INVESTIGATION OF MECHANISMS UNDERLYING ALTERED ALPHA-ADRENERGIC RECEPTOR-INDUCED CONTRACTILE RESPONSES IN THE STREPTOZOTOCIN-DIABETIC RAT HEART

by

TIANHAI GUO

B. Med., Sun Yat-sen University of Medical Sciences (Zhongshan University), China, 2002

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Abstract

Diabetic cardiomyopathy is one of the major chronic complications in diabetes mellitus. Alterations in the α_1-adrenergic receptor (α_1-AR)-induced positive inotropic effect (PIE) have been shown in the diabetic heart, but the results have not been consistent. The molecular signaling mechanisms underlying the α_1-AR-induced PIE in the heart, though still under debate, have been suggested to be related to two protein kinases, protein kinase C (PKC) and Rho kinase, both of which consist of several isoforms. Thus it was hypothesized that specific PKC and/or Rho Kinase isoforms play a role in the altered α_1-AR-induced PIE in the diabetic heart. The effects of chronic streptozotocin-induced diabetes on the basal contractile function and the α_1-AR-induced PIE, as well as the associated changes in four PKC isoforms (α, β_2, δ and ϵ) and two Rho kinase isoforms (ROCK 1 and ROCK 2) in the rat heart were investigated. Three cardiac contractile parameters, left ventricular developed pressure (LVDP), maximal rate of contraction (+dP/dt) and relaxation (-dP/dt), were measured using the isolated Langendorff-perfused isovolumic heart model. In the absence of adrenergic stimulation, all three contractile parameters were attenuated in hearts from 6~7 week and 12~15 week diabetic rats. The selective α_1-AR agonist, phenylephrine (PE), produced greater maximal increase (Rmax) values for LVDP and -dP/dt in both 6~7 week and 12~15 week diabetic hearts compared to age-matched controls. It also produced greater pD_2 (-log [ED_{50}]) values for +dP/dt in both 6~7 week and 12~15 week diabetic hearts, and greater pD_2 values for LVDP and -dP/dt in 12~15 week diabetic hearts compared to age-matched controls. In the presence of the non-isoform-selective PKC inhibitor, chelerythrine (CE), the increase in all three contractile parameters in response to PE was partially suppressed

in both diabetic and control hearts, and the increase to PE in LVDP and -dP/dt was not different in diabetic and control hearts. The non-isoform-selective Rho kinase inhibitors, Y-27632 and H1152, had no effect on the α_1-AR-induced PIE in either diabetic or control hearts. Western immunoblotting showed that in the absence of adrenergic stimulation, the basal levels of PKCδ and PKCε in the particulate fraction of 12~15 week diabetic hearts were increased compared to control, without any change in the soluble fraction. There was no change in the subcellular distribution of PKCα, PKCβ_2, ROCK 1 or ROCK 2 in diabetic hearts compared to control. PE produced a significant increase in levels of PKCδ and PKCε in the particulate fraction of both 12~15 week diabetic and control hearts, but without a corresponding decrease in the soluble fraction. The increase in particulate PKCδ over its own basal levels in diabetic hearts was significantly greater than control, whereas the increase in particulate PKCε over its own basal levels in diabetic and control hearts was not different. In the presence of CE, the PE-induced increase in levels of PKCδ and PKCε in the particulate fraction of both diabetic and control hearts was completely suppressed. PE had no effect on the subcellular distribution of PKCα, PKCβ_2, ROCK 1 or ROCK 2 in either diabetic or control hearts.

Activation of the renin-angiotensin system (RAS) has been suggested to contribute to diabetic cardiomyopathy. It has been shown that in isolated cardiomyocytes from diabetic rats, PKCε translocated from the soluble to the particulate fraction, while treatment with the angiotensin II type 1 receptor antagonist, L-158,809, normalized the alteration in PKCε. Thus it was hypothesized that treatment with this antagonist would improve the attenuated basal contractile function and normalize the enhanced α_1-AR-induced PIE, as well as the associated changes in PKC isoforms in the

diabetic heart. The results showed that treatment with L-158,809 significantly improved the basal contractile function of 12-week diabetic hearts. However, it did not normalize the enhanced α_1-AR-induced PIE. This antagonist also had no effect on the basal levels of PKCδ and PKCε in the particulate fraction of diabetic hearts, nor did it affect the PE-induced changes in these two PKC isozymes in either diabetic or control hearts.

The results from the present study suggest a role for PKCδ and/or PKCε in the PIE to α_1-AR stimulation in the heart, and that PKCδ may contribute to the enhanced α_1-AR-induced PIE in the diabetic heart. These two PKC isoforms appear to be activated under basal conditions in the diabetic heart. The present study does not support a role for Rho kinase in the α_1-AR-induced PIE in the heart or in diabetic cardiomyopathy. The activation of RAS contributes to cardiac contractile dysfunction in diabetes. However, this study does not support an involvement of PKC in this process.

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+dP/dt	Maximal rate of contraction
-dP/dt	Maximal rate of relaxation
ACE	Angiotensin-converting enzyme
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
ANP	Atrial natriuretic peptide
aPKC	Atypical protein kinase C
AR	Adrenergic receptor, adrenoceptor
AT_1 receptor	Angiotensin II type 1 receptor
AT_2 receptor	Angiotensin II type 2 receptor
BIM	Bisindolylmaleimide
β-MHC	β-myosin heavy chain
CE	Chelerythrine
cPKC	Conventional protein kinase C
CPP	Coronary perfusion pressure
CRC	Concentration-response curve
DAG	Diacylglycerol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ERK	Extracellular-regulated kinase
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GLUT	Glucose transporter
G-protein	Guanine nucleotide-binding protein
G_s	Stimulatory G-protein
HLA-D	Class-II major histocompatability complex
I_f channel	Hyperpolarization-activated cyclic nucleotide-gated cation channel
IP_3	Inositol 1,4,5-triphosphate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K-H buffer	Krebs-Henseleit buffer
LVDP	Left ventricular developed pressure
MAPK	Mitogen-activated protein kinase
MHC	Myosin heavy chain

MLC	Myosin light chain
MLC1	Essential myosin light chain
MLC2	Regulatory myosin light chain
nPKC	Novel protein kinase C
pD_2	$-\log[EC_{50}]$
PE	Phenylephrine
PIE	Positive inotropic effect
PIP_2	Phosphatidylinositol-4,5-biphosphate
pK_B	Apparent affinity constant
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PS	Phosphatidylserine
PTX	Pertussis toxin
RACK	Receptor for activated C-kinase
RAS	Renin-angiotensin system
Rho kinase	Rho-associated kinase
Rmax	Maximal response
RO	Ro318220, bisindolylmaleimide IX
ROCK	Rho kinase
SDS	Sodium dodecyl sulfate
SR	Sarcoplasmic reticulum
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
T_3	Triiodothyronine
TBS	Tris buffer saline
Tn	Troponin
TnC	Ca^{2+} binding subunit of troponin
TnI	ATPase inhibitory subunit of troponin
TnT	Tropomyosin binding subunit of troponin
Tris	Tris[hydroxymethyl]aminomethane
Tween 20	Polyoxyethylenesorbitan monolaurate
WHO	World Health Organization

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1 INTRODUCTION

1.1 Excitation-contraction coupling

Cardiomyocytes have the ability to contract and relax, which is the fundamental basis for the heartbeat. The heartbeat is initiated by the generation of electrical impulses in specialized cells in the sinoatrial node within the right atrium. The electrical impulse is then transmitted along a conduction system, which is made up of specialized cardiac cells, to individual cardiomyocytes. The cell membrane of cardiomyocytes, namely the sarcolemma, contains receptors, ion channels, ion pumps and transporters embedded in its lipid bilayer. This structure allows the cardiomyocyte to communicate with adjacent cardiomyocytes and the extracellular environment.

The intracellular mechanisms underlying each contraction-relaxation cycle of the cardiomyocyte are called excitation-contraction coupling (Korzick 2003). In response to the electrical impulse, voltage-dependent Na^+ channels in the sarcolemma open for several milliseconds and allow Na^+ entry. The sarcolemma is then depolarized, resulting in the opening of the L-type voltage-dependent Ca^{2+} channels. This permits the entry of extracellular Ca^{2+} down a concentration gradient, resulting in a small increase of intracellular Ca^{2+} concentration. This small elevation of intracellular Ca^{2+} levels allows the binding of Ca^{2+} to the sarcoplasmic reticulum (SR), which is an intracellular Ca^{2+} pool, resulting in the release of the stored Ca^{2+} via the Ca^{2+} releasing channels on the SR (a process named Ca^{2+}-induced Ca^{2+} release). As a result, the intracellular Ca^{2+} levels increase to a much greater extent. Ca^{2+} ions subsequently interact with the troponin-

tropomyosin complex, the contraction and relaxation unit of the cardiomyocyte. The complex is composed of tropomyosin and three troponin (Tn) subunits: the Ca^{2+}-binding subunit (TnC), the ATPase inhibitory subunit (TnI) and the tropomyosin-binding subunit (TnT). In the relaxed state, the troponin-tropomyosin complex lies between actin and myosin, preventing the interaction of the two. When the intracellular Ca^{2+} levels increase in response to the electrical impulse, Ca^{2+} ions bind to TnC, shifting away the troponin-tropomyosin complex, allowing the actin molecules to interact with myosin cross-bridges. The myosin-bound ATP is then hydrolyzed, providing energy for the persistent interaction of actin and myosin. As a result, the myosin cross-bridges make a "rowing" movement along the actin chain and the cardiomyocyte shortens. The magnitude of contractile force generated is dependent on the number of myosin cross-bridges interacting with actin. The more intracellular Ca^{2+} ions available to the interaction, and/or the higher affinity of the contractile proteins (i.e. troponin-tropomyosin complex, actin and myosin) for Ca^{2+} ions, the greater the contractile force can be obtained. Positive inotropic agents exert their effects either by elevating intracellular Ca^{2+} levels or sensitizing the contractile proteins to Ca^{2+}. During the relaxation of the cardiomyocyte, Ca^{2+} ions dissociate from the troponin-tropomyosin complex as a result of the decrease in intracellular Ca^{2+} concentration. The complex shifts back to its original location, preventing the interaction between myosin and actin. The decrease in intracellular Ca^{2+} levels is the result of three mechanisms: Ca^{2+} ions being taken up by the Ca^{2+} pumps on the SR, the Ca^{2+} pumps in the sarcolemma, and the Na^{+}-Ca^{2+} exchangers in the sarcolemma.

1.2 Overview of regulation of cardiac contractile function by the autonomic nervous system

Cardiac function is mainly regulated by the autonomic nervous systems (i.e. sympathetic and parasympathetic nervous systems), which act via adrenergic receptors (adrenoceptors, ARs) and muscarinic acetylcholine receptors, respectively. Activation of the sympathetic system results in elevation in intracellular cyclic AMP and intracellular Ca^{2+} levels or increase in myofibrillar Ca^{2+} sensitivity, leading to increased cardiac contractile performance; activation of the parasympathetic system decreases intracellular cyclic AMP and intracellular Ca^{2+} levels, thus attenuates contractile force. The endogenous neurotransmitter of the sympathetic nervous system is noradrenaline. At least nine adrenoceptor subtypes have been identified in mammalian tissues: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3 (Brodde *et al.* 1999). In the heart, noradrenaline acts on α_1-, β_1- and β_2-ARs to produce positive inotropic and chronotropic effects, increasing contractile force and heart rate, resulting in increased cardiac output (Brodde *et al.* 1999).

