INOS MEDIATES INCREASED RHOA EXPRESSION AND ALTERED CELL SIGNALING IN DIABETIC CARDIOMYOPATHY.

by

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ABSTRACT

The RhoA/ROCK pathway is a well established signaling pathway which regulates cellular contractility. Previous studies from this lab have shown that acute inhibition of ROCK improves heart function in 12 week STZ-diabetic rats. Here we wished to determine, 1) whether the RhoA/ROCK pathway was upregulated in the diabetic rat heart, 2) what factors may be contributing to altered activity of this pathway during diabetes, and 3) determine the consequences of the altered RhoA/ROCK signaling might be.

RhoA expression was significantly elevated in hearts and cardiomyocytes from rats with STZ-induced diabetes. Our preliminary studies also showed elevated iNOS expression in hearts from rats with STZ-induced diabetes. We hypothesized that NO from iNOS mediates elevated RhoA expression. This hypothesis is supported by a number of our observations. Elevated iNOS expression and activity was observed to be concomitant with increased RhoA expression in diabetic rat hearts. In cultured cardiomyocytes exposure to the NO donor, SNP, and the iNOS inducer, LPS, caused elevated RhoA expression. The iNOS inhibitor, L-NIL, blocked increased RhoA expression in LPS treated cardiomyocytes and in hearts from diabetic animals.

Increases in RhoA expression correlated strongly with increased levels of active RhoA in diabetic cardiomyocytes. We also observed increased phosphorylated LIMK, a marker for activation of the RhoA/ROCK pathway. LIMK phosphorylation was reduced to levels similar to control after chronic treatment of diabetic rats with L-NIL, suggesting that iNOS also contributes to increased activity of this pathway in the STZ-diabetic rat heart.
The actin cytoskeleton is a well established downstream target of the RhoA/ROCK pathway. The level of polymerized actin in diabetic rat cardiomyocytes was found to be significantly elevated, but was normalized after acute ROCK inhibition. Given this observation, we suggest that normalization of actin polymerization may contribute to ROCK inhibitor-mediated improvement of contractile function of hearts from diabetic rats.

The findings presented in this thesis indicate a central role for iNOS in the upregulation of the RhoA/ROCK pathway, which is believed to contribute to impaired contractility in the diabetic heart. Our findings also support the suggestion that ROCK is an excellent therapeutic target in the treatment of diabetic cardiomyopathy.
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>+dP/dT</td>
<td>maximal rate in rise of developed pressure</td>
</tr>
<tr>
<td>-dP/dT</td>
<td>maximal rate of decline in developed pressure</td>
</tr>
<tr>
<td>μ</td>
<td>micro (1 x 10^-6)</td>
</tr>
<tr>
<td>μm</td>
<td>micron (1 x 10^-6 metre)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>[Ca^{2+}]</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>inhibition constant, concentration for half maximal inhibition</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>L-NIL</td>
<td>L-N6-(1-iminoethyl)lysine</td>
</tr>
<tr>
<td>LVDP</td>
<td>left ventricular developed pressure</td>
</tr>
<tr>
<td>m</td>
<td>milli (1 x 10^-3), if used as a prefix</td>
</tr>
<tr>
<td>min</td>
<td>minute/s</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Y-27632</td>
<td>(R)-(+)<del>trans</del>4~-(1<del>Aminoethyl)<del>N</del>-(4</del>Pyridyl)~ -cyclohexanecarboxamide dihydrochloride</td>
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Thanks to my parents, for supporting my move to Canada and for all your advice and love.
1.0 INTRODUCTION

The last decade has seen growing public awareness of the prevalence of diabetes mellitus in the western world. A number of startling statistics and projections are in part responsible for this growing concern. Recent estimates suggest that 6.5% of the U.S. population have diabetes (American Diabetes Association, 2005). The number of people worldwide with diabetes, which was estimated to be around 135 million people in 1995, is projected to increase to 300 million in 2025 (King et al., 1998). Compared to many other diseases, the treatment of diabetes consumes a disproportionate share of health care resources (Winer and Sowers, 2004). This is because diabetic patients show heightened risk of development of a number of different medical complications, including retinopathy, nephropathy, neuropathy and cardiovascular disease. Of these complications cardiovascular disease (CVD) is the biggest cause of mortality in patients with diabetes. It is believed that a typical diabetic patient has an increased risk of development of cardiovascular disease by a factor of up to four times that of their healthy counterpart (Haffner, 2000). Cardiovascular diseases such as atherosclerosis, coronary artery disease and cardiac hypertrophy can ultimately lead to heart failure. In fact, heart failure is cited as the cause of death in 65% of people with diabetes (Geiss et al., 1995). Therefore there is tremendous interest in research that may help us to understand the factors contributing to the development of CVDs, and why diabetic patients in particular show increased risk of development of cardiovascular complications which can ultimately lead to heart failure.
1.1 Diabetes Mellitus.

Diabetes mellitus is a group of disorders characterized by persistent aberrations in carbohydrate, protein and lipid metabolism, as a result of impaired insulin action and/or insufficient insulin production. This is a metabolic disorder that requires medical diagnosis, treatment and lifestyle changes. It has been suggested that the history of diabetes can be traced to as far back as 1552 B.C. when the 3rd Dynasty Egyptian physician Hesy-Ra documented on papyrus a number of symptoms of diabetes (Canadian Diabetes Association, 2006). Diabetes was first specifically described by the ancient Greek physician Aretaeus, who in around 70 A.D. named the disease ‘Diabetes’. This name derived from an ancient Greek term meaning ‘passing through’ or ‘siphon’, a reference to one of diabetes’ major symptoms: polyuria (excessive urine production). In 1670 Thomas Willis, physician to King Charles, added ‘mellitus’, from the Latin word for honey, after he noted that a person with diabetes had urine and blood that had a sweet taste and aroma.

The discovery of the role of the pancreas in diabetes is generally ascribed to Joseph von Mering and Oskar Minkowski, who showed that pancreatectomized dogs developed hyperglycemia, and died shortly after surgery (Von Mehring and Minkowski, 1890). The Canadian researchers Sir Frederick Grant Banting and John James Richard Macleod expanded upon the work of Von Mering and Minkowski, when they demonstrated that they could reverse pancreatectomy-induced diabetes in dogs by giving them extracts from healthy pancreatic islets of Langerhans (Banting et al., 1922). Banting and MacLeod and their colleagues, went on to isolate the hormone insulin from bovine pancreata. In recognition of these findings Banting and MacLeod were awarded
the Nobel prize for medicine in 1923. The increased world-wide availability of insulin for the treatment of diabetes led to the distinction of insulin dependent and non insulin dependent forms of diabetes (Himsworth, 1936). The main forms of diabetes are now well identified and distinguished and are known as type 1 and type 2 diabetes.

1.1.1 Classification of Diabetes.

The clinical symptoms of diabetes are the primary factors used in the classification of this disease. Type 1, formerly known as insulin dependent diabetes mellitus (IDDM), makes up 5-10% of all diagnosed cases and is associated with insulin deficiency. It has been shown that Type 1 diabetes is the outcome of selective ablation of the insulin-producing β-cells of the pancreas (Foulis and Stewart, 1984). It is widely accepted that a T-cell mediated, immunological response is involved in the pathogenesis of type 1 diabetes. This is supported by histological examination of the pancreata from children at the onset of the disease and from a number of animal models (Bertera et al., 1999). However, despite this evidence, the precise mechanism triggering this immunologic β-cell loss is yet to be established and remains subject to debate. Studies have suggested that viral infection can act as an initiator of type 1 diabetes (Yoon et al., 1979), and infection of primates with Coxsackie viruses has been shown to induce abnormalities in glucose homeostasis (Yoon et al., 1986).

Despite lack of a specific mechanism describing the initiation of type 1 diabetes, evidence strongly supports the autoimmune nature of the pathogenesis of type 1 diabetes. Serum from patients with type 1 diabetes tests positive for antibodies raised specifically against pancreatic islet cells (Pietropaolo et al., 1998), temporary immunosuppression can delay the onset of type 1 diabetes (Bach, 1993), and there is
evidence that type 1 diabetes can be transmitted to non-diabetic patients via bone marrow transplants (Lampeter et al., 1993).

Type 2 diabetes represents 90-95% of all diagnosed cases. It is widely believed that type 2 diabetes develops in response to over-nutrition and an associated insulin resistance (see review by Prentki and Nolan, 2006). The normal β-cell response to over-nutrition and obesity-associated insulin resistance is compensatory insulin hypersecretion in order to maintain normoglycemia. Insulin hypersecretion leads to hyperinsulinaemia. Over time, the compensatory β-cell hypersecretion fails, resulting in a progressive decline in β-cell function. As a consequence, subjects progress to hyperglycemia and established type 2 diabetes. A number of different factors have been associated with a heightened incidence of type 2 diabetes within certain populations. It has been proposed that genetic predisposition, environmental factors, lifestyle choices and ethnicity may influence an individual's risk of acquiring type 2 diabetes (Pimenta et al., 1995).

A comprehensive explanation of the pathology of type 1 and type 2 diabetes is yet to be established. In order to further our understanding of the mechanisms and complications of diabetes mellitus further experimentation using appropriate animal models of diabetes are required.

1.1.2 The streptozotocin-induced diabetic rat model.

As described above, the historical experiments of von Mering demonstrated that surgical removal of the pancreas from an animal served as a useful model of diabetes mellitus. Since then non-surgical methods of inducing hyperglycemia by damaging the pancreas have been developed. This includes the administration of toxins such as
streptozocin (STZ). STZ was initially indicated as broad spectrum antibiotic isolated from the organism *Streptomyces achromogenes* (Vavra et al., 1959). Subsequently it was found that STZ was also a potent and selective toxin causing necrosis of pancreatic β-cells. This highlighted the potential use of the drug in animal models of diabetes (Mansford and Opie, 1968) and as a medical treatment for pancreatic carcinomas (Murray-Lyon et al., 1968).

The chemical structure of STZ consists of a glucose moiety conjugated to a nitrosourea side chain. The uptake of STZ by pancreatic β-cells is mediated by the glucose transporter GLUT2. Reduced expression of GLUT2 has been found to prevent the diabetogenic action of STZ (Schnedl et al., 1994, Thulesen et al., 1997). Once taken up by the pancreas, the highly reactive nitrosourea side chain of STZ is believed to mediate its cytotoxic effect. However, the exact mechanism by which STZ exerts its specific and selective toxic effect on β-cells remains a contentious topic. Studies have suggested that STZ-induced β-cell death is primarily as a result of DNA damage (Delaney et al. 1995; Elsner et al. 2000). It has been demonstrated that STZ is a powerful alkylating agent which can induce multiple DNA strand breaks (Bolzan and Bianchi, 2002). In support of this proposal previous studies have shown increased purine methylation in pancreatic tissue from STZ-diabetic rats (Bennett and Pegg, 1981). It has also been proposed that nitric oxide production (Kwon et al., 1994; Haluzik and Nedvidkova, 2000) and free radical generation (Oberley, 1988) also contribute to β-cell death after STZ administration.

When administered to rats, STZ’s action in β-cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Two hours after injection, hyperglycemia is observed with a concomitant drop in blood insulin. About six
hours later, hypoglycemia occurs with high levels of blood insulin. Within 24-48 hours, hyperglycemia develops and blood insulin levels decrease (Junod et al., 1969). This hyperglycemic and hypoinsulinemic effect is associated with the eventual onset of hyperlipidemia (Smith and Novotny, 1980). Thus this diabetic rat model demonstrates three of the classic hallmarks of untreated type 1 diabetes. The feasibility of using this model in chronic studies was assessed by Ar’Rajab and Ahren (1993). In a three month study it was shown that administration of STZ at doses exceeding 40 mg/kg resulted in a long-term, stable hyperglycemia. At these doses glucose-induced insulin release remained undetectable for the duration of the study. The STZ rat has been widely adopted as an experimental model for chronic, untreated, type 1 diabetes, and its use has been well reviewed by Rodrigues et al., (1999).

