018	
	Control	4	120.223	
	STZ	8	128.072	128.072
	STZ-N Combination	4		142.225
	Significance		.161	.157

Means for groups in homogeneous subsets are displayed. Means of one subset are considered significantly different at $P < 0.05$ from means of any other subset.
a-Uses Harmonic Mean Sample Size = 4.848.
b-The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Table 3-4: Summary of diastolic blood pressure.

	Treatment Group (mmHg)			
	Control (n=4)	STZ (n=8)	Nicotine (n=5)	STZ-N Combination (n=4)
Final Week of Observation	82 ± 9.9	72 ± 5.9	61[A] ± 9.3	89 ± 4.9
Treatment Duration Average	82 ± 2.2	76 ± 4.2	71 ± 5.3	87 ± 1.5

Values are expressed as means ± standard error of the mean. [A]n=4.

between any of the treatment groups. This was also the case for the mean diastolic pressure over the course of the treatment duration for the STZ-N combination group.

3.1.3 Heart Rate

Heart rate was assessed using the same trace used to measure systolic and diastolic blood pressures, as described in Section 2.3.1.1. During the final week of observation, the STZ-N combination group had a significantly higher ($P < 0.05$) heart rate (496 ± 9.42 bpm, n=4) than control group (385 ± 26.4 bpm, n=4), STZ group (320 ± 24.1 bpm, n=5) and nicotine group (349 ± 18.3 bpm, n=8) (Figure 3-2). However, the mean heart rate of rats in the four treatment groups did not differ significantly over the treatment duration for the STZ-N combination group.

3.2 Aortic Contractility

Concentration-effect curves of PHE, ACh and SNP were established using aortic ring segments from rats of the four treatment groups. From the concentration-effect curves, EC_{50} and E_{max} were derived. These observations are summarized in Table 3-6. Mean EC_{50} values are reported here in units of moles/litre (M), or a derivation thereof. Mean E_{max} values are expressed as a percentage on the basis of criteria described previously in Section 2.3.1.2.

3.2.1 Phenylephrine induced contraction

In Section 2.3.1.2, we described a method to establish a dose response of aortic ring segments to PHE. We found that the EC_{50} of aortic segments did not differ significantly between groups. As indicated in Table 3-6, the EC_{50} values for the control group, the

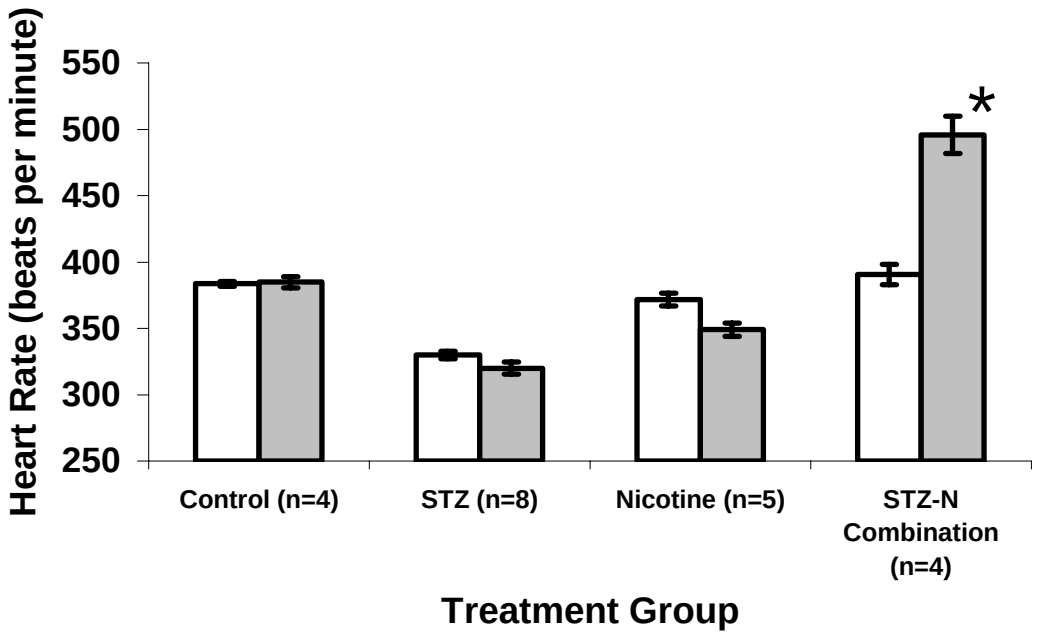

Figure 3-2: Summary and comparison of heart rate observed during the treatment duration average (white bars) and the final week of observation (grey bars). *Indicates significance at $P < 0.05$ as compared with all other groups according to Tukey's-HSD.

Table 3-6: Summary of aortic contractility.

	Phenylephrine		Acetylcholine		Sodium Nitroprusside	
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
Control	24 ± 7.0 (6)	95.1 ± 5.4 (6)	79 ± 2.4 (6)	84.1 ± 10.2 (6)	6.7 ± 1.6 (6)	86.5 ± 4.9 (6)
STZ	29 ± 3.5 (10)	143.8* ± 5.6 (10)	400 ± 130 (10)	65.4 ± 8.5 (10)	4.9 ± 9.2 (10)	65.7 ± 13.5 (10)
Nicotine	21 ± 8.8 (9)	106.7 ± 4.35 (9)	240 ± 86 (6)	58.7 ± 7.4 (6)	2.8 ± 4.0 (9)	112.2 ± 7.5 (9)
STZ-N Combination	20 ± 3.3 (8)	150.0* ± 5.8 (8)	310 ± 37 (7)	63.9 ± 9.6 (7)	1.7 ± 2.0 (7)	101.4 ± 2.5 (7)

Values are expressed as mean ± standard error of the mean (n in brackets). * $P<0.05$ vs. all other groups without a * in the same column.