1.3 Signaling mechanisms underlying β-AR-induced positive inotropic, lusitropic and chronotropic effects

There is ample evidence showing that β-ARs are the predominant ARs through which noradrenaline exerts its actions in the heart (Leone *et al.* 2002). β_1-ARs are more prominent than β_2-ARs, in terms of the number of receptors, and their positive inotropic and positive chronotropic effects (Lohse *et al.* 2003). Both β_1- and β_2-ARs couple to

stimulatory small guanine nucleotide-binding proteins (G_s proteins). Activation of both kinds of receptors leads to the activation of G_s and subsequently the activation of adenylyl cyclase, resulting in increased intracellular levels of cyclic AMP. This leads to the activation of protein kinase A, which regulates the phosphorylation of several cellular structures, including the L-type voltage-dependent Ca^{2+} channels in the sarcolemma, the Ca^{2+} releasing channels on the SR, TnI and phospholamban (Leone *et al.* 2002; Korzick 2003; Lohse *et al.* 2003). Phosphorylation of L-type Ca^{2+} channels increases their opening time, allowing more Ca^{2+} to enter the cardiomyocyte, and enhancing Ca^{2+}-induced Ca^{2+} release. Phosphorylation of the Ca^{2+} releasing channels on the SR allows more Ca^{2+} ions to be released from the SR. The overall outcome of these two mechanisms is an increase in intracellular Ca^{2+} levels, allowing more intracellular Ca^{2+} ions available for the interaction between myosin and actin, thus enhancing contractile force (positive inotropic effect). Phosphorylation of TnI by protein kinase A results in decreased Ca^{2+} sensitivity of contractile proteins, facilitating the dissociation of Ca^{2+} from the troponin-tropomyosin complex. When unphosphorylated, phospholamban inhibits the Ca^{2+} pumps on the SR and prevents intracellular Ca^{2+} uptake. Once phosphorylated by protein kinase A, phospholamban is inhibited, resulting in increased Ca^{2+} uptake into the SR. Phosphorylation of TnI and phospholamban accelerates the relaxation of the cardiomyocyte and shortens the diastolic phase (positive lusitropic effect). On the other hand, elevation of intracellular cyclic AMP levels by the activation of β_1- and β_2-ARs contributes to positive chronotropic effects. The heart rate is controlled by specialized cardiomyocytes that generate electrical impulses in the sinoatrial node. In the sarcolemma, the hyperpolarization-activated cyclic nucleotide-gated cation channels (I_f

channels), which are permeable to both K^+ and Na^+, determine the speed of depolarization, thus controlling heart rate. Upon the activation of β_1- and β_2-ARs, cyclic AMP binds to I_f channels and accelerates their activation kinetics (Biel *et al.* 2002), resulting in increased heart rate (positive chronotropic effect).

1.4 Signaling mechanisms underlying the α_1-AR-induced PIE

As early as the 1960's, a group of researchers reported an α-AR-mediated positive inotropic effect (PIE) in rat ventricular strips (Wenzel *et al.* 1966). Subsequently, similar observations have been confirmed in a number of investigations using different cardiac preparations from a variety of species (Li *et al.* 1997), even from humans (Schumann *et al.* 1978; Bruckner *et al.* 1984). Two types of α-ARs – α_1 and α_2 – have been found so far, but only α_1-AR mediates the PIE in heart, because selective α_2-AR agonists cause no positive inotropy (Williamson *et al.* 1987; Housmans 1990). Three subtypes of α_1-AR have been identified pharmacologically and through molecular cloning: α_{1A}, α_{1B} and α_{1D} (Hieble *et al.* 1995). Both α_{1A} and α_{1B} have been proposed to mediate PIE in adult rat hearts (Williamson *et al.* 1994a; Williamson *et al.* 1994b; Deng *et al.* 1996a). However, α_{1D} seems to play little role in the α_1-AR-mediated PIE (Deng *et al.* 1996b).

The mechanisms underlying the α_1-AR-mediated PIE in heart have been the target of intensive investigations during the last decade. Though not completely elucidated, several signaling pathways have been proposed. α_1-ARs couple to their signal transduction machinery mainly via pertussis toxin (PTX) – insensitive G-proteins of the $G_{q/11}$ family (Graham *et al.* 1996). Upon stimulation, α_1-ARs subsequently activate

phospholipase C (PLC) and this results in the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) after the cleavage of phosphatidylinositol-4,5-biphosphate (PIP_2) (Divecha *et al.* 1995). Both IP_3 and DAG are important second messengers upon α_1-AR activation. IP_3 binds to specific IP_3 receptors located on the SR, resulting in the release of stored Ca^{2+} into cytosol and elevation of intracellular Ca^{2+} (Divecha *et al.* 1995). However, in studies using saponin-skinned cardiomyocytes and isolated SR, there was no evidence showing IP_3-induced Ca^{2+} release from SR (Movsesian *et al.* 1985). Whether IP_3 can release Ca^{2+} from SR in cardiomyocytes, and even if IP_3 does modulate the mobilization of intracellular Ca^{2+}, whether this is associated with the α_1-AR-mediated PIE, is still controversial (Terzic *et al.* 1993).

1.4.1 Possible role of protein kinase C (PKC) in the α_1-AR-induced PIE

DAG activates protein kinase C (PKC), which consists of a family of serine-threonine kinases that play a critical role in signal transduction by phosphorylating a variety of substrates (Terzic *et al.* 1993). PKC isozymes are classified into three groups based on their structures and the cofactors bound upon activation (Mackay *et al.* 2001):

- The conventional PKC (cPKC) isozymes, comprising α, β_1, β_2 and γ, which are activated by Ca^{2+}, DAG and phosphatidylserine (PS);
- The novel PKC (nPKC) isozymes, comprising δ, ϵ, η and θ, which do not respond to Ca^{2+} but are activated by DAG, PS and unsaturated fatty acids;
- The atypical PKC (aPKC) isozymes, comprising ζ and τ/λ, which are unresponsive to Ca^{2+} and phorbol esters but can also be activated by PS and unsaturated fatty acids.

The presence of PKC isozymes is species-, tissue- and developmental stage-dependent. PKCα, δ and ϵ are consistently found in adult rat hearts (Puceat *et al.* 1994; Mackay *et al.* 2001; Das 2003). However, PKCβ_2 has not been consistently detected in adult rat hearts (Rybin *et al.* 1994; Mackay *et al.* 2001).

For the last two decades, translocation of PKC isoforms has been considered as a hallmark of their activation. In their inactive state, PKC isozymes are mainly in the soluble fraction of cells (Nishizuka 1992). Upon activation, catalytically competent PKC isozymes translocate from the soluble (cytosolic) to the particulate (membrane) fraction of cells where they are thought to bind to "receptors for activated C-kinase" (RACKs) and subsequently interact with their subcellular targets (Kraft *et al.* 1983; Mochly-Rosen *et al.* 1990; Mochly-Rosen *et al.* 1991). The translocation is rapidly followed by a return of PKC back to the soluble fraction (Feng *et al.* 1998b), a process referred to as reverse translocation and thought to require autophosphorylation (Feng *et al.* 1998a; Feng *et al.* 2000). Based on this attribute, and the available access to isoform-selective PKC antibodies, the activation of PKC isozymes can be detected and measured by immunoblot (Western blot) analysis, in which the protein levels in soluble and particulate fractions are quantified. Although total PKC activity (i.e. the activity of all isoforms) can be directly measured using radioactive or non-radioactive methods, it is difficult to measure the activity of individual isoforms due to the lack of selective substrates. The measurement of total PKC activity cannot be adopted in cases when the activity of a single isoform or several isoforms needs to be determined. Immunofluorescent studies showed that each PKC isozyme localizes to unique subcellular sites of cardiomyocytes upon stimulation (Disatnik *et al.* 1994; Johnson *et al.* 1996): PKCβ_2 is mainly found in fibrillar structures

in the unstimulated state and is translocated to the perinucleus and cell periphery after stimulation; on the other hand, PKCε translocates from the nucleus and perinucleus to cross-striated structures and cell-cell contacts upon stimulation. In addition, although still under debate, the involvement of different PKC isoforms has been proposed in specific physiological and pathological processes in the heart; moreover, even if several PKC isozymes participate in the progression of the same disease, each isoform may have its own contribution (Puceat *et al.* 1996; Koya *et al.* 1998; Mackay *et al.* 2001; Das 2003; Sabri *et al.* 2003). All these studies suggest different PKC isozymes may have unique functions, thus the measurement of the activation of individual isozymes is necessary. As a result, in current investigations, Western blot with isoform-selective antibodies to detect translocation from the soluble to the particulate fraction is still widely used as a measure of PKC isoform activation.

It has been well established that α_1-AR agonists increase myofibrillar sensitivity to Ca^{2+} (Endoh *et al.* 1988; Puceat *et al.* 1990). PKC-dependent regulation of contractile proteins (mainly the regulatory myosin light chain) may play a role in myofibrillar Ca^{2+} sensitization. Studies using pig (Morano *et al.* 1985; Morano *et al.* 1990) and human (Morano *et al.* 1988) cardiac preparations suggested that the phosphorylation of myosin light chain (MLC) increases the Ca^{2+} sensitivity of atrial or ventricular strips. Two types of MLC have been found in the heart: essential MLC (MLC1) and regulatory MLC (MLC2) (Morano 1999). MLC1 may act as a myosin heavy chain (MHC) / actin tether, imposing a load on the myosin cross-bridge. Relieving or weakening of this tether has been suggested to decrease this load, accelerate cross-bridge cycling and enhance the tension output per cross-bridge, thus increasing contractility (Morano *et al.* 1995). The

elimination of MLC2 has been shown to increase the attachment rate constant, leading to an increased number of force-generating cross-bridges at a given Ca^{2+} activation level and consequently to increased Ca^{2+} sensitivity of myosin (Brenner 1988). It has been suggested in another study that the elimination of MLC2 increases Ca^{2+} sensitivity of isometric tension generation (Hofmann *et al.* 1990). In all, MLC may act as an inhibitory factor in the unphosphorylated state; once phosphorylated, its inhibitory effect is relieved, resulting in increased Ca^{2+} sensitivity of the contractile proteins and cardiac contractile force (Morano 1999). There is evidence showing the phosphorylation of MLC2 is regulated by PKC. A group of researchers (Venema *et al.* 1993a; Venema *et al.* 1993b) showed that PKC incorporated phosphate stoichiometrically into MLC2 in cardiac myofibrils in vitro; direct activation of PKC by phorbol 12-myristate 13-acetate (PMA, a non-isoform-selective PKC activator) induced the phosphorylation of MLC2 in isolated cardiomyocytes. Besides these direct effects of PKC on MLC2, PKC has also been proposed to increase the phosphorylation of MCL2 via an action on MLC kinase. PKC was suggested to enhance the effect of MLC kinase on force development and ATPase activity (Clement *et al.* 1992). MLC2 phosphorylation by cardiac MLC kinase or by PKC has been suggested to increase actin-stimulated myosin MgATPase activity (Noland *et al.* 1993a). These investigations suggest PKC may play a role in the phosphorylation of MLC2, resulting in increased Ca^{2+} sensitivity of myofibrils (Morano *et al.* 1985) and the PIE. It should be noted that some of these experiments were performed in cell-free systems. The exact role for PKC in regulating the phosphorylation of MLC2 upon the stimulation of α_1-ARs in whole cardiomyocytes or in vivo is still not clear (Puceat *et al.* 1996). There are other contractile proteins that have been proposed to be possible

substrates for PKC in the heart, including C-protein, TnI and TnT. C-protein has been reported to be phosphorylated by PKC both in vitro (Lim *et al.* 1985) and in vivo (Venema *et al.* 1993a). Since the function of C-protein in the contractile process is not clear, the significance of its phosphorylation by PKC remains unresolved. There is ample evidence showing TnI and TnT can be phosphorylated by PKC, resulting in decreased Ca^{2+} sensitivity of myofilaments and attenuated contractile force (Katoh *et al.* 1983; Noland *et al.* 1991; Clement *et al.* 1992; Noland *et al.* 1993b). In summary, the regulation of cardiac contractile proteins by PKC is a complicated process, and the influences on contractile force may be opposite to each other. However, compared to other contractile proteins phosphorylated by PKC, the phosphorylation of MLC2 may be most prominent in the α_1-AR-mediated PIE (Puceat *et al.* 1996).

In studies using intact cardiomyocytes, among the three PKC isoforms (PKCα, ϵ and δ) that are consistently detected in adult rat hearts, only PKCϵ and δ translocate from the soluble fraction to the particulate fraction of cardiomyocytes upon the activation of α_1-AR by phenylephrine (PE, selective α_1-AR agonist) (Puceat *et al.* 1994; Wang *et al.* 2003). As already mentioned, PKCβ_2 has not been consistently detected in adult rat hearts (Mackay *et al.* 2001). The effect of α_1-AR activation on this isoform is still not clear.