1.1.3 Diabetic cardiomyopathy.

Although the discovery of insulin eliminated many acute complications of diabetes and prolonged the life of many patients with diabetes, it unfortunately highlighted a number of chronic complications in these patients. Cardiomyopathy progressing to heart failure is one of the most common chronic complications observed in diabetic patients (Mahgoub, 1998). The first description of diabetic cardiomyopathy was reported over thirty years ago (Rubler et al., 1972). This complication is characterized by impaired cardiac function and can be detected in the absence of hypertension and ischemic heart disease. Subsequent to the initial description of diabetic cardiomyopathy numerous epidemiological, animal, and clinical studies have supported the existence of diabetic cardiomyopathy as a distinct complication of diabetes (Hamby et al., 1974; Regan et al., 1977; Mizushige et al., 2000). Diabetic cardiomyopathy is associated with a series of morphological, biochemical and functional
abnormalities which are seen in both human patients and animal models of both type I and type II diabetes (Young et al., 2002; Taegtmeyer et al., 2002). Cardiac abnormalities, including diastolic dysfunction, manifested by decreased compliance, prolonged myocardial relaxation and impaired left ventricular filling, as well as an increased number of supraventricular and ventricular premature beats can be detected early in the course of diabetes in both humans and animal models (Dent et al., 2001; Mihm et al., 2001; Schanwell et al., 2002). Direct evidence of impaired cardiac function has been obtained both in vivo and in vitro. Echocardiography studies have shown that hearts from diabetic rats have impaired fractional shortening (an index of contractility) and defects in systolic and diastolic function (Mihm et al., 2001; Dent et al., 2001; Lin et al., unpublished data). Impaired contractility has also been demonstrated in the isolated whole heart and in isolated cardiomyocytes (Horackova et al., 1988; Fein et al., 1990), indicating that altered contractile function is due to changes occurring at the cellular and molecular level.

1.1.3.1 Biochemical and pathological changes in the heart during diabetes.

A series of biochemical and physiological abnormalities at the level of the heart, occurring during diabetes, have been established. These changes may contribute to the associated impaired cardiac performance. For example, alterations in ion homeostasis have been demonstrated in the diabetic heart, particularly alterations in calcium handling have been observed. These alterations include: impaired SR calcium release (Cannell et al., 1995; Choi et al., 2002), decreased expression of calcium release channels (Guner et al., 2004) and decreased expression of the SR Ca\(^{2+}\)-ATPase or Ca\(^{2+}\) pump (SERCA2) (Zhong et al., 2001). Any alterations in the regulation of the
intracellular calcium concentration would have the potential to impact dramatically on cardiac contractility. Na\(^+\)-K\(^+\)-ATPase activity has also been shown to be depressed and the depression appears to correlate with depressed atrial contractility (Tahiliani and McNeill, 1986). There is also evidence of altered regulation of contractile proteins in the diabetic heart. Several studies have demonstrated that myosin isoform expression shifts from V\(_1\) to V\(_3\) in diabetic rat hearts (Malhotra et al., 1981; Dillmann et al., 1982; Malhotra et al., 1985). These myosin isoforms differ in their ATPase activity, velocity of contraction and energy requirements (d'Albis et al., 1979; Schwartz et al., 1985). It has been suggested that very small myosin isoform shifts could result in marked changes in myofibrillar activity and shortening velocities of cardiac muscle (Malhotra and Sanghi, 1997).

1.1.3.2 Metabolic changes in the heart during diabetes.

Insulin insufficiency or insulin resistance results in compromised glucose utilization during diabetes. This change, along with an increase in circulating free fatty acids (Denton and Randle, 1964), results in a switch in cardiac energy metabolism in the heart during diabetes. Increases in fatty acid oxidation accompanied by decreases in glucose uptake and metabolism during diabetes are well established (Stanley et al., 1997; Lopaschuk, 2002). In fact, in the diabetic state energy supply to the heart is almost exclusively via fatty acids (Rodrigues et al., 1997). For some time it has been believed that this alteration in metabolism contributes to the impaired cardiac performance observed in the diabetic myocardium. Elevated concentrations of free fatty acids have been shown to impair cardiac function in control rat hearts (Henderson et al., 1970). Fatty acids are supplied to the heart via the hydrolysis of lipoproteins. In the STZ diabetic rat heart, increased lipoprotein hydrolysis and lipoprotein lipase activity have
been demonstrated (Sambandam et al., 1999; Pulinilkunnil et al., 2005). Increased fatty acid metabolism is also associated with impaired cardiac function in chronic diabetes (Borradaile et al., 2005). The involvement of abnormal cardiac metabolism in impaired cardiac function is further supported by studies in transgenic mice. Cardiac-specific overexpression of cardiac PPAR-α increases FA uptake and oxidation (Finck et al., 2002). Hearts from these transgenic mice, exhibited a metabolic phenotype similar to diabetic hearts (Finck et al., 2002). This was concomitant with systolic dysfunction and ventricular hypertrophy, suggesting altered cardiac metabolism can induce cardiac contractile dysfunction (Finck et al., 2002). A number of explanations have been proposed to explain how both increased FA metabolism and FA uptake impacts upon cardiac performance during diabetes, however, the exact mechanisms and the relative contribution of these two changes to impaired cardiac function remains unclear. One possibility is that FA metabolism decreases the energy efficiency of the heart compared to glucose metabolism. Oxidation of FA consumes more oxygen than glucose (2.58 vs. 2.33 ATP/oxygen atom). Cardiac efficiency, the ratio of cardiac work to myocardial oxygen consumption, is therefore reduced when FA oxidation is increased. An other effect of increased free fatty acid levels is lipotoxicity. It is believed that lipotoxicity is mediated via the accumulation of fatty acids, which can, either by themselves or via production of second messengers such as ceramides, provoke cell death (Listenberger and Schaffer, 2002). Lipotoxicity has been proposed to play an important role in the development of diabetic cardiomyopathy (Finck et al., 2003). The mechanisms by which lipotoxicity might mediate impaired cardiac function are not clear. It is possible that cell death reduces the number of viable cardiomyocytes in the heart, thus reducing the potential for the heart to generate force.
Interestingly treatment of diabetic rat hearts with agents that promote a shift from fatty acid metabolism to glucose oxidation (Tan et al., 1984; Nicholl et al., 1991) have been shown to acutely improve cardiac function in vitro. This evidence strongly suggests that alterations in metabolism contribute significantly to impaired cardiac function in the diabetic heart. Ultimately, maladaptations in the diabetic heart result in cardiomyopathy and can lead to heart failure (Fein and Sonnenblick, 1985; Sander and Giles, 2003). Although a number of factors have been identified, the biochemical basis of diabetic cardiomyopathy remains incompletely understood.

At the cellular level a group of proteins called Rho-GTPases have been studied for a number of years, and are well established as regulators of cell morphology and cellular contractility. These proteins have been implicated in the development of a number of cardiovascular complications including hypertension, atherosclerosis, and hypertrophic heart failure (Ren et al., 2005; Brown et al., 2006), and may therefore play a role in the pathology of diabetic cardiomyopathy.

1.2 The Rho-GTPases.

Rho GTPases are members of the Ras superfamily of monomeric 20-30 kDA GTP-binding proteins (Bishop and Hall, 2000). The first Rho family G-proteins were identified in the sea slug Aplysia (Madaule, 1988). Twenty two Rho family members have so far been identified (Wherlock and Mellor, 2002), which can further be subdivided into 8 subclasses. However, most research work has focused on the three members, Rac1, RhoA and Cdc42. This is most likely due to the fact that they were the first G-proteins to be described as playing an important role in the morphological responses of cells to extracellular stimuli around 14 years ago (Ridley and Hall, 1992).
The RhoGTPases are molecular switches that function to control complex cellular processes. Rho cycles between two conformational states (figure 1). In its active state guanosine-triphosphate (GTP) is bound. In its inactive state, guanosine-diphosphate (GDP) is bound. Once active, Rho G-proteins have the ability to bind target molecules and modulate the activity of these targets. Rho G-proteins possess intrinsic GTPase activity and will generate a response until GTP hydrolysis returns the G-protein to its inactive state (with GDP bound). The activity of Rho GTPases is highly modulated by several accessory proteins (figure 1). The binding and intrinsic hydrolysis of GTP is regulated by guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. In addition Rho can also be bound by guanine-nucleotide-dissociation inhibitors (GDIs), which alter activity by preventing the exchange of GDP for GTP, keeping Rho in an inactive state (Aspenstrom et al., 2004). The general mechanism of activation of Rho is well established. The inactive form of Rho (Rho.GDP) is present in the cytosol bound to a GDI. Receptor-induced activation of Rho can be mediated by Gαq and/or Gα13-coupled receptors via various RhoGEFs, which promote the exchange of GDP for GTP (Sahai and Marshall, 2002).

RhoA in particular has received much research attention. A vast number of studies have shown the importance of RhoA in regulating signaling cascades which affect cellular contractility (Hall, 1998; Nobes and Hall, 1999; Fukata and Kaibuchi, 2001; Etienne-Manneville and Hall, 2002; Somlyo and Somlyo, 2003; Aspenstrom et al., 2004). For some time, the literature has been unclear when discussing the role of the Rho proteins. Three subclasses of Rho G-proteins: RhoA, RhoB and RhoC have now been identified (Wheeler and Ridley, 2004). These three GTPases share 85% amino acid sequence identity, but despite their similarity,
Receptor Stimulus

GEF

Aktive

RhoA-GDP

Sequestration

Rho-GDI

GeRhoA-GDP

Inactive

RhoA-GTP

ROCK

GAP

Cytoskeletal Regulation

Figure 1: Rho GTPase regulation. GEFs promote the exchange of GDP for GTP; GDIs interact with GDP-bound Rho and inhibit exchange of GDP for GTP; GAPs increase the intrinsic GTPase activity of Rho GTPases. (adapted from: Nature Reviews Cancer 2; 133-142 [2002]).
regulators and effectors show preferential interaction with each of the three species, RhoA, B, or C. The three proteins exhibit distinct functions in cells (Wheeler and Ridley, 2004; Aspenstrom et al., 2004).

1.2.1 The cellular functions of RhoA.

The well-established functions of RhoA highlight this protein as an important target for research when investigating the molecular basis of complications such as cardiomyopathy, where cardiomyocyte contractility is significantly impaired. It has been shown that RhoA plays an important role in the regulation of a number of dynamic cellular processes including: fatty acid metabolism (Pulinilkunnil et al., 2005), hypertrophic responses to stimuli (Torsoni et al., 2005), regulation of intracellular Ca\(^{2+}\) concentration (Suematsu et al., 2002; Yatani et al., 2005), Ca\(^{2+}\) sensitization (Somlyo, 2002) and regulation of the actin cytoskeleton (Ridley and Hall, 1992b; Nobes and Hall, 1995).