STZ group, the nicotine group, and the STZ-N combination group are essentially equal. However, Table 3-7 indicates that the E_{max} of aortic segments from both the STZ-N combination (150 ± 5.8 %, n=8) and STZ (144 ± 5.6 %, n=10) groups were significantly higher than the E_{max} of segments from control (95 ± 5.4 %, n=6) or nicotine (107 ± 4.4 %, n=10) groups. A visualization of this result is presented in Figure 3-3.

3.2.2 Acetylcholine induced relaxation

In Section 2.3.1.2, we described a method to establish a dose response of aortic ring segments to ACh. The mean EC_{50} of aortic segments from each of the different treatment groups did not differ significantly in response to ACh (Table 3-6). Likewise, the mean E_{max} values of aortic segments from each of the treatment groups did not show any statistical difference between groups (Table 3-6).

3.2.3 Sodium nitroprusside induced relaxation

In Section 2.3.1.2, we described a method to establish a dose response of aortic ring segments to SNP. The mean EC_{50} of aortic segments from nicotine group (28 ± 4.0 nM, n=9) was significantly higher than that of control (6.7 ± 1.6 nM, n=6), STZ-N combination (16 ± 2.0 nM, n=8), and STZ (4.9 ± 0.9 nM, n=10) groups (Table 3-8, Figure 3-4). Tukey's-HSD post-hoc analysis was inconclusive when attempting to determine whether control group belonged in subset 1 or subset 2 (Table 3-8). However, the SNK test clearly places the mean of control group in subset 1. This clarification implies that the EC_{50} of STZ-N combination group is significantly higher than both control and STZ group, but still significantly lower than nicotine group (Table 3-8).

Finally, the E_{max} of aortic segments in response to SNP was not significantly different between groups (Table 3-6).

Table 3-7: Proposed subsets following Tukey's-HSD[a,b] post-hoc statistical analysis on E_{max} for phenylephrine in aortic ring segments.

Treatment Group	N	Subset for alpha = .05	
		1	2
Control	6	95.112	
Nicotine	9	106.660	
STZ	10		143.831
STZ-N Combination	8		149.965
Significance		0.454	0.857

Means for groups in homogeneous subsets are displayed. Means of one subset are considered significantly different at $P < 0.05$ from means of any other subset.
a-Uses Harmonic Mean Sample Size = 4.848.
b-The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

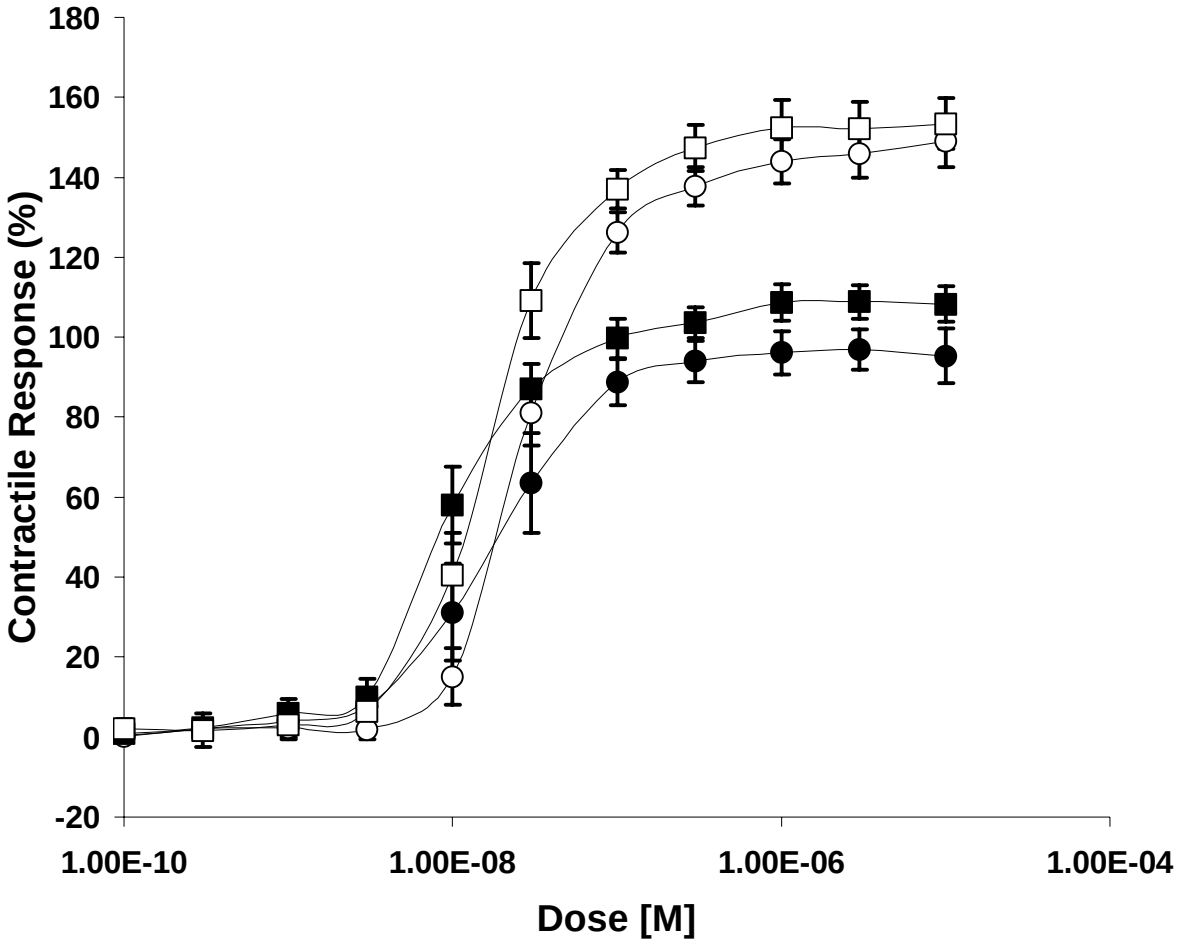

Figure 3-3: Contraction response to phenylephrine of aortic ring segments from the rats of the four treatment groups (●control, ○STZ, ■nicotine, □STZ-N combination). Contractile response due to phenylephrine is expressed as a percentage of a 60 mM KCl-induced contraction.