1.4.2 Possible role of Rho kinase in the α_1-AR-induced PIE

α_1-ARs not only couple to the $G_{q/11}$ family, but also couple to the $G_{12/13}$ family of G-proteins (Katoh *et al.* 1998). The activation of α_1-AR leads to the activation of RhoA,

which is a member of the small G-protein subfamily, Rho. Like other small G-proteins, inactive RhoA localizes in the cytosol and once activated, it translocates from the soluble fraction to the particulate fraction of cells (Bokoch *et al.* 1994). The activation and inactivation of RhoA are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Inactive RhoA binds to GDP and is activated by exchange of GDP for GTP. This process is enhanced and regulated by GEF. The innate GTPase activity of small G-proteins hydrolyzes bound GTP to GDP, returning them to the inactive state. This GTPase activity is stimulated by GAP (Kaibuchi *et al.* 1999).

The activation of RhoA results in the activation of downstream Rho kinase. Translocation of Rho kinase isoforms has also been considered as a hallmark of their activation. In their inactive state, Rho kinase isozymes mainly localize in the soluble fraction of the cell (Leung *et al.* 1995); once activated by RhoA, they translocate to the particulate fraction (Sin *et al.* 1998). Unlike PKC, there are no direct methods for the measurement of Rho kinase activity. However, there are isoform-selective Rho kinase antibodies commercially available. As a result, the activation of this kinase can be detected by Western blot analysis.

Two Rho kinase isoforms have been identified: ROKβ (ROCK 1) (Leung *et al.* 1996) and ROKα (ROCK 2) (Leung *et al.* 1995). The kinase domains of ROCK 1 and ROCK 2 are 92% identical and so far there is no evidence that they phosphorylate different substrates or have different functions (Riento *et al.* 2003).

Rho kinase has also been suggested to regulate MLC. Rho kinase has been proposed to promote the phosphorylation of MLC (Amano *et al.* 1996) by

phosphorylating the inhibitory subunit of myosin phosphatase, resulting in the inactivation of the latter (Kimura *et al.* 1996) and increased MLC phosphorylation. Y-27632 (a non-isoform-selective Rho kinase inhibitor) was reported to reduce the PE-induced PIE in rat left ventricular papillary muscles (Andersen *et al.* 2002) and it also blocked the PE-induced Ca^{2+} sensitization in isolated cardiomyocytes (Suematsu *et al.* 2001). It was also suggested that in failing hearts, α_1-AR-G_q-RhoA signaling is up-regulated, resulting in increased levels of activated Rho kinase, increased myofibrillar Ca^{2+} sensitivity and elevated contractility, which might be a compensatory mechanism in heart failure (Suematsu *et al.* 2001). All these investigations suggest a role for Rho kinase in the α_1-AR-mediated PIE.

1.4.3 Other possible mechanisms underlying the α_1-AR-induced PIE

It has been suggested that α_1-AR agonists increase intracellular pH by activating the Na^+-H^+ exchanger on the cell membrane (Terzic *et al.* 1993) possibly via α_{1A}-AR (Yokoyama *et al.* 1998). There is a correlation between the magnitude of the α_1-AR-mediated PIE and the degree of intracellular alkalinization (Vaughan-Jones *et al.* 1987; Gambassi *et al.* 1992; Terzic *et al.* 1992). Na^+-H^+ exchanger blockers inhibit the increase in PE-induced contractile force in cardiac myocytes (Otani *et al.* 1990; Gambassi *et al.* 1992). Intracellular alkalinization has been suggested to increase the affinity of TnC for Ca^{2+}, sensitize the actomyosin ATPase to Ca^{2+} and thus result in an increase in contractility (Fabiato *et al.* 1978). PKC has been proposed to modulate the

phosphorylation of Na^+-H^+ exchangers in the heart, resulting in a PIE (Wallert *et al.* 1992; Puceat *et al.* 1993).

α_1-AR agonists inhibit voltage-dependent K^+ current in isolated rat ventricular cardiomyocytes (Apkon *et al.* 1988). This results in the prolongation of action potential duration, leading to increased Ca^{2+} influx (Terzic *et al.* 1993) and PIE. However, this mechanism is still controversial because there is evidence suggesting that the α_1-AR-mediated PIE is associated with a decrease in action potential duration in ventricular papillary muscles (Arreola *et al.* 1994).

1.4.4 Summary of the signaling mechanisms underlying the α_1-AR-induced PIE in adult rat hearts

The mechanisms underlying the α_1-AR-mediated PIE in adult rat hearts may have the following components (Figure 1):

- An increase in intracellular Ca^{2+} due to the release of Ca^{2+} from SR by IP_3
- Ca^{2+} sensitization of contractile proteins, which may be related to PKC and/or Rho kinase activation
- Activation of the Na^+-H^+ exchanger and intracellular alkalization, which may be related to PKC activation
- Prolongation of action potential duration that increases Ca^{2+} influx into cardiomyocytes.

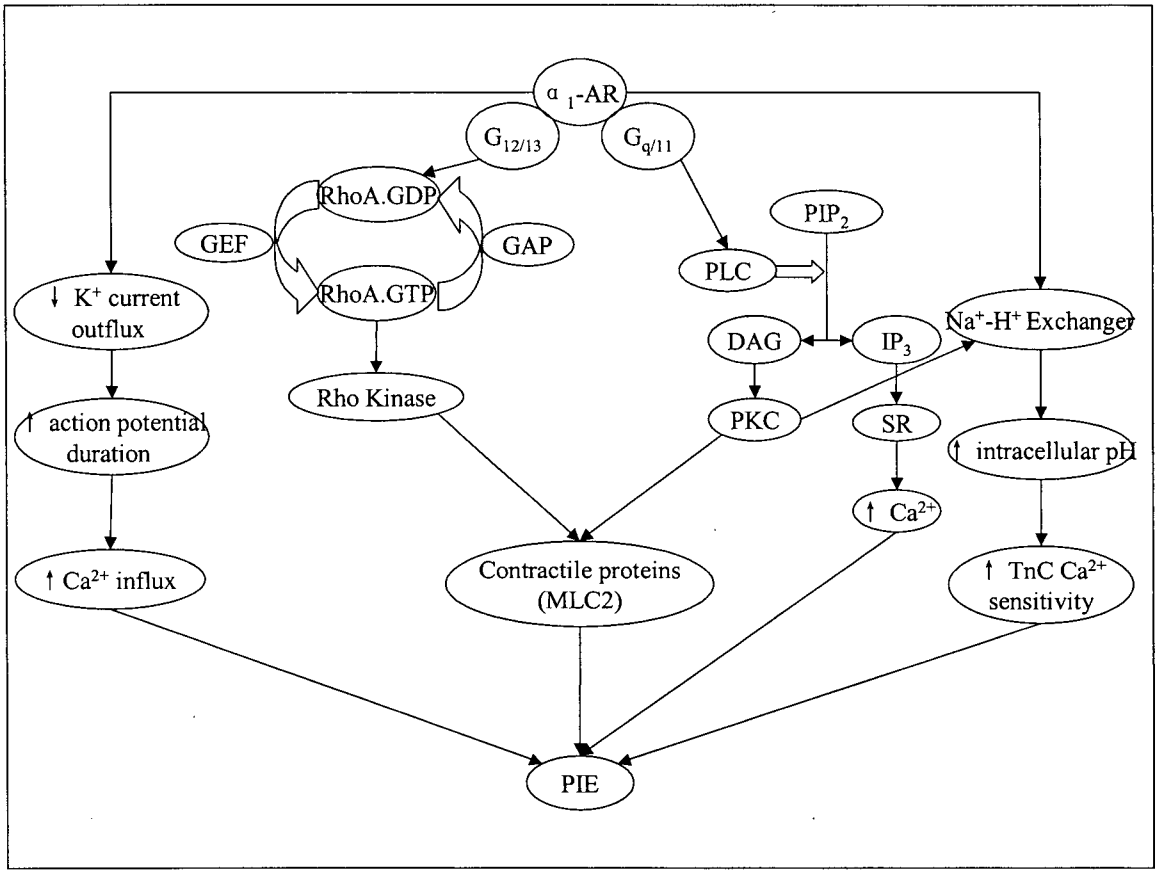

Figure 1 Possible signaling pathways underlying the α_1-AR-mediated PIE.

1.5 Diabetes mellitus

The earliest known record of diabetes mellitus is from 1552 B.C.. The 3rd Dynasty Egyptian physician Hesy-Ra mentioned frequent urination as a symptom of the disease in his papyrus. The first description is usually credited to Arataeus of Cappadocia in Asia Minor in the first century AD, who gave the disease its name, diabetes (the Greek word for siphon) mellitus (meaning honey). This was because the disease was thought to be like sweet water passing through a siphon (Medvei 1993). The discovery of insulin by Sir Frederick Banting and Dr. Charles Best and subsequently the

application of insulin in clinical treatment in the early 1920s were great medical triumphs of the last century (Ionescu-Tirgoviste 1996). The prevalence of diabetes is increasing rapidly. In 2000, the World Health Organization (WHO) estimated that over 177 million people have diabetes. This figure will go up to 300 million (5.4% of the world population) by 2025. In Canada, more than two million Canadians have diabetes. By the end of the decade, this number is expected to rise to three million (Leiter *et al.* 2001). Diabetic complications such as heart disease, stroke, kidney disease, blindness, amputation and erectile dysfunction are great threats to patients' health. Life expectancy for people with diabetes may be shortened by 5~15 years. On the other hand, the financial burden of diabetes and its complications on people with the disease and on the Canadian healthcare system is enormous. A person with diabetes incurs medical costs that are 2~3 times higher than that of a person without diabetes (data from the Canadian Diabetes Association, www.diabetes.ca).

1.5.1 Definition of diabetes mellitus

The term diabetes mellitus describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO 1999). Diabetes may have characteristic symptoms such as unusual thirst (polydipsia), frequent urination (polyuria), unexplained weight loss, extreme fatigue or lack of energy, blurred vision and slow healing. In more severe situation, ketoacidosis or a non-ketotic hyperosmolar state may occur, which lead to stupor, coma, and even death.

The long-term effects of diabetes include the progressive development of cardiovascular, peripheral vascular and cerebrovascular complications, resulting in cardiomyopathy, atherosclerosis, heart failure, nephropathy, skin ulcers, retinopathy and stroke, etc. The destruction of pancreatic β-cells with consequent insulin deficiency and tissue resistance to insulin action are the two pathogenetic processes of diabetes. The abnormalities of carbohydrate, fat and insulin metabolism are the consequence of lack of insulin and/or insensitivity of target tissues to insulin.