1.2.1.1 RhoA and the actin cytoskeleton.

Actin is a structural protein that is a vital component of the cardiomyocyte cytoskeleton. The individual subunits of actin are known as globular actin (G-actin). G-actin subunits polymerise to form a filamentous polymer (F-actin), also known as a microfilament. These microfilaments form the cytoskeleton, a three dimensional network found inside eukaryotic cells. The cardiomyocyte cytoskeleton provides structural support and compartmentalisation of intracellular components (Watson, 1991) as well as regulating intracellular vesicular transport and protein synthesis (Rogers and Gelfand, 2000). In both neonatal and adult cultured myocytes, distinct sarcomeric and costameric (cytoskeletal) actin have been identified (Sadoshima et al., 1992; Messerli and Perriard 1995; Larsen et al., 1999 and Larsen et al., 2000). Here we will focus
solely upon cytoskeletal actin, the functions it plays in the cardiomyocyte and regulation of the actin cytoskeleton by RhoA. The ability of the actin cytoskeleton to modulate both electrical and mechanical activity of the cardiomyocyte has been well reviewed (Calaghan et al., 2004). The microfilaments have been implicated in regulating the concentration of intracellular Ca\(^{2+}\) (Lange and Brandt, 1996), a key regulator of cardiomyocyte contractility (Bers and Guo, 2005). Lange and Brandt (1996) suggest that actin filaments can bind Ca\(^{2+}\) with high affinity and that actin polymerisation renders bound Ca\(^{2+}\) less available, effectively creating a Ca\(^{2+}\) store. This is supported by their evidence showing that the F-actin stabilizing agent, phalloidin, strongly inhibited ATP-dependent Ca\(^{2+}\) uptake and reduced the IP3-sensitive Ca\(^{2+}\) pool by 70%. Conversely, when actin filaments were depolymerised, for example following treatment with Cytochalasin-D, free Ca\(^{2+}\) was elevated from 50 nM to 500 nM (Lange and Brandt, 1996).

Evidence is emerging on the role played by the actin cytoskeleton in the regulation of plasma membrane Ca\(^{2+}\)-permeable channels as well as the intracellular calcium concentration. Disruption of the cytoskeleton by actin depolymerizing agents inhibits hormone-induced Ca\(^{2+}\) release from InsP3- and ryanodine-sensitive stores in NIH 3T3 cells (Ribiero et al., 1997), AR4-2J cells (Bozem et al., 2000), and hepatocytes (Wang et al., 2002). Moreover, actin depolymerization has been reported to reduce Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (Rueckschloss et al., 2001), while store-operated Ca\(^{2+}\) influx through transient receptor potential (TRP) ion channels is affected by both actin polymerization and depolymerization (Rosado and Sage, 2001; Wang et al., 2002). In cardiomyocytes increased actin polymerization has been associated with
decreased contractility due to disruption of intracellular Ca\textsuperscript{2+} release (Davani et al., 2004).

There is also evidence to suggest that in cardiomyocytes the actin cytoskeleton can regulate the metabolism of fatty acids (Pulinilkunnil et al., 2005), the preferred energy substrate of the heart (Randle et al., 1963; Rodrigues et al., 1995). Elevated levels of polymerised actin were shown to be associated with increased lipoprotein lipase activity in rat cardiomyocytes. This effect was blocked by actin depolymerising agents (Pulinilkunnil et al., 2005), strongly suggesting a role for the actin cytoskeleton in the regulation of fatty acid metabolism. Given the number of observations linking the actin cytoskeleton with factors known to influence myocardial contractility, it is not surprising that a number of studies have implicated the involvement of cytoskeletal abnormalities in conditions of impaired cardiac performance such as cardiomyopathy and heart failure (Hein et al., 2000; Kostin et al., 2000; Pyle, 2004).

RhoA and it’s downstream targets are known to be of great importance in maintaining the state of cellular actin polymerization (figure 2). Rho-kinase (ROCK), a well-known downstream target, which is directly activated by RhoA, includes two isoforms, ROCKI (also known as p160ROCK or ROKb) and ROCKII (ROKa) (Nakagawa et al., 1996). ROCK activity can be selectively blocked by the competitive inhibitor Y-27632, as well as the isoquinoline sulfonyl derivative HA1077 and its more potent and selective derivative, H1152 (Uehata et al., 1997; Sasaki et al., 2002; Breitenlechner et al., 2003). ROCK is a serine/threonine kinase which phosphorylates and activates its downstream target, LIM kinase, which in turn directly phosphorylates cofilin on the N-terminal Ser3 residue (Arber et al., 1998; Bamburg, 1999). Cofilin is an actin regulatory protein that plays a critical role in regulating actin filament dynamics via
its actin severing and depolymerizing activity (Huang et al., 2006). The phosphorylated form of cofilin is unable to bind and depolymerise F-actin (Agnew et al., 1995). In this way ROCK, via LIM kinase and Cofilin, inhibits actin depolymerisation, thus stabilizing filamentous actin.

A functional RhoA/ROCK/LIM-kinase pathway has been shown to be required for actin polymerization in lymphocytes (Lou et al., 2001) and in rat cardiomyocytes (Zeidan et al., 2006). Activation of RhoA, has been shown to result in significantly increased levels of polymerized actin in rat cardiomyocytes (Pulinilkunnil et al., 2005; Zeidan et al., 2006).
Figure 2: Mode of action of Rho/ROCK in cytoskeletal reorganization and cellular contractility. [Taken from: Takai et al., (2001)]
1.2.2 A pathological role for the RhoA/ROCK Pathway?

The RhoA/ROCK pathway is an important regulator of physiological function (Narumiya, 1996; Burridge and Warnerberg, 2004), and is believed to play a role in the pathophysiology of many diseases, including hypertension (Chitaley and Weber, 2001), myocardial hypertrophy (Kobayashi et al., 2001) and heart failure (Hu and Lee, 2003). The development of specific ROCK inhibitors has allowed extensive studies aimed at determining potential pathological roles for this signalling pathway. ROCK activity can be selectively blocked by the competitive inhibitor Y-27632, as well as the isoquinoline sulfonyl derivative HA1077, and its more potent and selective derivative, H1152 (Sasaki et al., 2002; Breitenlechner et al., 2003). Treatment with ROCK inhibitors has been shown to effectively reduce blood pressure in rats with spontaneous or angiotensin II-mediated hypertension (Uehata et al., 1997; Mukai et al., 2001), and in hypertensive patients in a clinical trial (Masumoto et al., 2001). The importance of Rho-mediated signalling in cardiac contractility was illustrated by Kobayashi et al., (2002). In this study Dahl salt-sensitive hypertensive rats were chronically treated with the specific ROCK inhibitor Y-27632. Inhibition of this downstream signaling target of RhoA resulted in significant improvement in cardiac function and helped prevent progression to heart failure.

A role for the RhoA/ROCK pathway in cardiac hypertrophy is supported by a number of studies. Mutational deletion of ROCK was shown to prevent pressure overload-induced hypertrophy in a mouse knock-out model (Zhang et al., 2006). Furthermore activation of RhoA by LPA has been shown to induce hypertrophy in cultured rat cardiomyocytes (Hilal-Dandan et al., 2004). This effect was blocked by the RhoA inhibitor C3-exoenzyme (Hilal-Dandan et al., 2004; Wei, 2004).
Increased activation of the RhoA/ROCK pathway has been demonstrated in vascular (Tang et al., 2006) and erectile tissue from animal models of type 1 and type 2 diabetes (Chang et al., 2003), but whether this also occurs in the heart and contributes to contractile dysfunction is not yet known. However, previous work in this lab has demonstrated that acute inhibition of ROCK with the inhibitors Y-27632 and H-1121 significantly improves cardiac function in 12 week STZ diabetic rats (unpublished data). This has been demonstrated both in vitro using isolated, perfused working hearts (figure 3) and in vivo using echo cardiography (figure 4).
Figure 3: Effect of Y-27632 on the function of isolated working hearts from control and diabetic rats. Control (black squares), control treated with inhibitor (black triangles), diabetic (white squares), and diabetic treated with inhibitor (white triangles). Hearts were perfused with $10^{-6}$ M Y-27632 (n=7 in each group), # significantly different from the diabetic treated with the inhibitor. *, P< 0.05 significantly different from all other groups. @ Three of seven diabetic hearts failed at this LAFP. [Taken from Lin et al., unpublished data].
Figure 4: Effect of H-1152 on percent fractional shortening (% FS) of control and diabetic left ventricle. %FS determined by transthoracic echocardiogram in vivo. Control before (C), control following treatment (C+H), diabetic before (D), and diabetic following treatment (D+H). #, significantly different (P<0.05) from control (C); and @, significantly different (P<0.05) from diabetic untreated (D) by repeated measure ANOVA followed by Newman-Keuls test (n=6 in each group). [Taken from Lin et al., unpublished data].
1.3 Nitric oxide and the heart.

Nitric oxide (NO) is a gaseous product generated from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). Three forms of NOS have so far been identified; endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS). nNOS and eNOS are constitutively active isoforms with calmodulin binding (and, consequently, enzyme activity) regulated by physiological \([Ca^{2+}]\), (Kelly et al., 1996). Unlike the other isoforms, iNOS activity is independent of \([Ca^{2+}]\), and is regulated primarily at the transcriptional level. Once expression is induced iNOS continuously produces NO until the enzyme is degraded (MacMicking et al., 1997). For some time NO has been regarded as an important bioregulatory molecule and its roles in the nervous, immune and cardiovascular systems have been well reviewed (Anggard, 1994; Blaise et al., 2005). It is well established that NO plays a regulatory role in the modulation of coronary vessel tone, thrombogenicity, and proliferative and inflammatory properties as well as the cellular cross-talk supporting angiogenesis. Numerous studies have suggested that NO is an important, indirect regulator of cardiac function (see review by Kelly et al., 1996). The recognition that all three isoforms of NOS are expressed in cardiac myocytes (Ballingand et al., 1994; Feron et al., 1996; Elfering et al., 2002) has raised several intriguing questions regarding the role of NO in the heart, and whether direct effects on myocardial contractility might exist. Studies in the past decade have provided extensive evidence to suggest that NO can in fact directly modulate myocardial contractility (Brady et al., 1992; Balligand et al., 1993; Massion et al., 2003; Belge et al., 2005). The exact mechanisms of these direct effects on myocardial contractility remain unclear. A number of studies have been contentious in that they suggest that NOS or NO can play both
cardio-protective as well as detrimental roles, though this appears to depend on the source of NO and the level of NO output (Cotton et al., 2005). Genetic knock-out models have helped our understanding of the role of the NOS and NO in pathological conditions such as heart failure (see review by Mungrue et al., 2002b). In eNOS knockout mice SR calcium handling is altered and cardiomyocyte contractility reduced (Sears et al., 2003). iNOS knock-out mice show improved cardiac performance and remodelling after myocardial infarction (Sam et al., 2001; Liu et al., 2005). It has also been shown that iNOS knockout mice have better survival rates after cardiac allograft surgery (Koglin et al., 1999). nNOS knockout mice have been shown to have significantly decreased life-span and cardiac performance (Li et al., 2004). It is important to appreciate that these genetic knock-out models may not truly reflect the cellular functions of the targeted protein or its product. Attributing physiological relevance to observations from knock-out models is difficult. Other experimental techniques have been adopted to assess the roles of the NOS isoforms. Using an iNOS inhibitor, S-methylisothiourea, Wildhirt et al., (1996), were able to improve left ventricular performance and survival rates after acute myocardial infarction in New Zealand rabbits. Using a different experimental approach, Mungrue et al., (2002), have developed a transgenic mouse model conditionally targeting the expression of human iNOS cDNA to the myocardium. These mice have been shown to display cardiac fibrosis, hypertrophy, and dilatation as well as a high incidence of sudden cardiac death due to bradyarrhythmia (Mungrue et al., 2002a). Studies have also looked for evidence of altered NOS expression under pathological conditions. It has been shown that iNOS expression is significantly elevated in biopsy samples taken from the myocardium of patients with heart failure (Haywood et al., 1996; Habib et al., 1996). In 12-week STZ
diabetic rats, iNOS expression is significantly elevated (Nagareddy et al., 2005), at time point when cardiomyopathy is well established in this model.