Table 3-8: Proposed subsets following Tukey's-HSD[a,b] and Student-Neuman-Keuls[a,b] post-hoc statistical analysis on EC_{50} for sodium nitroprusside in aortic ring segments.

	Treatment Group	N	Subset for alpha = .05		
			1	2	3
Student-Newman-Keuls (a,b)	**STZ**	10	4.95 E-09		
	Control	6	6.72 E-09		
	STZ-N Combination	8		1.60 E-08	
	Nicotine	9			2.76 E-08
	Significance		0.621	1.000	1.000
Tukey HSD (a,b)	**STZ**	10	4.95 E-09		
	Control	6	6.72 E-09	6.72 E-09	
	STZ-N Combination	8		1.60 E-08	
	Nicotine	9			2.76 E-08
	Significance		0.958	0.063	1.000

Means for groups in homogeneous subsets are displayed. Means of one subset are considered significantly different at $P < 0.05$ from means of any other subset.
a-Uses Harmonic Mean Sample Size = 4.848.
b-The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

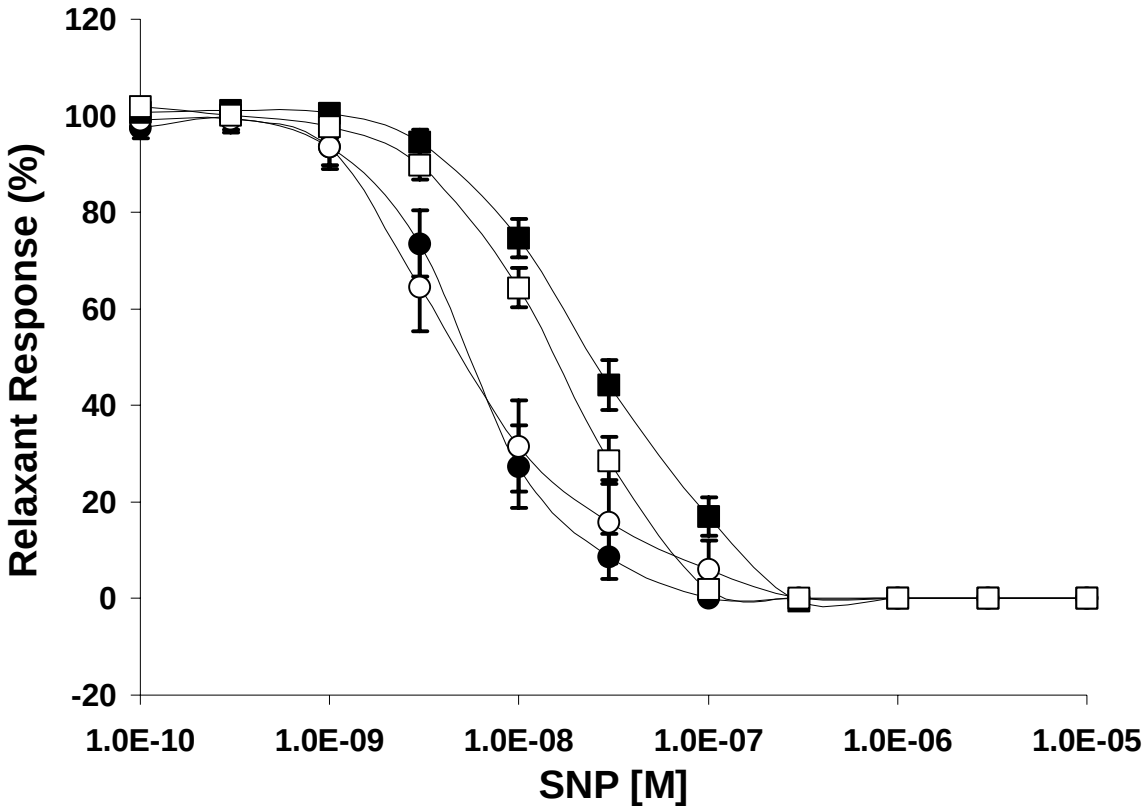

Figure 3-4: Relaxation response to sodium nitroprusside of aortic ring segments from the rats of the four treatment groups (●control, ○STZ, ■nicotine, □STZ-N combination). Data shown here have been normalized to illustrate changes in the EC_{50} of SNP in aortic ring segments. The relaxation elicited by SNP for each trial is taken as 100%. Then, the relaxant effect of each dose of SNP was divided by the maximum effect of SNP for that test, and expressed as a percentage. This percentage was subtracted from the 100% SNP response, and plotted against the corresponding dose of SNP to obtain the dose-response curves shown above. The result is a curve that depicts the changes in EC_{50}, which always reaches 100% for each test. This expression of the data enables us to illustrated potential changes in the EC_{50} of SNP in aortic rings segments due to the various experimental treatments.

3.3 Body Weight and Plasma Glucose

3.3.1 Body weight

Both STZ (372 ± 12.4 g, n=8) and STZ-N combination (337 ± 23.3 g, n=4) groups had a statistically lower ($P < 0.05$) body weight than control group (442 ± 9.0 g, n=8) at the end of experiment (20 weeks old). Conversely, nicotine group (527 ± 20.6 g, n=5) had a statistically higher ($P < 0.05$) body weight than control by the end of the experimental time course (Table 3-9).