1.5.2 Diagnostic criteria for diabetes mellitus

In 1997, The International Expert Committee On The Diagnosis And Classification Of Diabetes Mellitus revised the diagnostic criteria of diabetes, which were based on the 1979 publication of the National Diabetes Data Group (NationalDiabetesDataGroup 1979) and subsequent WHO study group (WHO 1985). The following diagnostic criteria are recommended by WHO for the diagnosis of diabetes:

- Fasting plasma glucose ≥ 7.0 mM/l
- Two-hour postprandial plasma glucose ≥ 11.1 mM/l during an oral glucose tolerance test with an oral glucose load of 75g
- For clinical purposes, the diagnosis of diabetes mellitus should be confirmed by repeating the test on another day

1.5.3 Etiological classification of diabetes mellitus

WHO has also revised the etiological classification of diabetes mellitus. The new classification (WHO 1999) contains four categories:

- Type 1 diabetes mellitus (β-cell destruction, usually leading to absolute insulin deficiency)
    - Autoimmune (major type, with identified autoimmune disorders that lead to β-cell destruction)
    - Idiopathic (rarely seen, without evidence of autoimmune disorders)
- Type 2 diabetes mellitus (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance)
- Other specific types of diabetes mellitus
    - Genetic defects of beta-cell function
    - Genetic defects in insulin action
    - Diseases of the exocrine pancreas
    - Endocrinopathies
    - Drug- or chemical-induced
    - Infections
    - Uncommon forms of immune-mediated diabetes
    - Other genetic syndromes sometimes associated diabetes
- Gestational diabetes (glucose intolerance during pregnancy; in most cases, this disorder is normalized after labor)

1.5.4 The two major types - Type 1 and Type 2 diabetes mellitus

Type 1 diabetes mellitus accounts for about 10% of all diabetic cases. It can occur at any age, but mostly starts to develop in youth. Three mechanisms have been proposed in the pathogenesis of Type 1 diabetes: genetic susceptibility, autoimmunity and environmental factors (Krolewski *et al.* 1987; Rossini *et al.* 1988). The genetic susceptibility, mainly a defect in the allele of the class-II major histocompatability complex (HLA-D), predisposes the individual to dysfunctioning of the antigen specific cytotoxic T-lymphocytes, resulting in a slow and progressive immunological attack on pancreatic β-cells. This process is augmented by cytokine release from macrophages and NK-cells. The autoimmunity can occur spontaneously, or can be triggered by a variety of environmental factors, such as viruses and chemicals. These three mechanisms interact with each other, leading to progressive destruction of β-cells. Symptomatic diabetes mellitus and insulin dependence occur only when the β-cell mass is reduced to 10% of normal. Exogenous insulin injection is the only effective treatment for patients with Type 1 diabetes mellitus, which was identified as "insulin-dependent diabetes mellitus" in an earlier classification.

Type 2 diabetes accounts for nearly 90% of all cases, and usually occurs over the age of 35. Obesity is prominent among 50~90% of all Type 2 diabetic patients (Valle 1997). The pathogenesis of Type 2 diabetes mellitus (in the earlier classification, non-insulin-dependent diabetes mellitus) is poorly understood. The primary defect is hepatic and peripheral insulin resistance. Subsequently, a compensatory hyperinsulinemia

occurs. With time, pancreatic β-cells fail to secret sufficient insulin and to overcome the insulin resistance (Valle 1997). As a result, hyperglycemia and Type 2 diabetes occur. Patients with this type of diabetes have a strong genetic predisposition, and environmental factors, such as imbalance of nutrition and lack of exercise, also contribute to the onset of the disease. Several cellular mechanisms have been proposed in insulin receptors and intracellular signaling pathways that may contribute to insulin resistance (Valle 1997). Multiple treatment choices, including changing life style and diet, medications and exogenous insulin supplementation, can be applied to patients with Type 2 diabetes. The exact treatment plan may vary from individual to individual. However, in the final stages of Type 2 diabetes, most patients require insulin injection, since endogenous insulin decreases to a very low level due to the dysfunction of pancreatic β-cells.

1.6 Diabetic cardiomyopathy

Diabetes mellitus can result in a host of acute and chronic complications. The most dangerous acute complication is ketoacidosis, a state of absolute or relative insulin deficiency aggravated by ensuing hyperglycemia (plasma glucose > 300 mg/dL), dehydration, and acidosis (plasma pH < 7.30). Ketoacidosis often occurs in severe Type 1 diabetic cases. The chronic complications are mainly macro- and microvascular diseases. Macrovascular diseases may occur in peripheral vessels leading to gangrene; in cerebral vessels leading to intracerebral bleeding and stroke; in cardiac vessels leading to coronary artery diseases, atherosclerosis and myocardial infarction (Uccella *et al.* 1991).

Microvascular diseases result in retinopathy and nephropathy. Autonomic neuropathy, another kind of chronic diabetic complication, may contribute to diabetic hypertension.

The diabetes-induced cardiac muscle disease, diabetic cardiomyopathy, is one of the chronic diabetic complications. Cardiovascular complications are responsible for about 80% of deaths among diabetic patients (Kannel *et al.* 1979; Valle 1997), most of which has been attributed to coronary artery disease. However, diabetic cardiomyopathy has gained intensive focus since the 1970's. It was first recognized in a study on diabetic patients with heart failure, but without evidence of vascular diseases, valvular heart diseases or congenital heart diseases (Rubler *et al.* 1972). Diabetic cardiomyopathy refers to a disease process that affects the myocardium in diabetic patients, causing a wide range of structural abnormalities, eventually leading to left ventricular hypertrophy and diastolic and systolic dysfunction or a combination of both. This disease can occur and be detected without the presence of any vascular diseases (Feuvray 2004).

The cellular mechanisms underlying diabetic cardiomyopathy, though not yet completely elucidated, have been investigated in a host of studies. Hyperglycemia leads to the excess formation of advanced glycation end-products and mitochondrial reactive oxygen species, resulting in myocardial collagen deposition and fibrosis (Singh *et al.* 2001). Hyperglycemia also leads to advanced glycation of the Ca^{2+} pumps on the SR, resulting in the inactivation of the latter and prolongation of cardiac relaxation (Bidasee *et al.* 2004). Hyperlipidaemia results in increased β-oxidation and mitochondrial accumulation of long-chain acyl carnitines, leading to uncoupling of oxidative phosphorylation (Stanley *et al.* 1997). Hypoinsulinemia decreases the utilization of glucose in cardiomyocytes, resulting in enhanced utilization of fatty acids and

perturbation of myocardial bioenegetics (Rodrigues *et al.* 1998). These lead to the dysfunction of contraction / relaxation coupling and apoptosis of cardiomyocytes (Zhou *et al.* 2000). In patients with hyperglycemia, aldosterone has been suggested to mediate cardiac fibrosis through the stimulation of myofibroblast growth (Neumann *et al.* 2002). In diabetes, the renin-angiotensin system is activated (Fein *et al.* 1985), leading to cardiac hypertrophy and apoptosis (Leri *et al.* 1999; Fiordaliso *et al.* 2000; Kajstura *et al.* 2001). The cardiac expression of vascular endothelial growth factor and its receptors is decreased in diabetes, resulting in inadequate angiogenic response to ischemia and poor collateral formation, thus the patients may have an increased propensity to infarction due to a reduced reparative response (Chou *et al.* 2002). In diabetic animals, depressed mRNA levels of the Na^+-K^+ ATPase and increased mRNA levels of the Na^+/Ca^{2+} exchanger have been found in cardiomyocytes, which may result in intracellular Ca^{2+} overload and contractile deficiency (Golfman *et al.* 1998). The mRNA levels and sarcolemmal protein density of the K^+ channels (Kv2.1, Kv4.2 and Kv4.3) in ventricular myocytes from diabetic animals are decreased, which may lead to cardiac arrhythmia (Qin *et al.* 2001). Cardiac autonomic neuropathy has been suggested in diabetic cardiomyopathy. Patients with Type 1 diabetes exhibit cardiac autonomic neuropathy and abnormal diastolic filling (Kahn *et al.* 1986). Sympathetic dysfunction has been related to both systolic and diastolic dysfunction in Type 2 diabetes (Annonu *et al.* 2001). In all, a range of molecular changes may underlie the development of diabetic cardiomyopathy.

1.7 Streptozotocin-diabetic animal models

Diabetic animal models are widely used as they provide a means to understand and explore the etiology, pathogenesis and treatment strategies in human diabetes. A variety of Type 1 and Type 2 diabetic animal models have been developed (Rodrigues *et al.* 1999b). Genetic Type 1 diabetic models include the diabetic biobreeding (BB) rats and the non-obese diabetic (NOD) mouse. In these models, diabetes occurs spontaneously, and the animals depend on exogenous insulin for survival. Chemically induced Type 1 models include alloxan-induced and streptozotocin (STZ)-induced diabetic rats. Both of the chemicals specifically destroy pancreatic β-cells. Since these models closely reproduce the lesions in human Type 1 diabetes, and they produce permanent and stable diabetes, they are of specific interest in diabetic research. STZ has replaced alloxan as the principal chemical to induce experimental diabetes because of its greater selectivity for β-cells, lower mortality rate and longer half-life (Rodrigues *et al.* 1999b). Genetic Type 2 diabetic models include db/db mice, fa/fa diabetic Zucker rats, etc. These models demonstrate some manifestations of human Type 2 diabetes, such as hyperglycemia, hyperinsulinemia and obesity. There are also some chemically induced Type 2 diabetic models. Although genetic models provide the possibility to investigate the genetic predisposition of diabetes and the influence of environmental factors on the pathogenesis of the disease, the use of these models is limited due to their high cost. Chemically induced models are less expensive. Moreover, their duration of diabetes and the severity of the disease can be better controlled. As a result, these models, especially STZ models, have been used widely in diabetic research.

STZ-diabetic rats exhibit characteristic symptoms similar to human Type 1 diabetes, such as ploydipsia, polyphagia (increased food intake) and weight loss. Hyperglycemia, hypoinsulinemia and increased levels of plasma lipids also occur in these models (Junod *et al.* 1969). The etiology of diabetic cardiomyopathy in STZ-diabetic rats appears to be similar to that in human Type 1 diabetes. The subcellular changes in the sarcolemma, the mitochondria, the SR and the contractile proteins are found in STZ-diabetic hearts (Rodrigues *et al.* 1999a). These eventually lead to left ventricular hypertrophy and diastolic and systolic dysfunction (Tahiliani *et al.* 1983; Mihm *et al.* 2001).

Depressed responses to noradrenaline, which subsequently result in attenuated cardiac contractile reserve, is one major type of cardiac dysfunction in STZ-diabetic animals (Gotzsche 1983a; Gotzsche 1983b; Smith *et al.* 1984). As mentioned previously, β-AR is the predominant adrenergic receptor through which noradrenaline exerts its actions on cardiac muscle (Leone *et al.* 2002). As a result, the depressed cardiac response to noradrenaline in these animals is possibly due to defects in the β-AR-mediated signaling cascade. Several studies have been reported in support of this hypothesis (Gotzsche 1983a; Gotzsche 1983b; Smith *et al.* 1984). On the other hand, alterations in the α_1-AR-mediated PIE in the STZ-diabetic heart have also been well-recognized.

1.8 Effects of STZ-diabetes on the α_1-AR-induced PIE and on the components of the signaling pathways coupled to α_1-AR

The α_1-AR-mediated PIE in right ventricular strips (Wald *et al.* 1988; Yu *et al.* 1991) and working hearts (Heijnis *et al.* 1992) isolated from STZ-induced diabetic rats was enhanced. Similar findings were shown in atria (Canga *et al.* 1986; Jackson *et al.* 1986; Durante *et al.* 1989; Brown *et al.* 1994) and left ventricular papillary muscles (Brown *et al.* 1994). These findings are intriguing because when β-AR-mediated responses in diabetic heart are diminished, the augmented α_1 responses have been proposed to help compensate to maintain cardiac performance (Corr *et al.* 1981; Milligan *et al.* 1994; Beaulieu *et al.* 1997; Skomedal *et al.* 1997). However, not all studies are in agreement with these findings, as in some investigations the α_1-AR-mediated PIE was reported to be attenuated in myocardial preparations from STZ-diabetic rats (Heyliger *et al.* 1982; Williams *et al.* 1983; Sunagawa *et al.* 1987). These discrepancies may be due to differences in the duration of diabetes or in experimental conditions between studies.