The evidence reviewed above suggests that iNOS plays a pathological role in cases of impaired cardiac function. However, the mechanistic and functional consequences of altered NOS expression and NO bioactivity in the failing human heart remain unclear.

1.3.1 Evidence for an interaction between NO and RhoA.

As described above (1.2, figure 1), the regulation of activation of RhoA and its role as a 'molecular switch' controlling cell signaling pathways has been well characterized. On the other hand, very few studies have addressed which factors regulate the expression of RhoA. However, recent evidence points towards an interaction between NO and RhoA. It has been shown that expression of RhoA in rat aortic smooth muscle cells is highly inducible by nitric oxide (Sauzeau et al., 2003). In this study, exposure of cultured vascular smooth muscle cells to the NO donor molecule, SNP, for 18 hours resulted in a significant increase in RhoA expression. Interestingly it has been shown that in STZ diabetic rat hearts, expression of iNOS is significantly elevated (Nagareddy et al., 2005). This is concomitant with significantly increased RhoA expression in the same model (preliminary data, not shown). Taken alone these are solely associated observations, nevertheless they provide us with the foundation for an interesting hypothesis to be tested using the STZ diabetic rat model.
1.4 Hypothesis and rationale for proposed experiments.

‘During diabetes, the elevated expression of iNOS in rat hearts results in increased RhoA expression and impaired cardiomyocyte contractility via alterations in actin cytoskeleton dynamics’.

As discussed above, our lab and others have shown that heightened iNOS expression is concomitant with increased RhoA expression (preliminary data, not shown), in the 12 week STZ diabetic rat model. Once induced, iNOS generates large amounts of NO until the enzyme is degraded (MacMicking et al., 1997). Evidence from vascular smooth muscle cells suggests that NO can induce increased levels of RhoA expression (Sauzeau et al., 2003). We therefore wish to investigate whether increased iNOS expression is responsible for the increased RhoA expression observed in the heart during diabetes.

Work conducted in this lab has shown that acute inhibition of the RhoA/ROCK signaling pathway causes rapidly improved cardiac performance in hearts from chronic diabetic rats (figures 3 and 4). This evidence suggests that the RhoA/ROCK signaling pathway may be upregulated in hearts from 12 week STZ diabetic rats. We therefore wished to assess whether increased expression of RhoA in the diabetic rat heart is associated with increased activity of the RhoA/ROCK pathway. It is also important to determine what consequences any change in activity of the RhoA/ROCK pathway might have in the diabetic rat heart. Given the well established role of RhoA in the regulation of the actin cytoskeleton in eukaryotic cells, we proposed to investigate whether any alterations in actin cytoskeletal dynamics could be detected in cardiomyocytes from diabetic hearts, and assess the effects of acute ROCK inhibition on the actin cytoskeleton in these cells.
1.5 Specific objectives.

1) Confirm that expression of RhoA and iNOS is up-regulated in diabetic cardiomyocytes.

2) Determine whether NO induces elevated expression of RhoA in isolated cardiomyocytes.

3) Determine if iNOS induction results in increased RhoA expression in isolated cardiomyocytes.


5) Determine if chronic inhibition of iNOS restores basal levels of expression of RhoA and activity of the RhoA/ROCK pathway in diabetic hearts.

6) Assess any changes in the dynamics of the actin cytoskeleton in diabetic cardiomyocytes, and the influence of ROCK inhibition on these changes.

The molecular mechanisms that underlie pathogenic changes in the heart during chronic diabetes remain poorly understood. In this study we will attempt to determine the cause of altered RhoA/ROCK signaling during diabetes and illustrate the pathological consequences of these changes. This investigation should provide insight
into the mechanisms contributing to the development of diabetic cardiomyopathy, and may potentially highlight new pharmacological targets for the treatment of this chronic complication of diabetes.
2.0 MATERIALS AND METHODS

2.1 Chemicals and materials

Chemicals and materials were purchased from or provided by the following sources:

Amersham International
ECL Western blotting detecting reagent, Ponceau S stain.

BioRad Laboratories
2-mercaptoethanol, Acrylamide (99.9%), glycine, polyvinylidene fluoride (PVDF), Precision Plus Protein™ Standards, tris base, 1.5M tris pH 8.8, 0.5M tris pH 6.8, sodium dodecyl sulfate (SDS), TEMED.

BD Biosciences
Mouse anti-iNOS primary antibody.

Boehringer Manheim GmbH
Bovine serum albumin (BSA).

Calbiochem Corp
DNAaseI-AlexaFluor®594, Phalloidin-AlexFluor®488.

Cayman Chemical
iNOS activity assay kit.

Cell Signaling Technology
Phospho-LIMK1 Antibody.

Cytoskeleton Inc.
G-actin/F-actin In Vivo Assay Kit, G-LISA™ RhoA Activation Assay Biochem Kit™.
Fisher Scientific Co  
Ethanol, Methanol.

GIBCO Life Technologies  
Sterile Phosphate Buffered Saline (PBS).

Medigas  
Carbogen (5% Carbon Dioxide, 95% Oxygen).

MTC Pharmaceuticals  
Somnitol® (Sodium Pentobarbital).

New England Biolabs Inc.  
L-N6-(1-iminoethyl)-lysine (L-NIL), Y-27632.

Organon Technika Inc  
Heparin Sulfate.

Roche Applied Science  
Laminin.

Santa Cruz Biotechnology Inc  
GAPDH antibody, β-Actin antibody, RhoA antibody, ROCKI antibody, all secondary HRP conjugated antibodies.

Safeway Stores  
Skim milk powder.

Sigma Chemical Co.  
Ammonium persulfate, aprotinin, calcium chloride, EDTA, EGTA, glucose (99.9%), halothane, HEPES, l-carnitine, leupeptin, lysopolysaccharides, magnesium sulfate, medium 199, penicillin, phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitor cocktail I, phosphatase inhibitor cocktail II, potassium chloride, potassium phosphate,
sodium chloride, sodium fluoride, sodium nitroprusside, streptomycin, Streptozotocin, taurine, Triton X-100, trypan blue solution.

Worthington Biochemical Corp
Collagenase (Type II, CLS II)

2.2 Animals.

Male Wistar rats were obtained from the UBC Animal Care Facility. Animals were housed in pairs. Water and standard rat chow was available ad libitum. Animal care was given in accordance with the principles and guidelines of the Canadian Council on Animal Care and the UBC Animal Care Committee.

2.2.1 Induction of diabetes.

Male Wistar rats weighing 180-200 g were allowed to acclimatize to the local vivarium for at least 3 days prior to induction of diabetes. Rats were allocated into groups randomly and made diabetic via a single tail vein injection of STZ dissolved in citrate buffer (55 mg/kg body weight). Control rats were administered equivalent volumes of citrate buffer via single tail vein injection. Blood glucose concentration was measured 72 hours after STZ administration. Induction of diabetes was confirmed by the presence of hyperglycemia (blood glucose $\geq 18$ mmol/L). For most experiments animals were kept for 12-15 weeks prior to sacrifice and collection of heart tissue and cardiomyocytes for analysis. At time of sacrifice blood samples were taken, and blood glucose levels were assessed. All diabetic animals exhibited blood glucose levels of $>18$ mmol/L. The STZ diabetic rat model is well established in our laboratory and all animals exhibited the typical characteristics and symptoms which we have previously
observed in this lab, this includes significant decreases in body weight, polyuria, polydipsia and diarrhea.

**2.2.2 Chronic inhibition of iNOS in control and STZ diabetic rats.**

Age-matched male Wistar rats were separated into two groups at random. One group was made diabetic using STZ, while the other was treated with vehicle as described above (2.2.1). One week after injection of STZ, control and diabetic rats were sub-divided into two groups; treated and untreated. Treated animals received the selective iNOS inhibitor, L-NIL at a dose of 3mg/kg/day by oral gavage (Ferrini et al., 2004; Lee et al., 2005). Untreated control and untreated diabetic animals received equivalent volumes of vehicle by oral gavage. Eight weeks later animals were sacrificed. Ventricular cardiac tissue was prepared as described below (2.3.2)

**2.3 Preparation of rat cardiac tissue.**

2.3.1 Preparation of isolated rat ventricular myocytes.

The isolation procedure was modified from the protocol of Huang et al., (2005). Rats were anaesthetized via a single intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight), co-administered with heparin (1000 units/kg body weight). The hearts were rapidly excised into ice-cold, pre-oxygenated Ca\(^{2+}\)-free Tyrode’s solution (containing 100 mM NaCl, 10mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 5 mM MgSO\(_4\), 50mM taurine, 20 mM glucose, 10 mM HEPES). Any extraneous tissue was quickly removed from the heart. Hearts were then mounted on Langendorff perfusion apparatus and perfused retrogradely at a constant flow of 8 ml/min with oxygenated Ca\(^{2+}\)-free Tyrode’s solution at 37 °C. After the coronary perfusate had cleared of blood, perfusion was continued for 4 min with Ca\(^{2+}\)-free Tyrode’s solution. Subsequently the hearts were perfused for 16
min with Tyrode's solution containing 0.05 mM Ca\(^{2+}\), 0.8 mg/ml collagenase and 0.1% BSA. After this time, the ventricles were excised from the heart, minced and gently shaken in the collagenase-containing isolation solution. This cell suspension was filtered through a 200 micron nylon mesh into a 50 ml culture tube. The solution was then centrifuged for 45 seconds at approximately 60g in a clinical centrifuge. The resulting supernatant was decanted and the cell pellet was resuspended in Tyrode's solution containing 0.2 mM Ca\(^{2+}\). Cells were allowed to settle for 10 minutes. This washing step was repeated twice with Tyrode's solution containing 0.5mM and then 1mM Ca\(^{2+}\). Cardiomyocyte counts were taken using a haemocytometer and viability was determined by assessing the percentage of cells that excluded trypan blue dye. Cell viability was greater than 65% in all groups. Cardiomyocytes were then used in experiments as described below or snap frozen in liquid nitrogen for future western blotting experiments.

### 2.3.1.1 Primary culture of isolated ventricular myocytes.

Cardiomyocytes were isolated and made calcium tolerant as described above. Myocytes were then resuspended in medium 199 supplemented with 1% BSA, 100 units/ml penicillin, 100 μg/ml streptomycin, 1.2 mM L-carnitine and 2.5 M HEPES (pH 7.4). Cells prepared from a single rat heart were plated on laminin coated culture-dishes. Cells were plated at a density of 0.75 x 10^6 cells per 100 mm dish. Culture dishes were placed in a 37 °C incubator with a maintained atmospheric CO\(_2\) level of 5%. Viable cells were given 3hrs to recover from the isolation process and adhere to the laminin-coated culture dish. After this incubation period the media and unattached cells were aspirated off. Fresh supplemented media 199 was then added to the culture dish. At this time any drugs were also added to the cells if required.
2.3.1.2 Induction of iNOS by LPS in cultured ventricular myocytes.