3.3.2 Plasma glucose

Plasma glucose was measured using blood samples from each of the experimental groups as per methods previously described (Table 3-10). The plasma glucose of both STZ-N combination (39.5 ± 2.32 mmol/L, n=4) and STZ (33.8 ± 0.84 mmol/L, n=7) groups was significantly higher ($P < 0.01$) than either control (7.8 ± 0.58 mmol/L, n=8) or nicotine (10.7 ± 0.40 mmol/L, n=2) groups (Table 3-11). Furthermore, STZ-N combination group was significantly higher ($P < 0.05$) than the STZ group (Table 3-11).

3.4 Physical Appearance

During the final week of the experiment, the STZ-N combination rats were visibly much less healthy compared with rats in the other three treatment groups. These animals seemed dehydrated, emaciated, weaker, and had diarrhea as the animal became weaker and less likely to groom.

Table 3-9: Summary of final rat body weight.

Treatment Group (grams)			
Control (n=8)	STZ (n=8)	Nicotine (n=5)	STZ-N Combination (n=4)
442 ± 9.0	372* ± 12.4	527* ± 20.6	337* ± 23.3

Values are expressed as means ± standard error of the mean. * Values are statistically different at $P < 0.05$ compared with control according to Tukey-HSD.

Table 3-10: Summary of plasma glucose concentration.

	Treatment Group			
	Control	STZ	Nicotine	STZ-N Combination
Plasma Glucose (mmol/L)	7.76 ± 0.58 (10)	33.76* ± 0.84 (7)	10.70 ± 0.40 (2)	39.53† ± 2.32 (4)

Values are expressed as means ± standard error of the mean. *,† Values are statistically different at $P < 0.05$ compared with all other groups according to Tukey-HSD. Sample size is indicated in brackets (n).

Table 3-11: Proposed subsets following Tukey's-HSD[a,b] post-hoc statistical analysis on plasma glucose level of rats from the main experimental group.

Treatment Group	N	Subset for alpha = .05		
		1	2	
Control	10	7.760		
Nicotine	2	10.700		
STZ	7		33.757	
STZ-N Combination	4			39.525
Significance		0.388	1.000	1.000

Means for groups in homogeneous subsets are displayed. Means of one subset are considered significantly different at $P < 0.05$ from means of any other subset.
a-Uses Harmonic Mean Sample Size = 4.848.
b-The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

CHAPTER 4

CONCLUSIONS

We concluded that STZ-N combination treatment significantly elevated systolic blood pressure, but not diastolic blood pressure or heart rate. Furthermore, we concluded that diabetes alone and STZ-N combination treatment increased the efficacy of PHE to contract aortic ring segments, and that nicotine treatment decreased the sensitivity of aortic segments to SNP. We made no new conclusions regarding the effect of streptozotocin, or the combination of STZ and nicotine, on body weight or blood glucose beyond what is already known about STZ rat models.

4.1 Blood Pressure and Heart Rate

4.1.1 Systolic blood pressure

We conclude that nicotine treatment in a rat model of diabetes leads to an increase in systolic blood pressure. We make this conclusion on the basis that the systolic blood pressure of STZ-N combination rats either at the final week of observation, or over the duration of the combination treatment, was higher than control rats. We also conclude that streptozotocin injection and nicotine treatment on their own do not affect the systolic blood pressure under these conditions.

4.1.2 Diastolic blood pressure

We conclude that there were no major statistical differences in diastolic blood pressure due to nicotine, streptozotocin injection, or STZ-N combination. Part of our result suggests that there might be a slight depression of diastolic blood pressure due to nicotine treatment during the first two-month period of our experiment, as compared with control.

However, the diastolic blood pressures of nicotine group and control group are not statistically different at the end of treatment.

4.1.3 Heart Rate

We conclude that there is no significant change in heart rate due to streptozotocin-injection, nicotine treatment, or the two combined. Although the heart rate of rats in the D-N combination group had a significantly higher rate than diabetic or nicotine group, their heart rate did not differ significantly from the control group. Also, the heart rate for the four groups was not significantly different from each other during the one month following streptozotocin injection.

4.2 Aortic Contractility

4.2.1 Phenylephrine induced contraction

We concluded that there was an increase in the efficacy of phenylephrine to contract aortic tissue from rats of the STZ or STZ-N combination groups. We concluded this on the basis that the E_{max} of phenylephrine in aortic ring segments from STZ-N combination and STZ group rats was significantly higher than segments from control or nicotine group rats.

4.2.2 Acetylcholine induced relaxation

We concluded that none of the treatment groups had an effect on the acetylcholine-induced relaxation of aortic tissue. Although there seemed to be slight reductions in the efficacy of acetylcholine to relax aortic segments from diabetic, nicotine and D-N combination groups as compared with control, ANOVA did not indicate any statistical difference in the E_{max} between all groups.

4.2.3 Sodium nitroprusside induced relaxation

We concluded that nicotine treatment decreased the sensitivity of aortic smooth muscle tissue to SNP induced relaxation. This conclusion was made on the basis that the EC_{50} of SNP in aortic ring segments from nicotine group rats was higher than the other three treatment groups. Although ANOVA detected significant difference in the E_{max} between groups, post-hoc analysis was unable to rank the treatment groups into distinct population subsets. Therefore, we concluded that our four treatment groups had no effect on the maximum effect of SNP to relax aortic ring segments.

4.3 Body Weight and Plasma Glucose

We confirmed that diabetic treatment models would lead to a decrease in body weight gain. This was concluded on the basis that the body weight of rats in either STZ or STZ-N combination group was lower than rats of either control or nicotine group. We further concluded that nicotine treatment alone lead to a body weight increase. This conclusion was based on the observation that rats from the nicotine group weighed more as compared with rats from the control group at the end of the experimental time course.