Besides the above functional studies, a number of investigations have suggested diabetes-induced changes in the components of the signaling pathways coupled to α_1-AR in the heart. Binding studies have consistently found that in diabetic cardiomyocytes, the number of α_1-AR-binding sites is reduced (Heyliger *et al.* 1982; Wald *et al.* 1988). This is associated with no change (Heyliger *et al.* 1982; Tanaka *et al.* 1992) or an increase (Wald *et al.* 1988) in their affinity constants. The enhanced α_1-AR-mediated PIE in the diabetic heart was associated with increased IP_3 production (Xiang *et al.* 1991), suggesting that α_1-AR-mediated stimulation of PLC is enhanced. However, opposite evidence suggested that IP_3 production in response to α_1-AR stimulation was decreased in diabetic cardiomyocytes (Tanaka *et al.* 1992; Tanaka *et al.* 1993). This discrepancy may be due to the different rat strains and different experimental protocols. A number of

investigations have indicated that STZ-diabetes affects PKC activity or levels of PKC isoforms in the particulate fractions in rat hearts, but the results are far from consistent. For instance, a high basal PKC activity was found in diabetic rat hearts, associated with a decrease in cell surface α_1-AR density and reduced IP_3 production in response to α_1-AR stimulation (Tanaka *et al.* 1992). Increased activity of particulate PKC along with elevated particulate levels of PKCβ_2, and increased intracellular levels of DAG were found in diabetic hearts, without any change in particulate levels of PKCα (Inoguchi *et al.* 1992). In another study, particulate PKCε was increased in diabetic cardiomyocytes while no change was found in PKCδ, however PKCβ was not even detected (Malhotra *et al.* 1997). On the other hand, Liu *et al.* (1999) reported that the total (soluble plus particulate) levels of PKC α, β and ε were increased in STZ-diabetic rat hearts, but the particulate levels of these isoforms were not changed (Liu *et al.* 1999). In another study, the total level of PKCα was increased, accompanied by reduced total levels of PKCε and no change in PKCβ_1, β_2 and δ (Kang *et al.* 1999). These varied results may be due to the difference in strains of rats, duration of diabetes, cardiac tissues and experimental conditions. Though there are discrepancies in these studies, they still suggest there may be changes in PKC activity and particulate levels of PKC isoforms in diabetic hearts. However, whether diabetes affects α_1-AR-induced changes in PKC in rat hearts has not been established.

1.9 Contribution of the activated renin-angiotensin system (RAS) to diabetic cardiomyopathy

The RAS is classically viewed as an enzymatic protein cascade (Volpe *et al.* 2002). The first component of the system is angiotensinogen, which forms angiotensin I in the presence of renin, an enzyme synthesized and released from kidney. Angiotensin I is subsequently transformed to angiotensin II by the action of angiotensin-converting enzyme (ACE). Angiotensin II is the terminal biologic effector of the system. Under physiological conditions, angiotensin II plays an important role in regulating the cardiovascular system to maintain homeostasis. It also participates in the regulation of salt and water balance and cellular growth (Volpe *et al.* 2002). Angiotensin II, which is a biologically active peptide, can bind to two receptor subtypes, type 1 (AT_1) and type 2 (AT_2) receptors (Bumpus *et al.* 1991). The principal actions of RAS in heart, vessels, kidney, brain, and other tissues and organs are mainly mediated by AT_1 receptors (Volpe *et al.* 2002).

A number of investigations suggest that the RAS is altered in STZ-diabetic animal models. Plasma renin concentration (Ubeda *et al.* 1988) and activity (Funakawa *et al.* 1983) are reduced, which may be due to hyalinization of the renin secreting structure in the kidney (Nakamura *et al.* 1978) and a reduction in renal prostaglandin production (Funakawa *et al.* 1983). Circulating levels of ACE are increased in STZ models, but the mechanisms for this phenomenon are not clear (Valentovic *et al.* 1987; Hartmann *et al.* 1988). Alterations in the other components of RAS are less obvious as compared to renin and angiotensin-converting enzyme. Plasma angiotensinogen levels have been found to be unchanged (Cassis 1992) or reduced (Brown *et al.* 1997). Circulating angiotensin II levels have also been found normal (Vallon *et al.* 1995) or reduced (Kigoshi *et al.* 1986). However, the existence of a local cardiac RAS comprising all components has been

shown (Dostal *et al.* 1992a; Dostal *et al.* 1992b; Silvestre *et al.* 1998). Although the circulating levels of angiotensin II may not increase in diabetes, left ventricles from STZ-diabetic rats have been reported to have higher ACE levels than normal (Goyal *et al.* 1998), and an up-regulation of the local RAS has been suggested in the diabetic heart (Fein *et al.* 1985; Rosen *et al.* 1995; Hayat *et al.* 2004).

Though there are uncertainties in the diabetes-induced changes in the RAS, the contribution of this system to diabetic cardiomyopathy, mainly through AT_1 receptors (Dzau 2001), has been suggested by several in vitro, in vivo, and even clinical studies. AT_1 receptor blockers prevent the attenuated contractile performance of isolated cardiomyocytes exposed to hyperglycemic medium (Privratsky *et al.* 2003). Incubation of diabetic cardiomyocytes with ACE inhibitors or AT_1 receptor blockers restores their depressed electrical properties (Shimoni 2001). Treatment of STZ-diabetic rats with AT_1 receptor blockers has been shown to prevent the decline of glucose transporters (GLUT4s) (Hoenack *et al.* 1996) and to improve glucose uptake in the heart (Raimondi *et al.* 2004). Treatment of diabetic rats with ACE inhibitors has been reported to improve cardiac function (Goyal *et al.* 1998; Al-Shafei *et al.* 2002). ACE inhibitors have also been shown to improve the outcome of heart failure in diabetic patients (Shekelle *et al.* 2003). AT_1 receptor blockers have been suggested to be a novel therapeutic approach for the treatment of diabetic cardiomyopathy and the prevention of sudden cardiac death (Taegtmeyer *et al.* 2002).

The molecular mechanisms underlying the role for RAS in diabetic cardiomyopathy, though still not clear, have been related to increased expression of AT_1 receptors (Sechi *et al.* 1994) and the up-regulation of the downstream signaling pathways,

which are very similar to those of α_1-ARs. AT_1 receptors also couple to $G_{q/11}$ proteins. Activation of the receptor also leads to the activation of PLC and subsequently the generation of DAG and IP_3 (Mattiazzi 1997). Thus PKC-related pathways may participate in this process. Malhotra *et al.* (1997) have found that PKCϵ translocated from the soluble to the particulate fraction in isolated cardiomyocytes from 4-week STZ-diabetic rats, accompanied by increased TnI phosphorylation. Treatment of the diabetic rats with the selective AT_1 antagonist, L-158,809, completely prevented both the change in the subcellular distribution of PKCε and the elevated phosphorylation of TnI. Since phosphorylation of TnI by PKC in vitro is associated with inhibition of the Ca^{2+}-stimulated MgATPase activity both of myofibrils and of reconstituted actomyosin complexes (Noland *et al.* 1993b; Venema *et al.* 1993a), and abnormalities in the regulatory proteins in myofibrils and myocytes from diabetic animals are associated with diminished Ca^{2+} sensitivity and impaired contractile performance (Hofmann *et al.* 1995; Malhotra *et al.* 1995), this PKC-dependent pathway is a possible mechanism that is involved in AT_1 receptor-mediated diabetic cardiomyopathy.

1.10 Experimental rationales and hypotheses

Although it has been shown that experimental diabetes affects the α_1-AR-induced PIE, results from previous studies have not been consistent. Because PKC and Rho kinase are downstream of α_1-ARs, and have been shown to contribute to the α_1-AR-induced PIE, in this study, it was hypothesized that PKC and/or Rho kinase play a role in the altered α_1-AR-induced PIE in diabetes.

Previous studies indicated that diabetes is associated with changes in PKC activity and the subcellular distribution of PKC isozymes; on the other hand, activation of the RAS contributes to diabetic cardiomypathy, in which a PKC-dependent pathway may be involved. In the present study, it was hypothesized that blockade of AT_1 receptors would improve the impaired cardiac contractile function, prevent the enhanced α_1-AR-induced PIE in diabetes and normalize the associated changes in PKC.

To test the first hypothesis, the isolated Langendorff-perfused isovolumic heart model was used to determine the effect of STZ-diabetes on the α_1-AR-mediated PIE. PKC and Rho kinase inhibitors were also used to clarify their role in diabetes-induced changes in the α_1-AR-mediated PIE. Moreover, Western blot was performed to measure the associated changes in the subcellular distribution of the isozymes of these two kinases, as an index of their activation.

To test the second hypothesis, chronic treatment with AT_1 receptor antagonists was performed in long-term STZ-diabetic rats. Basal cardiac contractile function and the PIE to α_1-AR stimulation were subsequently measured, and Western blot was used to determine the associated changes in PKC isoforms.

2 MATERIALS AND METHODS

2.1 Chemicals and materials

2.1.1 Langendorff heart studies

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): Streptozotocin, sodium chloride, calcium chloride, potassium chloride, potassium phosphate monobasic, magnesium sulfate, sodium bicarbonate, glucose, pyruvate, ethylenediaminetetraacetic acid (EDTA), timolol, phenylephrine, isoproterenol, chelerythrine chloride (CE), bisindolylmaleimide I (BIM I), bisindolylmaleimide IX (Ro318220, RO).

Sodium pentobarbital was purchased from MTC Pharmaceuticals (Cambridge, ON). H1152 and Y-27632 were purchased from Calbiochem Co. (Mississauga, ON). L-158,809 was purchased from Merck (Rahway, NJ).

2.1.2 Enhanced chemiluminescence Western blot studies

The following chemicals and materials were purchased from Bio-Rad (Hercules, CA): Tris[hydroxymethyl]aminomethane (Tris), 2-mercaptoethanol, sodium dodecyl sulfate (SDS), glycine, protein assay dye reagent, skim milk powder, nitrocellulose membrane.

The following chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO): polyoxyethylenesorbitan monolaurate (Tween 20), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), EDTA, sodium fluoride, leupeptin, aprotinin, deoxycholic acid, NP40.

4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) was purchased from CalBiochem Co. (Mississauga, ON). Enhanced chemiluminescence detection kit was purchased from Amersham Biosciences (United Kingdom).

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): PKC α, β_2, δ and ε (rabbit polyclonal), ROCK 1 and ROCK 2 (rabbit polyclonal), actin (goat polyclonal), goat anti-rabbit IgG-HRP, donkey anti-goat IgG-HRP.

2.2 Experimental protocols

2.2.1 Animals and blood samples

Male Wistar rats weighing 180-200g were obtained from the University of British Columbia Animal Care Unit and were housed and treated in accordance with the guidelines of the Canadian Council of Animal Care. STZ was dissolved in citrate buffer (pH 4.5) and diabetes was induced by injection of 60 mg/kg STZ into the lateral tail vein of rats lightly anesthetized with halothane. Control rats received the citrate buffer vehicle. STZ-treated rats with blood glucose levels of 13 mmol/L or greater, measured with an Ames glucometer one week after injection, were considered diabetic and were kept for

experiments. The diabetic state of the animals was confirmed at the time of the experiments, by measurement of plasma insulin and glucose levels. Six to seven or twelve to fifteen weeks later, animals were weighed and given an overdose of sodium pentobarbital. After the rat was deeply anesthetized, the heart was excised. Blood was collected from the chest cavity (in the presence of heparin) and spun in a centrifuge for 20 minutes at 17,000 x g for 15 minutes for the separation of plasma. The plasma samples were stored in -20°C for the later measurement of glucose and insulin levels.

2.2.2 Measurement of plasma glucose and insulin

Plasma glucose was determined using an assay kit from Roche (Laval, PQ). Plasma insulin was measured with a radioimmunoassay kit from Cedarlane (St. Charles, MO).

2.2.3 Langendorff heart studies

The excised heart was placed in ice-cold Krebs-Henseleit (K-H) bicarbonate buffer (95% O_2 – 5% CO_2) containing (in mM) 1.75 $CaCl_2$, 117.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.3 KH_2PO_4, 24.7 $NaHCO_3$, 11.0 glucose, 5.0 pyruvate and 0.5 EDTA. The non-selective β-AR anatagonist, timolol (1μM), was present in K-H buffer in all the following experiments (unless specified) to minimize PE effects through β-ARs. The heart was quickly cannulated via the aorta and perfused with the above K-H buffer (37°C). A pressure transducer was connected to the aortic cannula to monitor coronary

perfusion pressure (CPP). A balloon was inserted into the left ventricle via a cut in the left atria, and a second pressure transducer was connected to the balloon for the measurement of left ventricular developed pressure (LVDP), from which the maximal rate of contraction (+dP/dt) and relaxation (-dP/dt) were calculated using custom-written computer software. The balloon volume was adjusted to yield an end-diastolic pressure of 5 mmHg, which was then maintained constant throughout the experiment. The coronary flow rate was adjusted to give a CPP of 80 mmHg, which was determined in preliminary experiments to result in optimal basal contractile performance and PIE to α_1-AR stimulation, and then remained constant throughout the experiment. The heart was then paced at 300 beats/min, and was allowed to stabilize and equilibrate before the administration of adrenergic agonists.