Cardiomyocytes were isolated and plated on laminin coated culture dishes as described above. After attachment, media and unattached cells were aspirated off. Fresh supplemented media 199 was then added to the culture dish. Cells were then exposed to 50 μg/ml LPS for 18hrs. Preliminary studies had shown that a concentration of at least 40 μg/ml LPS was required for iNOS induction in this culture preparation (figure 5). After 18hrs cells were inspected to ensure that they had maintained viability and retained a rod shaped phenotype. At this time media was aspirated away and cell lysates prepared as described below (2.3.2)

2.3.2 Extraction of total protein from cultured myocytes.

After completion of the required incubation period, cultured myocytes were washed twice with ice-cold PBS. After being well drained, the cells, kept on ice, were treated with 200 μL of lysis buffer (100 μM Tris-HCl pH 7.5, 50mM NaF, 5mM Sodium Pyrophosphate, 0.5 mM EDTA, 2 mM EGTA, 1% (v/v) Glycerol, 2% Sodium Azide, 1% NP40, 0.1% SDS, 2μg/ml aprotinin, 25μg/ml leupeptin, 1% (v/v) phosphatase inhibitor cocktail). Cells were incubated with the lysis buffer on ice for 10 minutes. Cells were then scraped off the dish surface and the cell lysates were collected in Eppendorf tubes. Cells were homogenized via fine needle tituration and brief sonication on ice. To remove unbroken cells and nuclei, lysates were spun at 700g for 5 minutes in a Beckman Allegra® 21R centrifuge. At this point samples were taken for protein assay (see 2.4).
Figure 5: Induction of iNOS by LPS. (A) Representative western blot showing effect of varying concentrations of LPS on iNOS expression. (B) Band O.D. values were normalized by their corresponding GAPDH band value and then expressed relative to the mean control value. Results shown are means (± SEM). *, P< 0.05 significantly different from control group. Statistical analysis was performed using a one-way ANOVA.
2.3.3 Extraction of total protein from rat ventricular tissue.

Rats were anaesthetized as described above. Hearts were removed and rinsed in ice-cold PBS to remove excess blood. Ventricular tissue was cut away from the heart and snap frozen in liquid nitrogen. Frozen ventricular tissue was subsequently crushed in a liquid nitrogen cooled mortar and pestle. Heart powders were collected in cryovials and stored at -70 °C for future experiments. For homogenization, approximately 150 mg of the heart powder was collected in ice-cold polyethylene homogenization tubes. The tubes were kept on ice and 1.5 ml homogenization buffer was added to each tube. The composition of the homogenization buffer was as follows: 20 mM Tris-HCl (pH 7.5), 50 mM β-mercaptoethanol, 5 mM EGTA, 2 mM EDTA, 10 mM NaF, 1mM PMSF, 25 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1% NP40, 0.1% SDS, 0.1% deoxycholic acid, 1% phosphatase inhibitor cocktail. Heart powder was homogenized using a Kinematica Polytron homogeniser (3 x 10 second bursts, probe at setting 7, with the samples kept on ice). To remove unsolubilized protein and cells, homogenized samples were centrifuged at 700g for 5 minutes in a Beckman Model J2-21® centrifuge. Supernatants from this centrifugation were transferred to Eppendorf tubes and kept as ‘total protein samples’. Protein concentrations in these samples was then determined (see 2.4).

2.4 Protein concentration determination.

After harvesting protein samples from cells or tissues the concentration of protein in these samples was determined using the Bio-Rad Protein Assay™. This assay is based on the Bradford dye-binding procedure (Bradford, 1976) which measures the colour change of Coomassie Brilliant Blue G-250 dye when it binds to protein through basic and aromatic amino acid residues. To determine protein concentration the
manufacturers instructions were carefully followed. For each sample absorbance was read at 595 nm for comparison with a BSA standard curve (0.0625 mg/ml – 1 mg/ml).

2.5 SDS-Polyacrylamide gel electrophoresis and immunoblotting of proteins from rat ventricular tissue and rat ventricular myocytes.

Prior to electrophoresis all samples were made up to a final volume of 500 μL at a concentration of 2 mg/ml in sample buffer (SDS reducing buffer). The composition of the sample buffer was as follows: 2% (w/v) SDS, 120 mM tris-HCl pH 6.8, 10% (v/v) glycerol, 5% (v/v) β-Mercaptoethanol, 0.004% (w/v) bromophenol blue. The samples were then heated to 100 °C for 5 minutes. SDS-PAGE gels were cast following the method of Laemmli (1970). The resolving gel contained 10% (w/v) total acrylamide, 375 mM tris-HCl, 0.1% SDS (w/v), 0.08% (w/v) ammonium persulfate and 0.03% (w/v) TEMED. The stacking gel contained 4% (w/v) total acrylamide, 125 mM tris-HCl (pH 6.8), 0.1% SDS (w/v), 0.08% (w/v) ammonium persulfate and 0.05% (w/v) TEMED. The upper and lower tank was filled with running buffer (25 mM tris, 192 mM glycine, 0.1% (w/v) SDS, pH 6.8). Unless otherwise stated 25 μL of each sample or 15 μL of Precision Plus Protein™ standards were loaded into their appropriate wells in the stacking gel.

Proteins were electrophoretically separated through the gel under a constant voltage of 125 volts for 75 minutes using a Bio-Rad Protein II electrophoresis unit. After electrophoresis a Bio-Rad Criterion™ Blotter was used to transfer the resolved proteins onto a PVDF membrane. Transfer was conducted at a constant current of 250 mA for 1 hour in ice-cold transfer buffer. The transfer buffer composition was: 25 mM tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. After transfer, membranes were briefly stained
with Ponceau S stain to confirm successful protein transfer. Excess stain was subsequently removed by washing the membrane for 10 minutes in TTBS.

The PVDF membrane was treated for 2 hours at room temperature in blocking buffer (3% (v/w) skim milk powder in TTBS), to reduce non-specific antibody binding. The membranes were subsequently washed with TTBS (3 x 10 minutes). If more than one protein was being detected membranes were cut using a sterile surgical blade to isolate molecular weight regions containing the protein of interest. The membrane was then incubated overnight at 4 °C with the appropriate primary antibody. Primary antibodies were diluted in 5% (w/v) BSA in TTBS. The membrane was then washed (3 x 10 minutes) in TTBS and then incubated for 1 hour at room temperature with an appropriate secondary antibody. Secondary antibodies were diluted 1:10,000 in 5% milk powder in TTBS. Membranes were washed again with TTBS (3 x 10 minutes). Primary antibody binding was detected using the ECL technique. Equal amounts of reagent A (0.45 mg/ml Luminal in 0.1M tris, pH 8.5) and reagent B (0.2% H₂O₂ in 0.1 M tris, pH 8.5) were mixed thoroughly. The mixed reagents were incubated on the blots for 2 minutes before exposing the drained blots to the x-ray film.

2.6 iNOS Activity Assay.

A commercially available iNOS activity assay was used to determine iNOS activity in whole heart homogenates (Cayman Chemical MI). Extraction of protein from ventricular cardiac tissue was carried out following the manufacturers protocol. All samples were measured in triplicate.
2.7 **G-LISA™ RhoA Activation Assay.**

A commercially available RhoA G-LISA kit (Cytoskeleton Inc, CO) was used to determine the amount of active RhoA present in protein samples from ventricular myocytes. Myocytes were isolated as described previously (2.3.1). Cell lysates from freshly isolated myocytes were collected as described in the manufacturers protocol. Sample absorbances were read at 490 nm using a microplate spectrophotometer. Sample wells containing lysis buffer only were designated as the assay blank. A constitutively active Rho control protein was used as the assay positive control. All samples were measured in triplicate.

2.8 **G-actin / F-actin Assay.**

0.5 x 10^6 freshly isolated, viable cells were resuspended in 0.5 ml lysis and F-actin stabilization buffer (LAS). The LAS composition was as follows: 50 mM PIPES (pH 6.9), 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 5% (v/v) Glycerol, 0.1% NP40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% Antifoam C. Cells were gently homogenized via fine needle tituration. Lysates were then spun at 2000 rpm for 5 minutes to pellet unbroken cells. The supernatant from this spin was spun at 100,000g for 1 hour at 4 °C in a Beckman L8-60M® ultracentrifuge. The supernatants from this spin were collected in labeled eppendorf tubes and kept as the G-actin containing fraction. Pellets were resuspended to the same volume as the G-actin fraction using LAS. This was kept as the F-actin containing fraction. 40 μL of SDS sample reducing buffer was added to 160 μL of each of the F-actin and G-actin fractions. Samples were then heated to 100 °C for 5 minutes. Samples were then loaded onto a 10% SDS-polyacrylamide gel and electrophoretically separated following the protocol described previously (2.5). Gels were blotted onto PVDF membranes and actin immunoblots were
carried out as described previously (2.5). The optical density of each band was determined using a scanning densitometer. The ratio of G-actin to F-actin was calculated by dividing the optical density value of the appropriate F-actin band by its corresponding G-actin band.

2.9 Immunofluorescence labeling and Confocal Microscopy.

Myocytes were isolated from hearts as described previously (2.3.1). Cells were then plated on poly-L-lysine coated cover slips and rinsed with PBS. Myocytes were fixed for 10 min with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS for 3 min, treated with PBS containing 1% BSA for 20 minutes, and finally rinsed with PBS. Cells were double stained with DNAasel-AlexaFluor®594 and Phalloidin-AlexFluor®488 to colocalize monomeric globular actin (red, G-actin), and polymerized filamentous actin (green, F-actin) (Pullinikunil et al., 2005). The unbound fluorescent probe was rinsed with PBS buffer and slides were visualized using a Bio-Rad Radiance-600® Confocal Microscope at 1260X magnification.

2.10 Statistical Analysis.

Unless otherwise stated, all values are expressed as means ± SEM; n denotes the number of animals in each group. For all results the level of significance was set at P < 0.05. For multiple comparisons of more than two experimental groups a 2-way ANOVA followed by a Newman-Keuls post-hoc test was conducted using using NCSS statistical analysis system (NCSS). When comparing only two groups, for instance, control and diabetic samples, a one-way ANOVA was employed using GraphPad Prism® (GraphPad Software).
3.0 Results

3.1 Expression of RhoA in control and diabetic cardiomyocytes.

Previous work in this laboratory had shown that acute ROCK inhibition improved cardiac function in diabetic rats. Given this finding a major objective of this thesis was to determine any upregulation of the RhoA/ROCK pathway in hearts from 12 week STZ diabetic rats. Accordingly we isolated cardiomyocytes from 12 weeks STZ diabetic male rats and their age-matched controls, and determined the relative expression of RhoA in control and diabetic cardiomyocytes using western blotting. Primary RhoA antibody (mouse monoclonal) was used at a concentration of 1:250.

As shown in figure 6, when compared to control, expression of RhoA in freshly isolated diabetic cardiomyocytes was significantly elevated.
Figure 6: Relative expression of RhoA in control and diabetic cardiomyocytes, as assessed by western blotting. (A) A representative blot showing RhoA and Actin expression in freshly isolated control and diabetic cardiomyocytes. (B) RhoA band O.D. values were corrected by their equivalent actin band O.D. value and expressed relative to mean control value. Means are shown (± SEM), n = 8. *, P< 0.05 significantly different from control group. Statistical analysis was performed using a one-way ANOVA.
3.2 Expression of iNOS in control and diabetic cardiomyocytes.

An important objective of this thesis was to confirm the findings of Nagareddy et al., (2005), who had shown that iNOS expression was significantly elevated in whole hearts from 12 week STZ diabetic rats. We wished to confirm if this was also the case in isolated cardiomyocytes. The relative levels of iNOS expression in control and diabetic cardiomyocytes was determined using western blotting. Primary iNOS antibody (mouse monoclonal) was used at a concentration of 1:200.