We confirmed that diabetic treatment models would lead to an increase in plasma glucose concentration. This was confirmed by the observation that the plasma glucose level for rats in the STZ or STZ-N group was higher than the plasma glucose levels for rats in either the nicotine or control group. We have also observed a potential trend for the STZ-N combination treatment to elevate plasma glucose concentration significantly more than STZ treatment alone.

CHAPTER 5

DISCUSSION

Our study demonstrated that a combination of type-1 diabetic model and nicotine uptake would lead to chronically elevated blood pressure in the rat. We believe that this change is, at least in part, due to an interaction of high glucose concentration and nicotine to alter the function of vascular tissues. Although we still do not know the precise mechanism of this interaction, our observations confirm that functional alterations occur due to this combination treatment. Future studies should be designed to investigate the mechanism of blood pressure change due to the interaction of STZ and nicotine treatment in the rat.

5.1 Blood Pressure

We hypothesized that combining a model of type-1 diabetes and nicotine feeding in a rat would lead to chronic elevations of blood pressure in the rat. Although many studies have suggested that blood pressure change occurs, in either streptozotocin or nicotine treatment models, this has never been conclusively proven. Therefore, we designed a study that incorporated both nicotine feeding and streptozotocin injection in order to assess their effects both independently, and in combination.

5.1.1 Streptozotocin and blood pressure

We confirmed previous conclusions (Wang et al 2001) that streptozotocin treatment does not lead to chronic changes in blood pressure in the SD rat. Although the time course in this experiment for STZ injection was somewhat different, our previous experiments reported that STZ did not lead to significantly different blood pressure 3-months post injection as measured by cannulation of the femoral artery (Wang et al 2001). This

current experiment supplements this knowledge, and confirms that even in slightly older rats, streptozotocin injection does not lead to significant changes in blood pressure 1-month post injection as measured by the indirect tail-cuff method.

Our previous result (Wang et al 2001) confirms the result of Yamamoto et al (1988), which reports that a dose of 60 mg/kg STZ had no effect on the BP of either SD or WKY rats, respectively, as measured by direct cannulation. However, Yamamoto et al (1988) reports that the same dose of STZ results in an increase in BP of WKY rats 4 weeks post injection as measured by the tail-cuff technique. Our result contradicts Yamamoto's findings, as we did not observe an STZ-induced change in BP, 4 weeks post injection at the same dose. We believe the reason for this may be a major difference in protocol when measuring the BP using the tail-cuff technique. Yamamoto et al (1988) reported that the ambient temperature of the measurement chamber was 40 degrees Celsius. This is on average 12 degrees higher than the chamber temperature under our protocol at 28 degrees Celsius. It has been previously reported that an ambient air temperature of 40 degrees Celsius resulted in a significantly higher blood pressure observation as compared with 30 degrees Celsius (Kuwahara et al 1991). Kuwahara et al (1991) also stated that indirect tail cuff measurement was a reliable way to assess blood pressure levels at ambient temperatures of 30 degrees Celsius and below. Through optimization of our protocol, we found that 28 degrees Celsius was adequate enough to provide clear observations with the tail cuff, while maintaining a temperature only moderately above normal room temperature of 21 degrees Celsius. At any rate, we do not believe that our result can be compared with Yamamoto's result on the basis that they use a chamber

temperature much higher than what is acceptable for the indirect tail-cuff measurement technique.

5.1.2 Nicotine and blood pressure

We report that oral nicotine feeding has no effect on the blood pressure of male, Sprague Dawley rats over the course of three months of treatment. We believe this observation to be consistent with the literature which, although clearly demonstrates an acute and transient elevation of blood pressure due to nicotine, is inconclusive as to nicotine's chronic effect on blood pressure. A similar study to our own was published by Swislocki et al (1997), and claims that 'smokeless nicotine exposure leads to sustained but reversible hypertension' in 8-week old Sprague Dawley rats. However, we believe that Swislocki's paper does not demonstrate a truly 'chronic' effect of nicotine, and must be interpreted carefully in the context of hypertension. Although Swislocki et al (1997) reported an increase in blood pressure subsequent to the implantation of a nicotine pellet, their results are not directly comparable to our own due to the obvious differences in methodologies.

First of all, the concentration of nicotine in the report of Swislocki et al (1997) may not mimic moderate smoking as they suggest. Swislocki's protocol utilizes an implanted subcutaneous pellet to deliver nicotine to their experimental animals with a daily dose of about 10 mg/kg. In our study, 2 oral doses of nicotine a day were given with a daily dose of 3 mg/kg. Swislocki et al (1997) cited Andersson et al (1993) who used a nicotine dose of 7.2 mg/kg/day. However, the dosage level in Swislocki's study is clearly higher than that used by either Andersson et al (1993) or by us. These authors did not convincingly relate their nicotine dosage level to a clinically relevant concentration of nicotine that one

might find in a moderate smoker. In Section 2.2.2, we have provided a rationale for a dosage level of nicotine that we feel correlates more closely to the daily dose obtained by a moderate, 1-pack per day, smoker. Our dosage level of nicotine was approximately 1/3 that of the level used by Swislocki et al (1997). Therefore, although Swislocki's protocol appeared to result in an elevated systolic blood pressure level due to nicotine pellet implantation, this result may not be as relevant to the moderate smoker as they have suggested.