2.2.3.1 Preliminary experiment to determine the coronary perfusion pressure (CPP) at which the heart developed optimal basal contractile performance and α_1-AR-induced PIE

Two groups of normal hearts (four in each group) were used, one of which was perfused with a CPP of 50 mmHg (CPP50 group) and the other with a CPP of 70 mmHg (CPP70 group). After a brief equilibration, the basal LVDP, +dP/dt and -dP/dt were measured. Subsequently a single dose of PE (10^{-5} M) was added to the perfusion buffer, and the increases in these contractile parameters in response to PE were measured.

2.2.3.2 Preliminary experiment to confirm that PE selectively activates α-ARs

Although PE is a selective α_1-AR agonist, it could have potential effects through other ARs in the heart, such as β-ARs. To ensure that the PIE was produced by the stimulation of α-AR alone, not by the stimulation of β-ARs, the following experiment was performed.

Two groups of normal hearts (three in each group) were used. Phentolamine (an α-AR antagonist; 10^{-5} M) was added to the K-H buffer in one group, while the other group was perfused with normal K-H buffer. Timolol (10^{-6} M) was added to the K-H buffer in both groups. After 20 minutes of equilibration, cumulative PE concentration-response curves (PE CRCs) were performed in both groups. Subsequently an apparent affinity constant (pK_B value) for phentolamine was calculated using the equation pK_B = log (concentration ratio – 1) – log [molar antagonist concentration], where the concentration ratio = EC_{50} of PE in the phentolamine group / EC50 of PE in the control group (Arunlakshana *et al.* 1959; Bowman *et al.* 1980; Kenakin 1987). The pK_B value was then compared to the pK_i values of phentolamine for α_1-AR obtained from previous binding studies.

2.2.3.3 Cardiac Function Study #1: Investigation of the effects of diabetes on the α_1-AR-mediated PIE

Hearts from two sets of diabetic rats (6~7 weeks and 12~15 weeks, respectively) and age-matched control rats were used (n = 9~10 hearts in each group). After a brief

equilibration period, the basal LVDP, +dP/dt and -dP/dt were measured. Subsequently PE concentration-response curves (PE CRCs, 10^{-9} M ~ 10^{-4} M) were determined in each group. The maximal response (Rmax) values and $-\log[EC_{50}]$ (pD_2) values of the PE CRCs were calculated using GraphPad Prism 4 computer software. In some hearts from the second set of rats (12~15 week diabetic and control), the heart weight and coronary perfusion flow rate (at a CPP of 80 mmHg, in the absence of PE) were measured, and the ratios of flow rate / heart weight were calculated.

2.2.3.4 Cardiac Function Study #2: Investigation of the role for PKC in the α_1-AR-induced PIE

To clarify the role for PKC in the PIE to the stimulation of α_1-ARs, the effects of a PKC inhibitor on PE-induced PIE were determined.

2.2.3.4.1 Choice of PKC inhibitor

Three non-isoform selective PKC inhibitors were used: bisindolylmaleimide I (BIM I), bisindolylmaleimide IX (Ro318220, RO) and chelerythrine (CE). Eight normal hearts were divided into four groups (two in each group): no PKC inhibitor was present in the control group, while BIM I ($3*10^{-6}$ M), RO ($2*10^{-6}$ M) and CE (10^{-5} M) were present in the other three groups, respectively. The hearts were perfused with K-H buffer or K-H buffer containing the PKC inhibitor for 20 minutes, at the end of which the

LVDP, +dP/dt and -dP/dt were determined. Subsequently a single dose of PE (10^{-5} M) was added to the perfusion buffer. At the end of a 2-minute perfusion with PE, the increases in these parameters were measured.

2.2.3.4.2 Effect of chelerythrine on the PIE induced by β-AR stimulation in normal hearts

To investigate whether CE had any nonspecific effects on other signaling cascades, the effect of this PKC inhibitor on the PIE induced by the β-adrenoceptor agonist, isoproterenol, was determined.

Nine normal hearts were divided into two groups, a control group (n = 5) and a CE-treated group (10^{-5} M CE was present in the perfusion buffer, n = 4). The hearts were perfused with K-H buffer or K-H buffer containing CE for 20 minutes, at the end of which the LVDP, +dP/dt and -dP/dt were determined. Subsequently a single dose of isoproternol (10^{-6} M) was added to the perfusion buffer. At the end of a 1-minute perfusion with isoproternol, the increases in these parameters were measured. It should be noted that in this experiment, all the hearts were perfused at a basal CPP of 70 mmHg and paced at 350 beats/min before the administration of isoproterenol. As a result, a lower basal contractile performance as compared to previous experiments was obtained (section 3.1.5.2).

2.2.3.4.3 Effect of chelerythrine on basal contractile performance and the α_1-AR-induced PIE in hearts from 12~15 week diabetic and age-matched control rats

Hearts from 12~15 week diabetic and age-matched control rats were divided into groups and treated as follows:

- Basal group (B) – hearts were perfused with K-H buffer for 22 minutes;
- CE group (CE) – hearts were perfused with K-H buffer containing CE (10^{-5} M) for 22 minutes;
- PE group (PE) – hearts were perfused with K-H buffer for 20 minutes, followed by treatment of a single dose of PE (10^{-5} M) for 2 minutes;
- CE + PE group (CE+PE) – hearts were perfused with K-H buffer containing CE (10^{-5} M) for 20 minutes, followed by treatment with a single dose of PE (10^{-5} M; in the presence of 10^{-5} M CE) for 2 minutes.

The basal LVDP, +dP/dt, -dP/dt and the increase in these parameters in response to PE (at the end of the 2-minute perfusion) were measured. Following the treatment period the hearts were quickly removed from the cannula and the aorta, atrium and right ventricles were removed and discarded. Left ventricles (including the left ventricular walls and the septa) were snap frozen in liquid nitrogen and stored at -70°C for Western blot analysis.

2.2.3.5 Cardiac Function Study #3: Investigation of the role for Rho kinase in the α_1-AR-induced PIE

To clarify the role for Rho kinase in the PIE to α_1-AR stimulation, the effects of two non-isoform-selective Rho kinase inhibitors, Y-27632 and H1152, on the PE-induced PIE were determined.

2.2.3.5.1 Effect of Y-27632 on basal contractile performance and the α_1-AR-induced PIE in normal hearts

Six normal hearts were divided into two groups, a control group (n = 3) and a Y-27632-treated group (10^{-6} M Y-27632 was present in the perfusion buffer, n = 3). The hearts were perfused with K-H buffer or K-H buffer containing Y-27632 for 20 minutes, at the end of which the LVDP, +dP/dt and -dP/dt were determined. Subsequently PE CRCs (10^{-8} M ~ 10^{-4} M) were performed and the Rmax and pD_2 values were calculated.

2.2.3.5.2 Effect of H1152 on basal contractile performance and the α_1-AR-induced PIE in hearts from normal and 12-week diabetic rats

H1152, which is also a non-isoform-selective Rho kinase inhibitor, is more potent than Y-27632. Nine hearts obtained from normal rats weighing 400~450 g were divided into two groups, a control group (n = 4) and an H1152-treated group (10^{-6} M H1152 was

present in the perfusion buffer, n = 5). The hearts were perfused with K-H buffer or K-H buffer containing H1152 for 20 minutes, at the end of which the LVDP, +dP/dt and -dP/dt were determined. Subsequently a single dose of PE (10^{-5} M) was added to the perfusion buffer. At the end of a 2-minute perfusion with PE, the increases in these parameters were measured.

In order to investigate whether Rho kinase also plays a role in the PIE to α_1-AR stimulation in diabetic state, nine hearts obtained from 12-week diabetic rats were divided into two groups, a control group (n = 4) and an H1152-treated group (n = 5). The treatment protocol was the same as above.

2.2.3.6 Cardiac Function Study #4: Investigation of effects of AT_1 receptor blockade on basal contractile performance and the α_1-AR-induced PIE in hearts from 12-week diabetic and age-matched control rats

An AT_1 receptor antagonist, L-158,809, was used. One week after STZ or vehicle injection, diabetic and control rats were divided into two groups. One group of diabetic or control rats was treated with L-158,809 (1 mg/kg/day) orally in their drinking water for eleven weeks. The other group of diabetic or control rats remained untreated. At the time of termination, the rat hearts were further separated into 2 subgroups:

- Basal group (B) – hearts were perfused with K-H buffer for 22 minutes;
- PE group (PE) – hearts were perfused with K-H buffer for 20 minutes, followed by treatment with a single dose of PE (10^{-5} M) for 2 minutes.

The basal LVDP, +dP/dt, -dP/dt and the increase in these parameters at the end of PE perfusion were measured. Following the treatment period the hearts were quickly removed from the cannula and the aorta, atrium and right ventricles were removed and discarded. The left ventricles (including the septum) were snap frozen in liquid nitrogen and stored at -70°C for Western blot analysis.

2.2.4 Enhanced chemiluminescence Western blot studies

The frozen left ventricle preparations were powdered, homogenized and sonicated in EGTA buffer containing Tris-HCl (20 mM), 2-mercaptoethanol (50 mM), EGTA (5 mM), EDTA (2 mM), NaF (1 mM), AEBSF (1 mM), leupeptin (25 μg/ml) and aprotinin (2 μg/ml). The homogenized samples were spun in a centrifuge at 600 x g for 3 minutes to precipitate unbroken cardiomyocytes and organelles and the supernatant was then centrifuged at 100,000 x g for 1 hour. The resulting supernatant was retained as the soluble fraction, and the pellets were re-suspended in EGTA buffer containing (v/v) 1% NP40, 0.1% SDS and 0.5% deoxycholic acid. Following centrifugation at 100,000 x g for another 1 hour, the supernatant was collected and used as the particulate fraction. The protein content of each fraction was determined using the Bradford protein assay. Equal amounts of protein (50 μg) from each fraction were subjected to SDS-PAGE on 11% polyacrylamide gels. This amount of protein was shown to fall within the linear range of densitometric detection in preliminary experiments (section 3.2.1). The resolved proteins were electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk in 0.05% (v/v) Tween-20/TBS (tris buffer saline containing

20 mM tris and 250 mM NaCl) solution and incubated with the appropriate PKC isoform-specific primary antibodies [α, β_2, δ and ε; rabbit polyclonal, 1:500 (v/v)] or Rho kinase isoform-specific primary antibody [ROCK 1 and ROCK 2; rabbit polyclonal, 1:180 (v/v)] overnight at 4°C. Actin was used as an internal control and membranes were incubated with actin primary antibody [goat polyclonal, 1:200 (v/v)] in the manner described above. Immune complexes were detected following incubation of membranes with horseradish peroxidase conjugated anti-rabbit or anti-goat secondary antibody [1:20,000 (v/v); in 3% skim milk] for two hours at room temperature using an enhanced chemiluminescence detection kit. Band intensity was analyzed by densitometry and normalized for actin on the same membrane using a method similar to that described by a study (Ping *et al.* 1997). In brief, the average density of actin bands for each fraction (control soluble, control particulate, diabetic soluble and diabetic particulate) was calculated. The density of actin in each lane was divided by the corresponding average density, generating a correction value. The density of PKC or Rho kinase isoform band in each lane was then adjusted by dividing it by the corresponding correction value.

2.2.4.1 Preliminary experiment to determine the appropriate amount of protein that should be loaded

This preliminary experiment was done to determine the appropriate amount of protein that should be loaded into each lane to ensure that this amount of protein falls within the linear range of densitometric detection. Increasing amounts of cardiac protein (20, 30, 40, 50, 60 μg) were loaded onto the same polyacrylamide gel and the

densitometric values of soluble and particulate PKCδ, PKCα, ROCK 1 and ROCK 2 were obtained. The densitometric reading was then plotted against the amount of protein and subjected to linear regression analysis, from which the R^2 values were calculated.