It was noticeable that iNOS was expressed, albeit weakly, in freshly isolated control cardiomyocytes (figure 7B). There was a strong increase in iNOS expression in diabetic cardiomyocytes. Densitrometry data (figure 7A), confirmed that the expression of iNOS in diabetic cardiomyocytes was significantly elevated compared to control.
Figure 7: Relative expression of iNOS in control and diabetic cardiomyocytes, as assessed by western blotting. (A) A representative blot showing iNOS and actin expression in freshly isolated control and diabetic cardiomyocytes. (B) iNOS band O.D. values were corrected by their equivalent actin band O.D. value and expressed relative to mean control value. Means are shown (± SEM), n = 8. *, P< 0.05 significantly different from control group. Statistical analysis was performed using a one-way ANOVA.
3.3 Effect of SNP on the expression of RhoA in cultured cardiomyocytes.

Sauzeau et al., (2003) demonstrated that when exposed to SNP, cultured vascular smooth muscle cells exhibited a significant increase in the expression of RhoA. We wished to determine whether the same effect occurred in cultured primary rat cardiomyocytes.

Isolated cardiomyocytes were cultured for 18 hours in the presence or absence of 10 μM SNP. After this time lysates were collected and expression of RhoA was compared in control and treated cells using western blotting. Primary RhoA antibody (mouse monoclonal) was used at a concentration of 1:250.

As shown in figure 8, there was a significant increase in RhoA expression in SNP treated cells compared to control.
Figure 8: Effect of 10 μM sodium nitroprusside (SNP) treatment for 18hrs on RhoA expression. 0.75 x 10⁶ viable myocytes were cultured per plate. Expression levels were assessed using western blotting. Band O.D. values were normalised to the mean control value. Results shown are normalised means (± SEM). *, P< 0.05 significantly different from control group. Statistical analysis was performed using a one-way ANOVA.
3.4. Effect of iNOS induction on the expression of RhoA in cultured cardiomyocytes.

When induced, iNOS catalyzes the production of large amounts of NO from L-arginine and molecular oxygen (Palmer et al., 1988). Cells were exposed to 50 μg/ml LPS for 18hrs. Myocytes were also co-treated with 50 μg/ml LPS and 100 μM N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL), a selective iNOS inhibitor. Previously, this concentration of L-NIL had been shown effectively inhibit iNOS in vitro (Moore et al., 1994). A control group treated with 100 μM L-NIL alone was also implemented.

After this incubation period western blotting was performed to confirm induction of iNOS and to analyze the level of expression of RhoA in these cells. Primary antibodies were used at the concentrations described previously (see 3.1 and 3.2). As shown in figure 9A, induction of iNOS by LPS resulted in a significant increase in RhoA expression. Co-treatment of cells with LPS and L-NIL resulted in the induction of iNOS, however, RhoA expression in these cells was reduced to levels not different from control (figure 9B.). Treatment with L-NIL alone had no effect on RhoA expression. There was no evidence of iNOS induction in cells treated with L-NIL alone (figure 9A).
Figure 9A: Effect of LPS treatment on RhoA and iNOS expression. A representative blot showing iNOS, RhoA and GAPDH expression in control (1), LPS treated (2), LPS and LNIL treated (3), and LNIL treated cardiomyocytes (4).
Figure 9B: Effect of LPS treatment (50 μg/ml for 18hrs) on RhoA expression. Representative Band O.D. values were normalised by their corresponding GAPDH band value and then expressed relative to the mean control value. Results shown are means (± SEM). *, P< 0.05 significantly different from control group. Statistical analysis was performed using a one-way ANOVA.
3.5 Effect of chronic iNOS inhibition on expression of RhoA in control and diabetic rat hearts.

Control and diabetic male Wistar rats were chronically administered L-NIL as described (2.2.2). It was important to determine whether treatment with the chosen dose of L-NIL had been effective in inhibiting iNOS activity. iNOS activity was found to be significantly increased in hearts from untreated diabetic rats (figure 10B). Treatment of diabetic rats with L-NIL at 3mg/kg/day for nine weeks was effective at reducing iNOS activity to levels not significantly different from control. Elevated iNOS expression in untreated diabetic hearts was confirmed using western blotting (figure 10A). Chronic treatment with L-NIL appeared to normalise iNOS expression in diabetic rat hearts (figure 10A). Concomitant with elevated iNOS expression, RhoA expression in hearts from 9 week diabetic rats was significantly elevated. Hearts from diabetic rats treated with L-NIL will showed normalized RhoA expression (figure 11). L-NIL had no effect on RhoA expression in hearts from control rats.
Figure 10: iNOS expression and activity in control and diabetic cardiac ventricular tissue following chronic iNOS inhibition. (A) A representative blot showing iNOS and GAPDH expression in ventricular tissue. Treatment was with L-NIL at 3mg/kg/day for 9 weeks via oral gavage. (B) Graph displaying iNOS activity values (counts per min per 80μg heart tissue). Activity values are expressed relative to the mean control value. Data shown are means ±SEM. Statistical analysis was performed using a one-way ANOVA followed by a Newman-Keuls post hoc test. *, P< 0.05 significantly different from control group (n = 6). C = Control, D = Diabetic, CT = Treated Control, DT = Treated Diabetic.
Figure 11: Relative RhoA expression levels in control and diabetic cardiac ventricular tissue following chronic iNOS inhibition. (A) A representative blot showing RhoA and GAPDH expression in ventricular tissue. Treatment was with L-NIL at 3mg/kg/day for 9 weeks via oral gavage. (B) Band O.D. values were corrected by their loading control band O.D. value and expressed relative to the mean control. Data shown are means ±SEM. Statistical analysis was performed using a one-way ANOVA followed by a Newman-Keuls post hoc test. *, P< 0.05 significantly different from control group (n = 6). C = Control, D = Diabetic, CT = Treated Control, DT = Treated Diabetic.
3.6 Effect of chronic iNOS inhibition on activity of the RhoA/ROCK signaling pathway.

To date no commercially available ROCK assay has been established. However, it is well established that, once activated by RhoA, ROCK phosphorylates and activates its downstream target, LIMK (Bamburg, 1999). Therefore, phosphorylated LIMK (LIMK-P) was used as a marker for activity of RhoA/ROCK in this experiment. Protein samples from ventricular tissue (from 3.4) were prepared for western blotting. Western blotting was performed using an antibody specific for LIMK-P. This rabbit polyclonal primary antibody was used at a concentration of 1:1000.

We observed evidence for increased activation of the RhoA/ROCK pathway in diabetic rat hearts (figure 12). There was a significant elevation in the levels of LIMK-P in diabetic samples, chronic treatment with L-NIL reduced LIMK-P to levels that were not significantly different from control.
Figure 12: Relative levels of LIMK-P in cardiac ventricular tissue. (A) A representative blot showing LIMK and GAPDH expression in ventricular tissue. C = Control, D = Diabetic, CT = Treated Control, DT = Treated Diabetic. Treatment was with L-NIL at 3mg/kg/day for 9 weeks via oral gavage. (B) Band O.D. values were normalized by their corresponding GAPDH band value and expressed relative to the mean control value. Data shown are means ±SEM. Statistical analysis was performed using a one-way ANOVA followed by a Newman-Keuls post hoc test. *, P < 0.05 significantly different from control group (n = 6).

It was important that as well as determining an increase in expression of RhoA we also confirm whether the activity of RhoA is altered in diabetic cardiomyocytes. To this end, we measured levels of active RhoA in freshly isolated myocytes from control and 12-week STZ diabetic rat hearts. To assess activation of ROCK, we used western blotting to measure the levels of phosphorylated LIMK relative to total LIMK in cells lysates from control and diabetic cardiomyocytes.

Compared to control cardiomyocytes there was a significantly increased amount of active RhoA in diabetic cardiomyocytes (figure 13). Concomitantly, there was a significant increase in the levels of phosphorylated LIMK in diabetic cardiomyocytes compared to control (figure 14).
Figure 13: Relative amount of active RhoA in Control and 12 week STZ diabetic rat ventricular myocytes. 0.75 x 10^6 cells per sample. n = 12 in both groups. All data shown are means ± SEM. *Significantly different from control sample (P<0.05), as determined by a one-way ANOVA.
Figure 14: LIMK-P/Total LIMK in Control and 12 week STZ diabetic rat ventricular myocytes. (A) A representative blot showing LIMK and LIMK-P in ventricular tissue. (B) LIMK-P band O.D. values were normalized by their corresponding LIMK band value and expressed relative to the mean control value. Data shown are means ±SEM. Statistical analysis was performed using a one-way ANOVA followed by a Newman-Keuls post hoc test. *, P< 0.05 significantly different from control group (n = 6).
3.8 **Effect of ROCK inhibition on the actin cytoskeleton in control and diabetic cardiomyocytes.**

The actin cytoskeleton is a well-established downstream target of RhoA/ROCK in eukaryotic cells. It was therefore of interest in this study to determine the state of actin cytoskeleton dynamics in control and diabetic cardiomyocytes, and to determine the effect of acute ROCK inhibition on cytoskeletal actin in these cells. A commercially available assay kit was used to isolate and determine the free globular actin (G-actin) and filamentous actin (F-actin) content from freshly isolated control and diabetic cardiac myocytes. Cells from control and diabetic animals were also treated with the ROCK inhibitor, Y-27632 (1 μM, 15 minutes). For each sample, actin was extracted from 0.5 x 10^6 viable cells.

We also used a qualitative fluorescence confocal microscopy approach to assess the levels of filamentous actin in control and diabetic cells in the absence and presence of Y-27632.

It was found that there was a consistent, significant elevation in the F-actin/G-actin ratios in diabetic cardiomyocytes (figure 15). Acute treatment with Y-27632 reduced the F-actin/G-actin ratios in diabetic cells to levels which were not significantly different from control. Acute treatment of control cells with Y-27632 had no effect on the F-actin/G-actin ratio.

Using fluorescence microscopy it was found that fluorescence from the labeled F-actin (green, Phalloidin-AlexFluor®488) appeared to be much more intense in diabetic cells than in controls (figure 16). The levels of F-actin fluorescence (green) in Y-27632 treated diabetic cells was not noticeably different from that in control and control-treated cells.
Figure 15: Comparison of the F-actin to G-actin ratio in freshly isolated myocytes. Myocytes were isolated from control (C), 12 week STZ diabetic rats (D). The effect of treatment with Y-27623 (1μM, 15 mins) on control (CT) and treated diabetic (DT) myocytes was also assessed. (A) The western blot shown is representative of all samples assayed. Western blot bands are labeled as follow: (1) = Control F-Actin, (2) = Control G-Actin, (3) = Diabetic F-Actin, (4) = Diabetic G-Actin, (5) = Treated control F-Actin, (6) = Treated control G-Actin, (7) = Treated diabetic F-Actin, (8) = Treated diabetic G-Actin. (B) Results are mean F/G-actin ratios ± SEM (n=6 in each group), *, P<0.05 different from all other groups. Statistics were performed by performing a one-way ANOVA followed by a Newman-Keuls post-hoc test.
Figure 16: Confocal microscopy images of fluorescently labeled cardiomyocytes. Freshly isolated myocytes from control and 12-week STZ diabetic rats were untreated or treated with 1μM Y-27632 for 15 mins. Myocytes were fixed, permeabilized and double stained with AlexaFluor®594-DNAsel and AlexaFluor®488-Phalloidin to colocalize G-actin (red) and F-actin (green). Slides were visualized using a Bio-Rad Radiance confocal microscope at 1260x magnification. Control (A), Diabetic (B), Control-Treated (C), Diabetic-Treated (D).
4.0 DISCUSSION

4.1 Increased RhoA and iNOS expression in STZ diabetic rat cardiomyocytes.

The compelling finding that acute inhibition of ROCK improves heart function in 12 week STZ diabetic rats (figures 3 and 4) was a major inspiration for conducting this research project. On the basis of these observations we wished to determine what factors may be contributing to any change in activity of the RhoA/ROCK pathway during chronic STZ diabetes and determine what the cellular consequences of altered RhoA/ROCK signaling might be.