Secondly, the method of nicotine delivery employed in Swislocki et al (1997) was different from ours and might not be the best model to mimic the behaviour of smokers and smokeless tobacco users. The continuous nicotine delivery by a subcutaneous pellet may not have the same clinical/physiological relevance as does discrete doses. The method used to deliver nicotine to our experimental system could certainly account for differing observations between Swislocki's study and our own. Although we did not measure the blood pressure of our rats immediately following nicotine dosage, it is entirely possible that the blood pressure of rats was elevated following the dose. One would expect a transient increase in blood pressure and heart rate under these conditions according to the well-established transient effect of nicotine on the cardiovascular system (Gebber 1969). However, we were more interested in the chronic effect of nicotine, which is why we chose to measure blood pressure at times well outside any potential transient effect which would have occurred due to one of our dosages. Swislocki's protocol does not allow for blood pressure measurement to occur outside of a time where nicotine is being delivered to the system due to the nature of the continuous infusion of the nicotine pellet. There is some doubt as to whether or not the observation of elevated

blood pressure in Swislocki's study is due to a transient effect of nicotine, as demonstrated by Gebber (1969), or a chronic modification of the cardiovascular system by nicotine.

Thirdly, the overall duration of nicotine treatment is different between the study of Swislicki et al (1997) and ours. Compared with Swislocki's approximate 3-week nicotine delivery, our nicotine treatment lasted for a period of 3 months. Although we cannot disprove that the effect of nicotine in Swislocki's study is truly chronic in nature, our protocol represents a more realistic long-term nicotine consumption.

Lastly, the reliability of the blood pressure observations reported by Swislocki et al (1997) is questionable. Over their experimental time course, Swislocki's observations certainly seem to indicate a trend for higher blood pressure in the rats implanted with nicotine pellets. The last blood pressure observation prior to pellet exhaustion is significantly higher for nicotine treated animals as compared with placebo treated animals. At the next time point after pellet exhaustion the blood pressure of the nicotine treated animals decreases and is no longer significantly different than that of placebo treated animals. It is important to point out that most of Swislocki's blood pressure observations were reported using a line graph; therefore it is impossible to know the exact value corresponding to the various experimental time points. The only time point for systolic blood pressure reported with actual values is at the time that oral glucose tolerance testing was performed, 2.5 weeks post pellet implantation. At the time of oral glucose tolerance testing the systolic blood pressure of rats with the nicotine pellet was 150.5 ± 1.66 mmHg as compared with those that received the placebo, 121.0 ± 8.54 mmHg. It should also be noted that the number of animals used for each group in

Swislocki's study is not reported. Swislocki describes the whole phenomenon as a 'sustained but reversible hypertension' because the increase in blood pressure attributed to the nicotine pellet seems to subside following the exhaustion of the nicotine pellet. One troublesome aspect of Swislocki's protocol is that during the time of approximate pellet activity, blood pressure is measured many times weekly. However, this frequency of observation stops just prior to the approximate time of pellet exhaustion, and another observation is not reported for another 2 weeks. We are still left with many unanswered questions about the mechanism of nicotine's action in this experimental system.

In summary, our result of no chronic change in blood pressure after nicotine treatment is more reliable and closer to the nicotine consumption by smokers. Our result is consistent with that of Bui et al (1995) that reported no significant difference of systolic blood pressure in 7-week old WKY rats that were implanted with similar nicotine tablets over a period of 6 weeks.

5.1.3 STZ-N combination and blood pressure

We supposed that one of the reasons that elevations in blood pressure were not consistent between studies is that there was some level of threshold metabolic disturbance that either nicotine or STZ treatment may or may not achieve independently, and were highly dependent upon confounding variables. Our hypothesis essentially suggests that nicotine and STZ treatments, when put together, will cause sufficient metabolic disturbance to meet this threshold. Our results seem to confirm this hypothesis, as we concluded that rats from the STZ-N combination group had significantly higher blood pressures than either of the other two treatments independently, or compared with control rats. Although we had a relatively low number of animals remaining in the STZ-N

combination group at the end of our experiment, the statistics we used were fairly conservative with respect to groups of different size, and smaller degrees of freedom. What is quite apparent is that rats from this STZ-N combination group were visibly much less healthy that their counterparts in any of the other three experimental groups. These experimental observations would seem to parallel well established clinical observations that report an acceleration of cardiovascular sequelae in diabetic patients who smoke (Schwamberger and Flora 1975). Whatever the reason, we feel confident in concluding that the combination of hyperglycaemia and nicotine feeding in an experimental animal leads to significant pathophysiology. We feel that this experiment is a strong suggestion that human conditions which lead to high plasma glucose and significant plasma nicotine levels have the potential for accelerated negative cardiovascular outcomes.

5.1.4 Limitations of the tail-cuff method for determining blood pressure

Although the tail-cuff method for determining blood pressure has certain advantages over other blood pressure measurement techniques, it has various limitations that should be considered when interpreting data. The tail-cuff method is non-invasive, inexpensive, good for large populations of animals, and for repeated measurement over a long experimental period (Van Vliet et al 2000). The limitations include restraint (Van Vliet et al 2000) and the tail-cuff method's inability to directly assess diastolic blood pressure.

Physical restraint is the major limitation of the tail-cuff method according to Van Vliet et al (2000). Van Vliet et al (2000) point out that even with limited external pre-heating, restraint stress may combine with this limited warming to produce significant increases in core body temperature as described by Bunag (1984). Restraint stress seemed to play a role in the observed blood pressure in baroreceptor denervated rats (Norman et al Dent

1981). Findings from Normal et al (1981) showed that in this particular experimental model, the blood pressure measured by the tail-cuff method and direct catheter under restraint, differed from the blood pressure measured by direct catheter without restraint. As a result of such observations, Van Vliet et al (2000) suggest the possibility that blood pressure observed with the tail-cuff method may be a product of the animal's reaction to treatment combined with their response to restraint stress. Consequently, the measured blood pressure may not accurately reflect the real level.