2.2.4.2 Effect of diabetes, α_1-AR stimulation and PKC inhibition on the subcellular distribution of PKC and Rho kinase isoforms

In this experiment, the left ventricle preparations were from the previous experiment (section 2.2.3.4.3). The soluble and particulate protein levels of four PKC isoforms (α, β_2, δ and ε) and two Rho kinase isoforms (ROCK 1 and ROCK 2) were measured.

2.2.4.3 Effect of L-158,809 treatment on the levels of PKCδ, PKCε, ROCK 1 and ROCK 2 in the particulate fraction in unstimulated and PE-stimulated hearts from 12-week diabetic and age-matched control rats

In this experiment, the left ventricle preparations were from the previous experiment (section 2.2.3.6). The particulate protein levels of two PKC isoforms (δ and ε) and two Rho kinase isoforms (ROCK 1 and ROCK 2) were measured.

2.3 Statistical analyses

All data were presented as mean ± standard error of mean, unless specified. PE CRCs were analyzed by non-linear regression for calculation of pD_2 ($-\log[EC_{50}]$) values and maximum responses (Rmax) values. Statistical significance was evaluated by one-way or two-way ANOVA followed by Newman-Keuls post-hoc test for multiple comparisons in NCSS 2000 computer software. A P-value < 0.05 was considered statistically significant.

3 RESULTS

3.1 Langendorff heart studies

3.1.1 Preliminary experiment to determine the CPP at which the heart developed optimal basal contractile performance and α_1-AR-induced PIE

As shown in Figure 2, the basal LVDP, +dP/dt, -dP/dt and the increase in these parameters in response to PE in hearts perfused at a CPP of 70 mmHg (CPP70) were all significantly higher than those in hearts perfused at a CPP of 50 mmHg (CPP50). This suggests that a higher CPP produces a better basal cardiac performance and a greater response to PE. However, there is a limit to the increase in CPP, because the load on the heart increases with the increase in CPP, eventually resulting in heart failure. In order to determine the optimal CPP at which the best basal cardiac performance and the greatest PE response could be obtained, several normal hearts were perfused with a CPP of 90 mmHg. Unfortunately all these hearts failed quickly (i.e. the diastolic pressure was not steady and increased gradually). However, at a CPP of 80 mmHg the hearts did not fail (section 3.1.2). Therefore, in all the following experiments, the hearts were perfused at a basal CPP of 80 mmHg, unless specified.

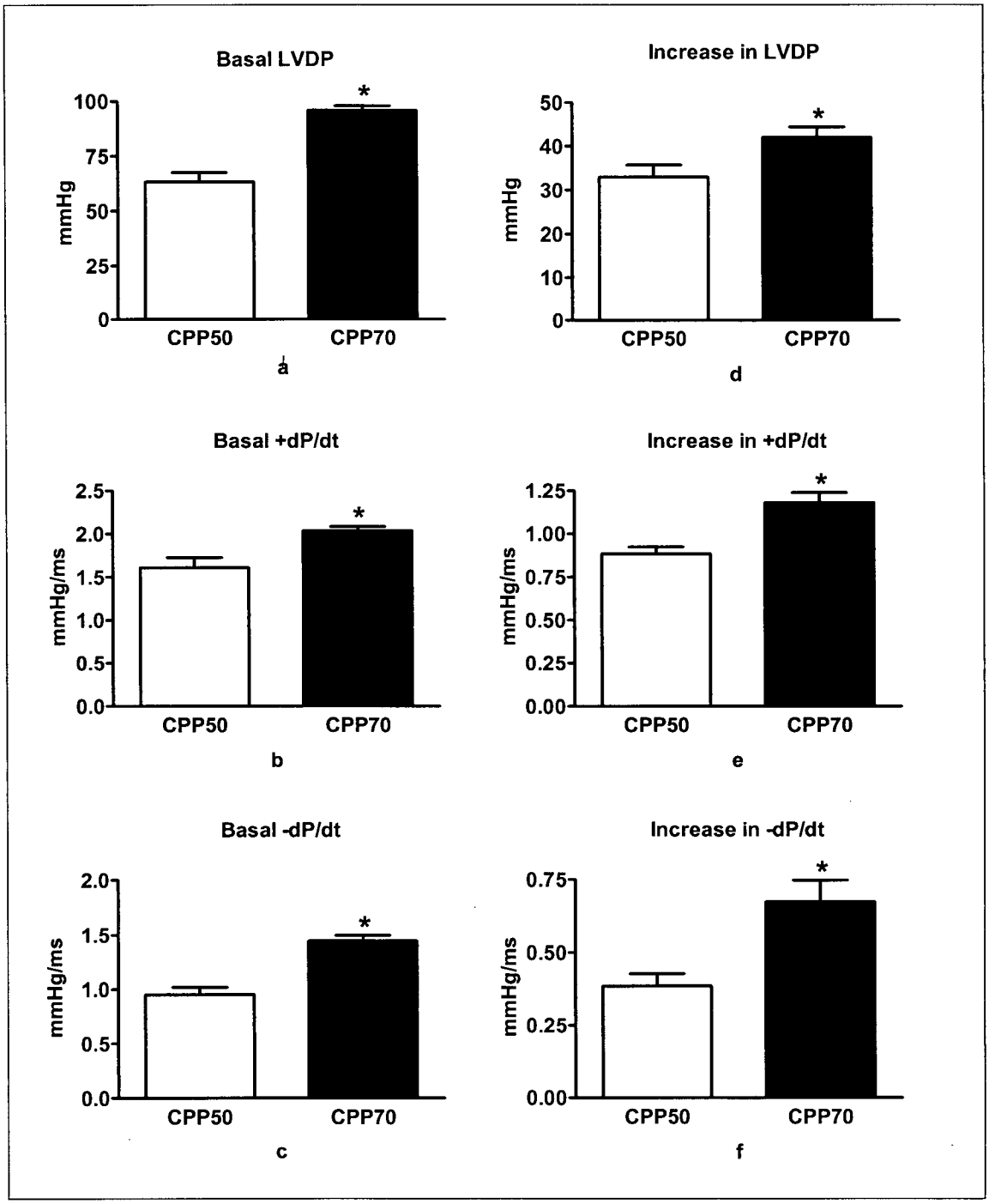

Figure 2 Basal LVDP (a), +dP/dt (b), -dP/dt (c) and the increase in these parameters (d, e, f) in response to PE (10^{-5} M) in the CPP50 group (in which the hearts were perfused at a basal CPP of 50 mmHg) and the CPP70 group (in which the hearts were perfused at a basal CPP of 70 mmHg). n = 4 hearts in each group.

* Significantly different from corresponding CPP50 groups. $P<0.05$

3.1.2 Preliminary experiment to confirm that PE selectively activates α-ARs

As shown in Figure 3, 10^{-5} M phentolamine produced a significant rightward shift of the PE CRC. The calculated pK_B values of phentolamine were 7.77, 7.66 and 7.66 for the increase in LVDP, +dP/dt and -dP/dt, respectively. The mean pK_i values of phentolamine for cloned human α_{1A}-, α_{1B}- and α_{1D}-ARs were reported to be 8.17, 7.20 and 7.48, respectively (Yan *et al.* 2001); while the mean pK_i value of phentolamine for rat cardiac α_1-ARs was found to be 8.25 (Asano *et al.* 1990). Therefore, the results from the current study were within the range of the values reported for phentolamine acting at α_1-ARs in other studies. It should be noted that only one concentration (10^{-5} M) of phentolamine was used in the experiment and no Schild graph was plotted. However, the observation that the pK_B values were close to the pK_i values in the literature, suggests that in the presence of 10^{-6} M timolol, the PIE to PE was produced by the stimulation of α-AR alone.

In this preliminary experiment, the concentration range of the PE CRCs in the control group was 10^{-9}~10^{-4} M (Figure 3). In all three contractile parameters, 10^{-9} M PE produced a small PIE, while the PIE produced by 10^{-5} M PE was nearly maximal. This suggests that 10^{-9}~10^{-4} M is the appropriate concentration range of PE CRCs. In the following experiments with PE CRCs, this concentration range was used, unless specified.

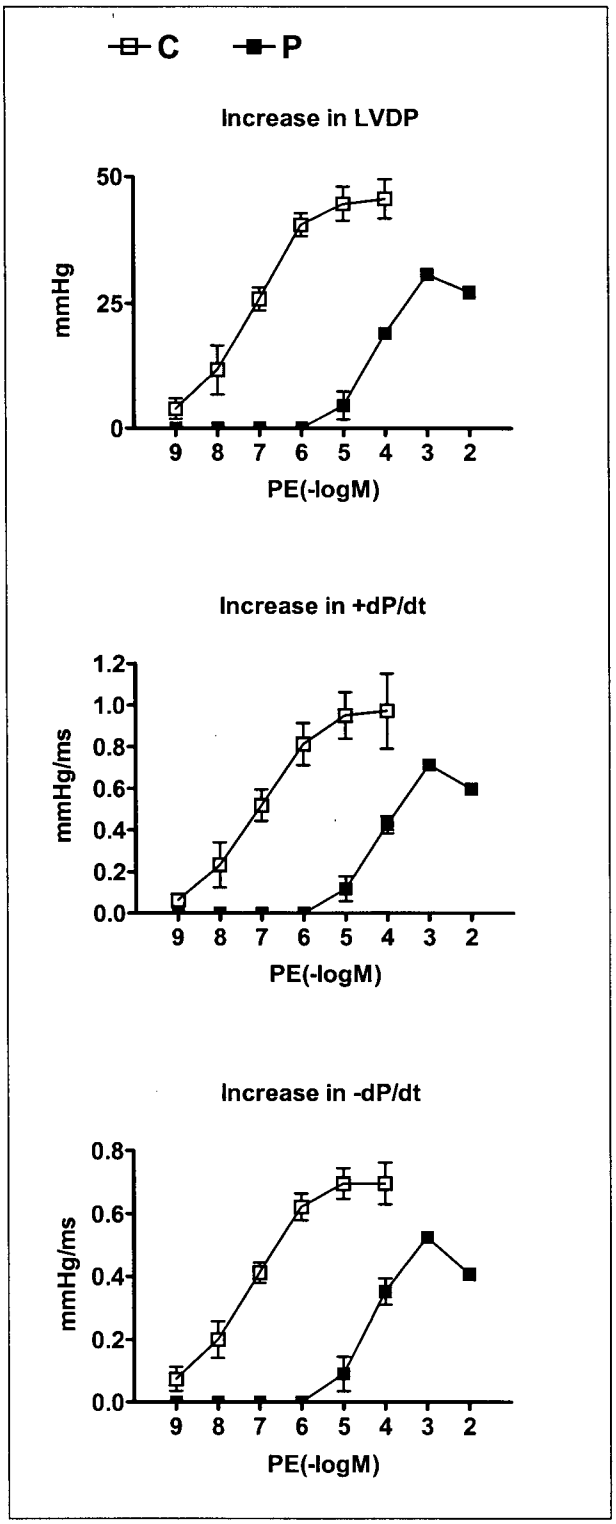

Figure 3 Increase in LVDP (top), +dP/dt (middle) and -dP/dt (bottom) produced by cumulative addition of PE in phentolamine (10^{-5}M) treated hearts (P) and control hearts (C). n = 3 hearts in each group.

3.1.3 Body weight, plasma glucose level and plasma insulin level of 6~7 week and 12~15 week diabetic and age-matched control rats

The body weights, plasma glucose levels and plasma insulin levels of the 6~7 week and 12~15 week diabetic and control animals are presented in Figure 4. In both cases the diabetic animals weighed significantly less than the age-matched controls. The 12~15 week control animals weighed significantly more than the 6~7 week controls while there was no difference in body weight between the two diabetic groups. The plasma glucose levels were significantly higher in 6~7 week and 12~15 week diabetic animals compared to control, while the plasma insulin levels were significantly lower in both groups of diabetic rats.

3.1.4 Cardiac Function Study #1: Investigation of the effects of diabetes on the α_1-AR-mediated PIE

3.1.4.1 Heart weight, coronary perfusion flow rate and ratio of heart weight / flow rate from 12~15 week diabetic and age-matched control hearts

As shown in Figure 5, the diabetic hearts weighed significantly less than control. When perfused at the same basal CPP of 80 mmHg, the coronary perfusion flow rate in the diabetic hearts was also significantly lower than the control. However, when normalized for heart weight, the ratio of flow rate / heart weight in the diabetic hearts was not different from the control.