In this study we found that expression of RhoA was upregulated in hearts from 12 week STZ diabetic rats (figure 6). This observation is at odds with a recent report that RhoA mRNA and protein expression are decreased in hearts from diabetic rats (Tang et al., 2005). The reason for this difference is not clear, but it may be due to differences in the duration of diabetes between the two studies. The study by Tang et al., (2005) was conducted in hearts from 3 week STZ diabetic rats, a time which is prior to the development of overt impaired contractile function in most investigations. It should be noted that in further studies conducted in this laboratory we were not able to detect altered expression of RhoA in whole heart tissue from 3 week STZ diabetic rats, and in 5-6 week STZ diabetic rats we have observed increased RhoA expression (data not shown). It is interesting that the increase in expression of RhoA in the heart correlates well with the onset of diabetic cardiac dysfunction. Taken together with evidence that cardiac specific over-expression of RhoA leads to diminished ventricular contractility (Sah et al., 1999), these findings suggest a strong association between increased RhoA expression and impaired myocardial function in the chronic STZ diabetic rat.
The observation that iNOS expression is elevated in diabetic cardiomyocytes is in agreement with Nagareddy et al., (2005), who showed increased expression of iNOS in whole heart tissue from 12 week STZ diabetic rats. In this thesis we have shown that iNOS and RhoA expression are concomitantly elevated in cardiomyocytes and heart tissue from STZ diabetic rats (figure 6, figure 7). Interestingly, this is not the first study which has shown concomitant increases in RhoA and iNOS expression in a pathological model of impaired cardiac function. Dong et al., (2005), investigated the effects of iron-deficiency on cardiac ultrastructure and found that iron-deficient rats exhibited significantly elevated RhoA and iNOS expression. Unfortunately Dong et al., (2005) did not assess cardiac function in these animals. However, a previous investigation using the same model has shown that impaired myocardial function develops in iron-deficient rats (Goldstein et al., 1996). Interestingly Goldstein et al., observed impaired L-type Ca\(^{2+}\) currents and depressed rates of ventricular contraction and relaxation. These pathological observations are similar to those observed in hearts from animals with chronic type 1 diabetes. These findings strongly support an association between elevated iNOS and RhoA expression the pathology of impaired cardiac function.

4.2 NO and induction of iNOS elevates RhoA expression in vitro.

Given the association of elevated iNOS and RhoA expression in whole heart tissue and cardiomyocytes from 12 week STZ diabetic rats, we wished to determine if an interaction existed between these two proteins. Sauzeau et al., (2003), demonstrated that the NO donor, SNP, caused significant increases in RhoA expression in cultured vascular smooth muscle cells. In agreement with this work, we have shown that using the same concentration of SNP, a significant increase in RhoA expression can be induced in cultured cardiomyocytes over an 18 hour period.
The mechanism by which NO might be elevating RhoA expression is still not fully understood. The elevated levels of RhoA that we have observed could be due to transcriptional and translational upregulation of the RhoA gene or decreased degradation of the RhoA protein. There is very limited published data on the regulation of stability or expression of Rho GTPases. Sauzeau et al., (2002) suggest that that NO activates PKG, which phosphorylates RhoA on the C-terminal domain, thus protecting the protein from degradation and resulting in RhoA accumulation in the cell. Sauzeau et al., have shown elevated PKG activity and RhoA phosphorylation in cells exposed to SNP. They also show that co-treatment of cells with SNP and the PKG inhibitor (Rp)-8-Br-PET-cGMP-S blocks the SNP-induced elevation of RhoA, supporting the author's hypothesis. It should be appreciated that with other proteins, cell-type-specific expressional regulation mechanisms have been established (Park et al., 2003). It is therefore possible that the mechanisms proposed in fibroblasts and vascular smooth muscle cells by Sauzeau et al., (2003) may not necessarily be mirrored in cardiomyocytes.

The activity of iNOS is primarily regulated at the level of protein expression. Unlike eNOS and nNOS, regulation of protein activity is uncommon for iNOS (Kleinert et al., 2003). After expressional induction, iNOS continuously produces NO until the enzyme is degraded (MacMicking et al., 1997). In cultured cardiomyocytes we were able to induce iNOS expression using LPS. This observation is supported by a number of reports which have indicated that cytokines including LPS induce iNOS and NO production in cardiomyocytes (see review by Kelly et al., 1996). Previous studies in rats have used neonatal cardiomyocytes and have demonstrated a significant induction of iNOS after LPS exposure (Kinugawa et al., 1997a; Kinugawa et al., 1997b; Keira et al.,
A difference between our studies and previous ones is that in the previous studies a lower LPS concentration (10 µg/ml) was required for iNOS induction. In our experiments a concentration of at least 40 µg/ml LPS was required for induction of iNOS. These differences may reflect a difference in responsiveness to LPS between cultured neonatal cardiomyocytes and primary cultured adult rat cardiomyocytes. It should be noted that in all experiments described here cardiomyocyte cultures were examined carefully and appeared to be free of any contamination from other cell types. This is an important point as other cell types, such as macrophages, are highly responsive to LPS (Fujihara et al., 2003). Although we cannot completely rule out the possibility of a very low level of contamination by macrophages, given the number of cardiomyocytes cultured in each experiment, it is unlikely that this would impact on the total iNOS or RhoA pool.

The results presented here suggest that the increased NO produced by iNOS results in increased RhoA expression. Induction of iNOS resulted in a concomitant increase in RhoA expression. This effect was blocked by co-treatment of cardiomyocytes with LPS and L-NIL (figure 9). L-NIL is an L-arginine analogue which acts as a selective, irreversible inhibitor of iNOS (Moore et al., 1994). Our results strongly support the hypothesis that induction of iNOS results in increased RhoA expression.
4.3 Chronic iNOS inhibition results in decreased iNOS expression in diabetic rat hearts.

The expression and activity of iNOS in heart tissue from diabetic rats was significantly elevated compared to control. Treatment of diabetic rats with L-NIL at a dose of 3mg/kg/day for 8 weeks was effective at reducing iNOS activity in whole heart tissue to levels not different from control (figure 10B). Interestingly, in diabetic hearts, chronic treatment with L-NIL also appeared to reduce iNOS expression (figure 10A). Given that iNOS is a selective, irreversible inhibitor of iNOS (Moore et al., 1994), it was not anticipated that L-NIL treatment would affect iNOS expression. This data suggests that auto-regulation of iNOS expression may be occurring. However our in vitro experiment using cultured cardiomyocytes, there was no detectable effect on iNOS expression after exposure to L-NIL (or LPS and L-NIL) for 18 hours (figure 9A). There are conflicting reports as to whether treatment with iNOS inhibitors affects iNOS expression. Using rat mesangial cells, Muhl and Pfeilschifter (1995), showed that iNOS inhibitors acutely reduced IL-1 beta-induced iNOS expression. In rat synovial tissue and peripheral blood leukocytes, iNOS expression was reduced after chronic administration of L-NIL at 3 mg/kg/day (McCartney-Francis et al., 2001). However Behr-Roussel et al., (2000), did not detect a change in iNOS expression in aortas from rats chronically treated with L-NIL. Taken together the results shown in this thesis and by others suggest that the differences may exist in the regulation of iNOS expression depending upon cell and species type and the duration of treatment with iNOS inhibitors.
4.4 Chronic inhibition of iNOS normalizes expression of RhoA and activity of the RhoA/ROCK pathway in heart tissue from rats with STZ induced diabetes.

Concomitant with elevated iNOS expression, RhoA expression in hearts from 9 week diabetic rats was significantly elevated, while hearts from diabetic rats treated with L-NIL showed normalized RhoA expression (figure 11). This observation agrees with results from our in vitro experiment (figure 9) and supports the proposal that elevated iNOS expression induces increased RhoA expression.

We also wished to determine if the activity of the RhoA/ROCK pathway was affected by the observed changes in RhoA expression in these tissues. There was a significant elevation in the levels of LIMK-P in diabetic rat hearts and chronic treatment with L-NIL reduced LIMK-P to levels that were not different from control (figure 12). LIMK is a direct downstream target of ROCK. Once activated ROCK phosphorylates and activates LIMK. These results suggest that there is increased activity of ROCK and LIMK in heart tissue from rats with diabetes. Currently there have been no identified kinases other than ROCK which phosphorylate LIMK, and other studies from this laboratory have shown that acute inhibition of ROCK normalizes LIMK phosphorylation in 12 week STZ diabetic rats (Lin et al., unpublished data). Given that chronic inhibition of iNOS reduced the levels of RhoA expression and the levels of phosphorylated LIMK in heart tissue from rats with diabetes, these results suggest that iNOS induces increased expression and activity of RhoA in the diabetic rat heart.
4.5 Increased activity of RhoA in diabetic rat cardiomyocytes.

As discussed in the introduction it was important that we assessed the levels of active RhoA in diabetic cardiomyocytes. Given the elevated LIMK-P observed in whole heart tissue from diabetic rats, it was expected that levels of active RhoA would also be elevated in diabetic cardiomyocytes.

Our RhoA activity assay showed that, compared to control, there were significantly higher levels active RhoA in cardiomyocytes from diabetic hearts. There was an approximate 50% increase in the levels of active RhoA in diabetic cardiomyocytes compared to control. This correlated strongly to the relative fold-increase observed in expression of RhoA in diabetic cardiomyocytes. Using western blotting, elevated LIMK-P was also observed in diabetic cardiomyocytes. This strongly supports the evidence that RhoA activity is upregulated in diabetic cardiomyocytes, leading to increased activity of ROCK.

Elevated expression of RhoA may be contributing to elevated levels of active RhoA in diabetic cardiomyocytes, by increasing the 'total RhoA pool' available for activation. In addition, it has recently become apparent that GPCR signalling through heterotrimeric G-proteins can lead to the activation of Rho family GTPases (Sah et al., 2000; Marinissen and Gutkind, 2001) (figure 17). Increased Ag-II and angiotensin receptor levels have been shown in an in vivo study in streptozotocin-induced diabetic rat hearts (Fiordaliso et al., 2000). Ag-II has been shown to activate RhoA in rat cardiomyocytes (Aoki et al., 1998).
Figure 17: GPCR mediated activation of Rho GTPases. The binding of agonist to a GPCR promotes the exchange of GDP for GTP on the G-protein Ga subunit, which leads to dissociation of the Ga and Gβγ subunits. The activated proteins Ga and Gβγ can positively or negatively regulate various downstream effectors. GPCRs can also signal to, and activate and Rho GTPases (via Ga12/Ga13). [Adapted from: Bhattacharya et al., 2004].
It has also been proposed that increased stimulation by endothelium-derived vasoconstrictors, such as endothelin-1 and thromboxane-A2, contribute to increased RhoA/ROCK activation in the diabetic heart (Sharma and McNeill, 2006). In streptozotocin-diabetic rats, plasma concentrations of endothelin-1 (Benrezzak et al., 2004) and thromboxane-A2 (Valentovic and Lubawy 1983) have been shown to be elevated compared to control rats. The elevated expression of RhoA is likely to amplify the effects of increased circulating factors such as endothelin-1 and Ag-II which are known to activate RhoA, resulting in heightened activation the RhoA/ROCK pathway in diabetic rat heart. However, this would not account for our observation that RhoA activity was increased in isolated cardiomyocytes where these factors would be absent.