Measurement of the diastolic blood pressure via tail-cuff method is done indirectly through observation of the mean blood pressure. The mean blood pressure is taken as 'the first maximum amplitude' in the pulse channel after occlusion (systolic) pressure, as described in 'OPERATING INSTRUCTIONS FOR THE MODEL 59, 29, & 29SSP AMPLIFIER' as provided by IITC Life Sciences Inc. The evidence that 'the first maximum amplitude' corresponds to the mean blood pressure in rats is reported by Bunag and Butterfield (1982). They show that 'the first maximum amplitude' has a high statistical correlation to the mean blood pressure as measured using direct femoral artery cannulation (Bunag and Butterfield 1982). However, 'the first maximum amplitude' also has a certain degree of correlation to the diastolic blood pressure as well, although not as significantly. For now, 'the first maximum amplitude' has been widely accepted as the mean blood pressure for that animal in numerous laboratories in the world. However, this will continue to be a contentious issue until such time as a physiological basis can be given to describe why the mean blood pressure occurs at 'the first maximum amplitude'.

Although the tail-cuff method has many advantages compared to other methods of blood pressure measurement, its limitations should be considered when interpreting the data

obtained. These limitations may necessitate validation of results obtained with the tail-cuff method by another reliable method blood pressure measurement. Therefore, a secondary method of assessing blood pressure in animals treated with streptozotocin and nicotine, specifically one that does not include restraint, may help to confirm the result of this treatment on the blood pressure in rats.

5.2 Body Weight

Our result showed a marked weight gain in rats that received nicotine treatment compared with control. This is contrary to other studies where nicotine has been shown to decrease food intake and body weight in rats (Grunberg et al 1984, Bowen et al 1986). Similar studies also show that cessation of nicotine treatment leads to and increase in food intake and body weight (Levin et al 1987). Recent studies have begun to explore the mechanism behind nicotine's effect on body weight, which may be related to set point (Frankham and Cabanac 2003). Unfortunately, we cannot explain the difference in body weight change between our experimental animals and the literature reports. Certainly, we would need to repeat this experiment in order to confirm our result. Differences in nicotine delivery, as well as gender and species of the animal tested, may play a role in nicotine's effect on body weight.

5.3 Aortic Contractility

We hypothesized that changes in blood pressure due to streptozotocin, nicotine or STZ-N combination treatment occurred due to changes in vascular reactivity. We have observed an increase in the maximum contractile response of aortic tissue to phenylephrine due to both streptozotocin and STZ-N combination treatment. Although we have not clearly demonstrated why hypertension exists in the STZ-N combination group, and not the STZ

group, we feel this phenomenon is dependent upon the combination of the two stressors. We also observed a decrease in the sensitivity to sodium nitroprusside due to the nicotine treatment in aortic tissues. Also, the sensitivity to sodium nitroprusside due to STZ-N combination treatment decreased slightly, although not significantly, in aortic ring segments. Previous literature has reported that cigarette smoke (Mayhan and Sharpe 1996), nicotine treatment (Mayhan and Sharpe 1999a), and diabetes mellitus (Johnstone et al 1993) lead to impairment of endothelium-dependent vasodilation. Although our result is somewhat consistent with these previous reports, we found no alteration of acetylcholine induced relaxation of vascular tissues, but did see an alteration of sodium nitroprusside induced relaxation. This leads us to speculate that perhaps the mechanism of altered vascular reactivity in our model is due to alteration of vascular smooth muscle and not the vascular endothelium. Whatever the mechanism, it seems quite likely that damage to vascular tissues, and resulting altered vascular reactivity, is part of the underlying mechanism of chronic blood pressure change in a combination STZ/nicotine treatment model.

5.4 Mechanism for Changes in Aortic Contractility

Our result shows an increase in blood pressure, as well as changes in vascular reactivity to certain compounds, in animals treated with STZ-N combination. Therefore, we speculate that changes in blood pressure due to the STZ-N combination treatment occur, at least in part, due to altered peripheral vascular reactivity. An increased tendency for vascular contraction seems to be evident in our contractility result. Although we still do not know the specific mechanism associated with this increased vascular contraction, our

result allows us to speculate about a few potential pathways which could serve as a basis for further experimentation.

Both PHE and SNP concentration-effect relationships suggest an increase in tissue contraction, whereas the result of our ACh data is normal. This suggests that an increase in tension in these tissues may not be a result of modifications to the normal relaxation pathways governed by the release of NO from vascular endothelial cells. Therefore, given that the normal relaxation response does not seem to be affected, one could speculate that the changes seen here are due to enhanced response of the contractile elements. However, it is also possible that our treatment had an effect on the nature of the interaction between the drugs we selected and vascular tissues.

5.4.1 Increased EC_{50} of SNP

An increase in the EC_{50} of SNP in rat thoracic aorta, in the absence of any change in the ACh EC_{50}, is difficult to explain. The reason these two vasorelaxants are used together is usually to elucidate whether or not a change in the relaxation of vascular tissues is due to either the endothelial or vascular smooth muscle component of the tissue. Logically, if there were an increase in the EC_{50} response of ACh and SNP, this would suggest that impaired relaxation is occurring at the level of the vascular endothelial cells and/or smooth muscle cells. An increase in the EC_{50} of ACh with no change in SNP would suggest that impaired relaxation is occurring due to a problem with vascular endothelial cells. As indicated, our result shows a change in SNP induced relaxation without an accompanying change in ACh induced relaxation. This change in SNP response could potentially be due to how it functions as an NO donor, particularly with respect to our

experimental system. There are at least two possibilities that might explain our result related to SNP as an NO donor.

The first possibility is that the liberation of nitric oxide from SNP is affected in our experimental system. Bates et al (1991) showed that production of NO from SNP is dependent upon light exposure, and is promoted by the presence of vascular tissue. Bates et al (1991) also showed that the effect of vascular tissue to promote NO liberation from SNP could be mimicked by reducing agents. Because our result indicated that the EC_{50} of SNP was decreased, this may suggest that the reducing properties of the vascular tissue of our experimental rats are somehow affected by treatment with nicotine. Therefore, we may have stumbled upon an indirect method of uncovering nicotine's role in the oxidative status of vascular smooth muscle tissue. Furthermore, we cannot draw any definite conclusions as to the EC_{50} of NO in these experimental tissues. Further studies would do well to test alternate NO donors in order to attribute our result to the NO releasing properties of SNP.

The second possibility is that the NO that is liberated from SNP is less effective than that which is produced from vascular endothelium in response to an ACh stimulus. There is weak evidence in the literature that suggests that this might be possible. In a 2001 review article on the cardiac effects of NO, Sarkar, Vallance and Harding (2001) point out that the NO species generated by different NO donors are not always the same. Sarkar and colleagues (2001) further state that the different forms of NO (namely $NO^{\bullet}$, NO^{-} and NO^{+}) have the potential to react differently with other biomolecules. The species most commonly associated with release from SNP in vascular tissues is the uncharged free radical ($NO^{\bullet}$) form (Bates et al 1991). However, it is possible that the major species

released from SNP could be shifted depending on the redox state of the surrounding tissues. Therefore, the redox state of vascular tissue will potentially change the amount, and the type of NO being liberated from SNP. This could certainly have an effect on the amount (EC_{50}) of SNP required to produce the same level of relaxation in tissue that has not been treated with nicotine or D-N combination.

We speculate that our SNP result is not a product of the tissue's sensitivity to NO, but rather, due to a change in the redox state of vascular tissues due to nicotine treatment. Such a change in redox state could affect both the amount and kind of NO being liberated from a donor such as SNP. The use of another NO donor with a much different mechanism of release could help us to clarify whether or not our result is due to altered contractility, or altered release of NO from SNP. However, if it can be demonstrated that nicotine leads to changes in the redox state of vascular tissue, this could serve as a basis for nicotine's role in the development of hypertension due to D-N combination treatment.

5.4.2 Increased E_{max} of PHE

From our result we see that the maximum ability of PHE to contract vascular smooth muscle tissue is increased in both treatment models that included STZ treatment. Although we are still uncertain as to precise mechanism of this change, we will consider one possibility that has some support in the literature. The demonstrated ability of hyperglycaemia to induce PK-C was discussed in Chapter 1. This phenomenon could be resulting in vascular remodeling, which could account for the change in the response of rat thoracic aorta to PHE.

Vascular remodeling may account for a change in the response of vascular tissues to PHE. Cunha et al. (2000) observed that the one-kidney, one-clip (1K1C) model of

hypertension lead to hypertrophy of aortic tissue, and an increase in sensitivity to PHE. Cunha and colleagues (2000) also report that under the same conditions there was no change in the aortic response to either ACh or SNP induced relaxation. If we suppose that our own SNP result is not attributed to the function of aortic tissues, but rather due to decreased NO liberation as previously discussed, then our result might be comparable to that of Cunha and colleagues (2000). If we consider that the response of aortic tissue to PHE was altered in both of our treatment models that included STZ treatment, it is plausible to hypothesize that part of the mechanism of hypertension in this model is due to vascular remodeling of arterial tissue. Although our result differs from Cunha and colleagues (2000) in that we saw a change in the maximum effect of PHE, not the EC_{50} (sensitivity), it is possible that the precise mechanism of functional change is different in our model.

As discussed in Chapter 1, it has been demonstrated that hyperglycaemia leads to the production of ROS, and subsequently to the induction of PK-C (Koya and King 1998). Further to this, vascular remodeling is associated with induction of PK-C (Koya and King 1998). This evidence would suggest that both vascular remodeling occurs due to the induction of PK-C. This proposed mechanism may constitute the contribution that hyperglycaemia has in the development of hypertension in our STZ-N combination group.

5.5 Future Directions

It may also be possible that other metabolic stressors play a part in the development of chronic elevation of blood pressure in this model. Future studies should attempt to investigate whether different stressors combine with a diabetic model to accelerate

cardiovascular pathology. Furthermore, assaying for other markers of physiological stress, such as cortisol, could help to understand the role that non-specific stress has on the development of cardiovascular pathology in our model.

We would also like to confirm whether or not the combination of nicotine and STZ lead to a greater increase in plasma glucose concentration as compared with STZ treatment alone. We have observed a potential trend in the worsening of plasma glucose level due to STZ-N combination treatment as compared with a STZ only treatment. If true, the worsening of plasma glucose level as a result of the combination of STZ and nicotine treatment could serve as a basis for the worsening of cardiovascular pathology of diabetes mellitus. A phenomenon like this might help to confirm the theory that hyperglycemia and nicotine act via very similar pathways to damage vascular tissues. More work is needed to repeat these treatment groups and increase the sample size for plasma glucose measurement.

We would like to further investigate the cellular and molecular mechanisms of the STZ + nicotine combination on chronically elevated systolic blood pressure. Our evidence suggests that changes in vascular reactivity might be involved in at least part of this mechanism. Vascular contractility studies should be designed to test the effect of biochemical agents suspected to play a role in diabetic cardiovascular disease. More importantly, experiments should be designed to investigate whether the effects of streptozotocin and nicotine to increase blood pressure are concentration dependent. Further to this, if the STZ + nicotine treatment model is repeated, special care should be given to ensure that sample size is sufficient enough to avoid type II statistical error. It may also be advantageous to design the experiment according to a true two-way (factor)

analysis in order to statistically test for interaction between our two independent variables.

5.6 Summary

We have shown that a combination of STZ and nicotine will lead to a chronic elevation of systolic blood pressure in the rat. Although we still do not know the precise mechanism of this interaction, our results suggest that altered vascular reactivity may contribute to blood pressure change in our model. More work needs to be done to confirm this treatment model, and further characterize its metabolic features and changes to vascular reactivity.

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