4.6 Effects of acute RhoA/ROCK inhibition on the actin cytoskeleton in diabetic cardiomyocytes.

The evidence presented in this thesis strongly suggests increased activation of RhoA, ROCK and LIMK in diabetic rat hearts and cardiomyocytes. Previous findings from this laboratory have shown that acute inhibition of ROCK significantly improved heart function in diabetic rats. These findings implicate increased activation of the RhoA/ROCK pathway in mediating impaired cardiac function in the diabetic heart. An important aim of this thesis was to determine the consequences of RhoA/ROCK upregulation in diabetic cardiomyocytes, and attempt to shed more light on the mechanism by which acute ROCK inhibition improves cardiac function in the STZ-diabetic rat.

As described in section 1.1, signaling from RhoA to the actin cytoskeleton through ROCK and LIMK is well established. Maekawa et al., (1999) demonstrated that
phosphorylation of LIMK by ROCK and consequently increased phosphorylation of cofilin by LIMK contribute to RhoA-induced actin polymerization in N1E-115 neuroblastoma cells.

Here we have shown that there is a significant increase in the levels of polymerized actin in cardiomyocytes from 12-week STZ diabetic rats. Acute inhibition of ROCK decreased polymerized actin in diabetic cardiomyocytes to levels that were not different from control cardiomyocytes. Evidence from both in vitro actin polymerization assays and confocal microscopy supported this finding.

The ability of the actin cytoskeleton to modulate the mechanical activity of the cardiomyocyte has been well reviewed (Calaghan et al., 2004) and a number of studies have implicated a role for the actin cytoskeleton in conditions of impaired cardiac performance (Hein et al., 2000; Kostin et al., 2000). A number of mechanisms could explain this interaction. The cardiomyocyte actin cytoskeleton has been shown to be an important regulator of the metabolism of fatty acids (Pulinilkunnil et al., 2005), the preferred energy substrate of the heart (Rodrigues et al., 1995). Increased actin polymerization, induced by RhoA activation, can promote the exocytosis of lipoprotein lipase and increase fatty acid metabolism (Pulinilkunnil et al., 2005), which is associated with impaired cardiac function and lipotoxicity in chronic diabetes (Tan et al., 1984). Increased filamentous actin within the cardiomyocyte may also increase cell rigidity, contributing to the increased myocardial stiffness which is a component of impaired cardiac performance in hearts from diabetic rats (Joffe et al., 1999; Dent et al., 2001).

It is possible that normalization of actin polymerization may contribute to the ROCK inhibitor-mediated improvement in contractile function of hearts from diabetic rats, observed in previous studies conducted in this laboratory (figures 3 and 4). One of
the few specific mechanisms previously known to acutely improve the contractile function of the diabetic heart was treatment with agents that promote a shift from fatty acid metabolism to glucose oxidation (Tan et al., 1984; Nicholl et al., 1991). Given these unique previous findings, a possible mechanism by which ROCK inhibitors improve cardiac function, is via normalization of actin polymerization and reduction of fatty acid metabolism, thereby improving the metabolic state of the heart during chronic diabetes. However, since the completion of this research thesis, initial studies conducted in collaboration with the laboratory of Dr. Michael Allard at the James Hogg iCapture Centre for Cardiovascular and Pulmonary Research, were unable to detect significant changes in fatty acid or glucose metabolism in 12 week STZ diabetic rat hearts acutely treated with Y-27632. It is possible that normalization of actin polymerization in diabetic cardiomyocytes may not be able to acutely normalize fatty acid metabolism.

The actin microfilaments have also been implicated in regulating the concentration of intracellular Ca\(^{2+}\) (Lange and Brandt, 1996), a key regulator of cardiomyocyte contractility (Bers and Guo, 2005). Increased actin polymerization in cardiomyocytes has been associated with decreased contractility due to disruption of intracellular Ca\(^{2+}\) release (Davani et al., 2004). Acute changes in intracellular [Ca\(^{2+}\)] are likely to manifest as acute changes in cardiomyocyte contractility. It would be therefore interesting to determine the effects of ROCK inhibition on intracellular [Ca\(^{2+}\)] and calcium homeostasis in diabetic cardiomyocytes.

The evidence supports a role for increased actin polymerization in impaired cardiac function in the 12 week STZ diabetic rat. Given that acute inhibition of ROCK has been shown to normalize both actin polymerization and cardiac performance in diabetic rat hearts, we conclude that there is a strong association between elevated
activity of the RhoA/ROCK pathway, increased actin polymerization and decreased cardiac performance in the diabetic rat heart. However further studies are required to determine the mechanisms by which increased actin polymerization affects cardiac contractility in the diabetic rat heart.

4.7 Further Studies.

The following outlines briefly describe some studies that would be interesting to pursue given the outcomes of this research thesis.

To determine if inhibition of ROCK improves cardiomyocyte contractility, and if this effect is achieved via normalization of calcium transients.

Previous work in this laboratory has established that acute ROCK inhibition improves cardiac function both in vivo and ex vivo. It would be interesting to determine if acute ROCK inhibition can improve contractility at the cellular level, and whether this is mediated via normalization of calcium handling.

Previous studies have confirmed impaired contractility and calcium handling in myocytes isolated from STZ diabetic rat hearts (Choi et al., 2003). Cell shortening and intracellular [Ca$^{2+}$] can be measured in cardiomyocytes using fluorescence microscopy as described previously (Huang et al., 2005). This investigation could provide useful information on the pathology of diabetic cardiomyopathy at the cellular level, as well as an insight into the possible mechanisms by which acute ROCK inhibition improves cardiac function in the STZ diabetic rat heart.
To determine whether increased RhoA expression is induced directly by NO in isolated cardiomyocytes.

In this thesis we have shown that when treated with 10 μM SNP, a potent NO donor, cardiomyocytes exhibit significantly elevated RhoA expression levels. However, we have not proven causally that NO directly mediates this change. It would be interesting to observe if this effect could be blocked with nitric oxide scavengers. Furthermore, NO strongly interacts with superoxide to form peroxynitrite (Stamler, 1994). This highly reactive nitrogen-containing radical can cause oxidative and nitrosative stress, and may lead to the activation of stress-response pathways (Klotz et al., 2002). This may ultimately affect the expression levels of a broad range of proteins. The effect of NO (from SNP) on the expression of RhoA in primary culture cardiomyocytes could therefore be direct or indirect. To help clarify this, cells could be treated with 10 μM SNP and 5 μM FeTMPyP (5,10,15,20-tetrakis(N-methyl-4'-pyridyl) porphyrinato-iron III), a peroxynitrite decomposition catalyst. Cells treated with FeTMPyP alone would serve as a treatment control. If FeTMPyP blocks the SNP-induced increase in RhoA this would strongly suggest that the effect of NO on RhoA expression is mediated via peroxynitrite. This experiment would be important in clarifying how NO from iNOS results in altered RhoA expression in the diabetic cardiomyocyte.

To determine if chronic iNOS inhibition improves cardiac function in STZ diabetic rats.

The data provided in this thesis indicates that chronic inhibition of iNOS restores basal levels of expression of RhoA as well as basal levels of phosphorylated LIMK in hearts from STZ diabetic rats. Given the association between elevated activity of the
RhoA/ROCK pathway and impaired cardiac function it would be interesting to determine whether chronic inhibition of iNOS improves cardiac function in the STZ diabetic rat. In this study control and diabetic rats would be chronically administered L-NIL and subsequently heart function would be measured either using echocardiography or by using the isolated work heart model. Normalization of RhoA expression and activity of the RhoA/ROCK pathway concomitant with improved cardiac function would be extremely compelling evidence to support a role for iNOS upregulation in altered RhoA/ROCK signaling and impaired cardiac function in the 12 week STZ diabetic rat heart.

To determine the status of the RhoA/ROCK pathway in iNOS knockout mice.

To further strengthen our proposal that iNOS mediates elevated RhoA expression and upregulation of the RhoA/ROCK pathway, we would like to examine this pathways characteristics in the well-established iNOS knockout mouse (MacMicking et al., 1995). It would be interesting to investigate whether mice made diabetic, in the absence of iNOS, would still exhibit upregulation of the RhoA/ROCK pathway. Furthermore, it would be important to assess cardiac function in these animals and determine if iNOS knockout mice developed diabetic cardiomyopathy. If iNOS knockout mice did not exhibit diabetic cardiomyopathy, this would be compelling and conclusive evidence that iNOS mediated upregulation of the RhoA/ROCK pathway, played a central role in the development of diabetic cardiomyopathy.
5.0 SUMMARY AND CONCLUSIONS

This thesis has provided strong evidence that RhoA expression, and the activity of the RhoA/ROCK pathway are significantly elevated in hearts and cardiomyocytes from rats with STZ-induced diabetes. Given that exposure to the NO donor, SNP, elevated RhoA expression in cultured myocytes we propose that NO from iNOS is mediating elevated RhoA expression in diabetic rat hearts. The conclusion that iNOS mediates increased RhoA expression in the STZ-diabetic rat heart is strongly supported by a number of other observations in this thesis. Western blotting and iNOS activity assays confirmed increased expression and activity of iNOS, concomitant with increased RhoA expression in diabetic rat hearts. Chronic treatment of diabetic rats with the specific iNOS inhibitor, L-NIL, blocked increased RhoA expression in hearts from these animals. In cultured cardiomyocytes LPS evoked increased iNOS and RhoA expression. Co-treatment of cells with LPS and the specific iNOS inhibitor, L-NIL, blocked increased RhoA expression, with no detectable change in expression of iNOS. Taken together these findings strongly support the proposal that induction of iNOS results in increased RhoA expression in diabetic cardiomyocytes.

Increases in RhoA expression correlated strongly with increased levels of active RhoA in diabetic cardiomyocytes. We also observed increased levels of phosphorylated LIMK, a marker for activation of the RhoA/ROCK pathway, in diabetic cardiomyocytes. LIMK phosphorylation was reduced to levels similar to control after chronic treatment of diabetic rats with L-NIL, suggesting that iNOS contributes to increases activity, in addition to increased expression, of RhoA in the STZ-diabetic rat heart. The actin cytoskeleton is a well established downstream target of the RhoA/ROCK pathway. Phosphorylated LIMK acts as a positive regulator of actin polymerization, and the levels
of polymerized actin in cardiomyocytes from diabetic hearts were found to be significantly elevated. The attenuation of this change via acute inhibition of ROCK implicates increased activation of the RhoA/ROCK pathway in mediating these changes in the diabetic heart.

In conclusion, the present study demonstrates, for the first time to our knowledge, that the RhoA/ROCK pathway is upregulated in hearts, and cardiomyocytes, from rats with chronic STZ-induced diabetes. Despite the diverse biochemical and physiological changes that occur in the diabetic heart, acute inhibition of the RhoA/ROCK pathway has been previously shown in this laboratory to improve cardiac function, both in vitro and in vivo. Given that acute ROCK inhibition was also shown to normalize the levels of polymerized actin in diabetic cardiomyocytes, it is possible that normalization of actin polymerization may contribute to ROCK inhibitor-mediated improvement of contractile function of hearts from diabetic rats.

Finally, the findings presented in this thesis suggest a central role for iNOS in the upregulation of the RhoA/ROCK pathway which has been shown to contribute to impaired contractility in the diabetic heart. These findings also support the suggestion that ROCK is an excellent therapeutic target in the treatment of diabetic cardiomyopathy.
BIBLIOGRAPHY