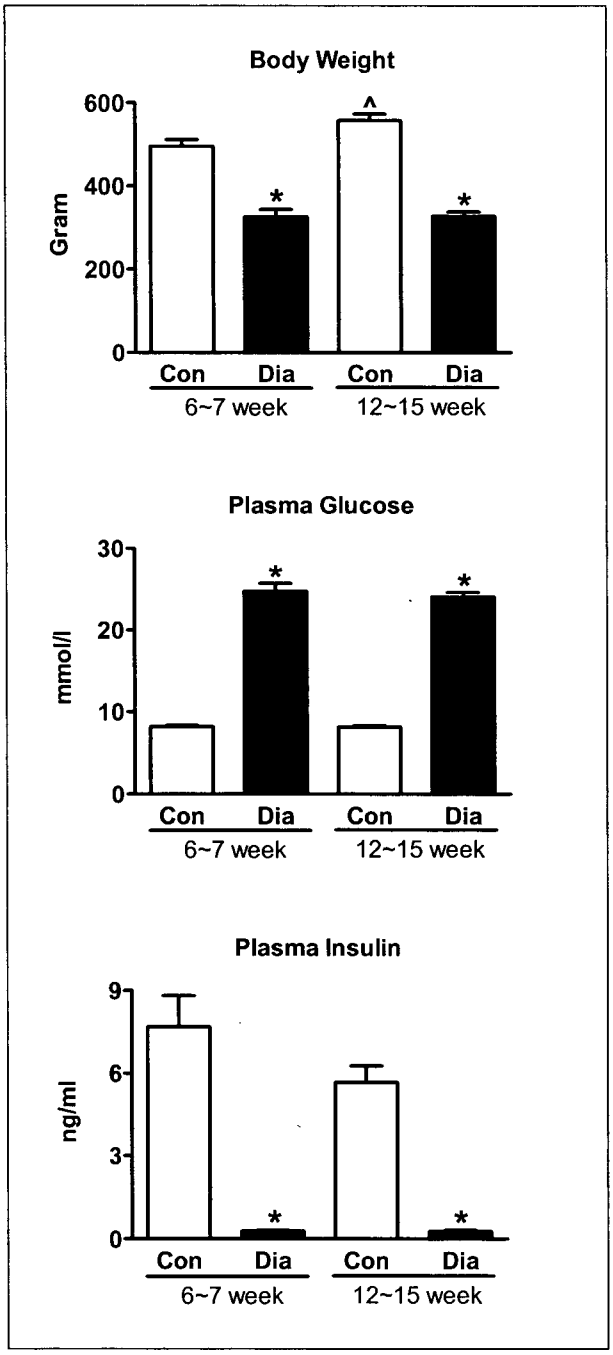

Figure 4 Body weight (top), plasma glucose level (middle) and plasma insulin level (bottom) of the 6~7 week and 12~15 week diabetic (Dia) and age-matched control (Con) rats. n = 9~10 animals in each group.

* Significantly different from age-matched controls. $P<0.05$

^ Significantly different from 6~7 week controls. $P<0.05$

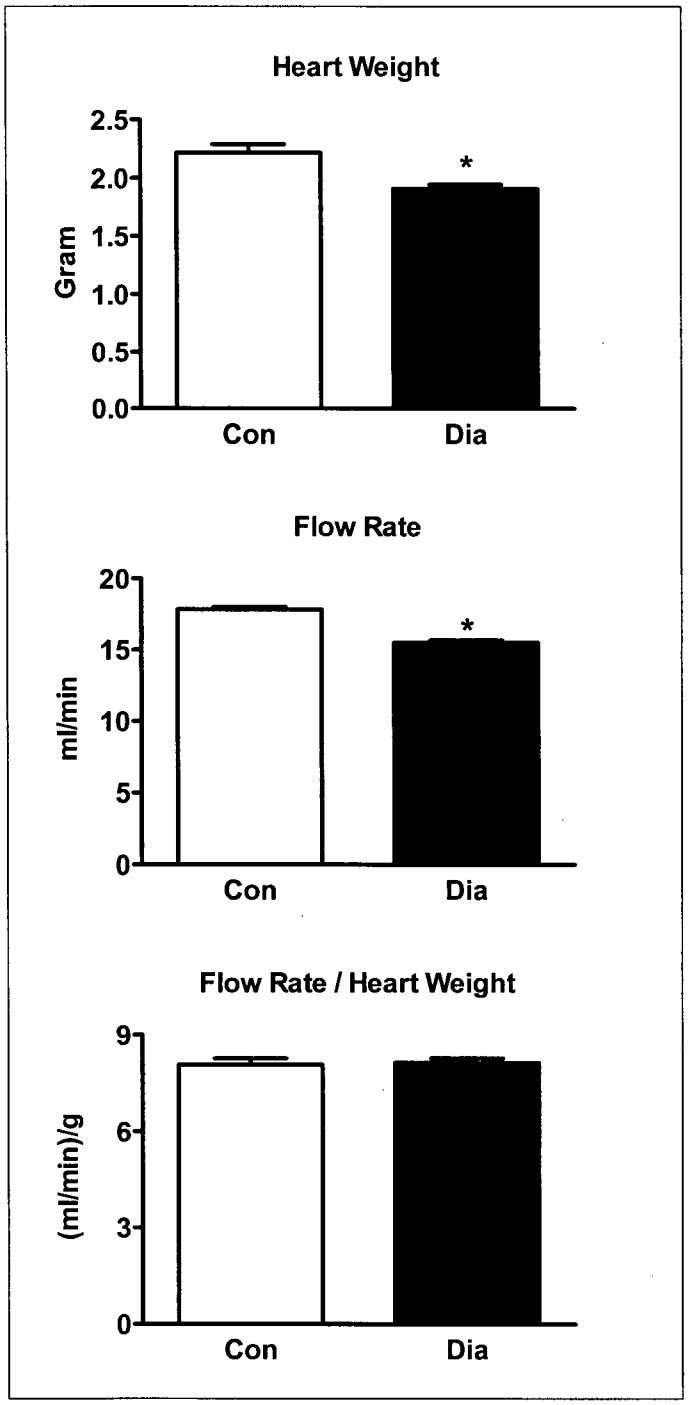

Figure 5 Heart weight (top), coronary perfusion flow rate (middle) and ratio of heart weight / flow rate (bottom) from the 12~15 week diabetic (Dia) and age-matched control (Con) hearts. n = 6 hearts in each group.

* Significantly different from age-matched controls. $P<0.05$

3.1.4.2 Basal contractile performance of hearts from 6~7 week and 12~15 week diabetic and age-matched control rats

In the absence of PE, the basal LVDP, +dP/dt and -dP/dt in both the 6~7 week and 12~15 week diabetic hearts were all significantly attenuated compared to the age-matched controls (Figure 6).

3.1.4.3 Effect of chronic diabetes on the α_1-AR-induced PIE

PE CRCs ($10^{-9} \sim 10^{-4}$ M) were obtained in 6~7 week and 12~15 week diabetic and age-matched control hearts (Figure 7). The increase in LVDP, +dP/dt and -dP/dt produced by the cumulative addition of PE is shown in Figure 8. The corresponding Rmax (maximal response) and pD_2 (-log[EC_{50}]) values for the PE-induced PIE are shown in Table 1.

In 6~7 week diabetic hearts, the maximal increases in the PE CRCs and Rmax values for both LVDP and -dP/dt in response to PE were significantly greater than control, although there was no significant change in the PE pD_2 values (Figure 8 a, c; Table 1). In contrast, while Rmax value for +dP/dt produced by PE was similar in control and diabetic hearts, the PE CRC for this parameter was shifted to the left, resulting in a significant increase in the PE pD_2 value in the diabetic hearts (Figure 8 b; Table 1). In 12~15 week diabetic hearts, similar changes in response to PE were seen. The Rmax values for LVDP and -dP/dt but not +dP/dt produced by PE were significantly

increased, but in addition, the PE pD_2 values for all three parameters were significantly greater at this time (Figure 8 d, e, f; Table 1). Therefore, in hearts from 12~15 week diabetic rats, there was not only an increase in maximal response to PE, but also an increase in sensitivity to this agonist.

Despite the impairement in the basal contractile performance of the 6~7 week diabetic hearts, the maximal LVDP, +dP/dt and -dP/dt that these hearts attained in the presence of PE were not different from control (Figure 7 a, b, c). However, since the basal contractile performance of the 12~15 week diabetic hearts was far lower than control, the maximal LVDP, +dP/dt and -dP/dt that these hearts attained in the presence of PE remained below those in control despite the greater PIE to PE (Figure 7 d, e, f).

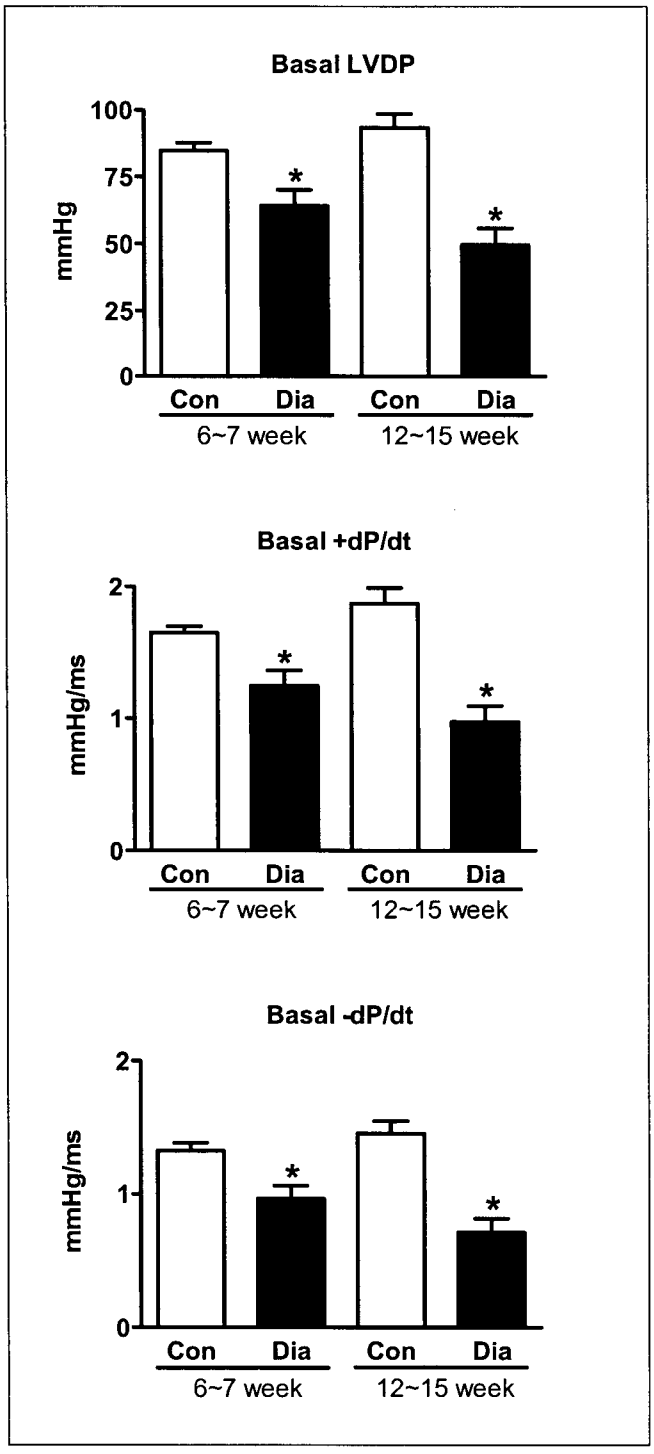

Figure 6 Basal LVDP (top), +dP/dt (middle) and -dP/dt (bottom) of 6~7 week and 12~15 week diabetic (Dia) and age-matched control (Con) hearts. n = 9~10 hearts in each group.

* Significantly different from age-matched controls. $P<0.05$

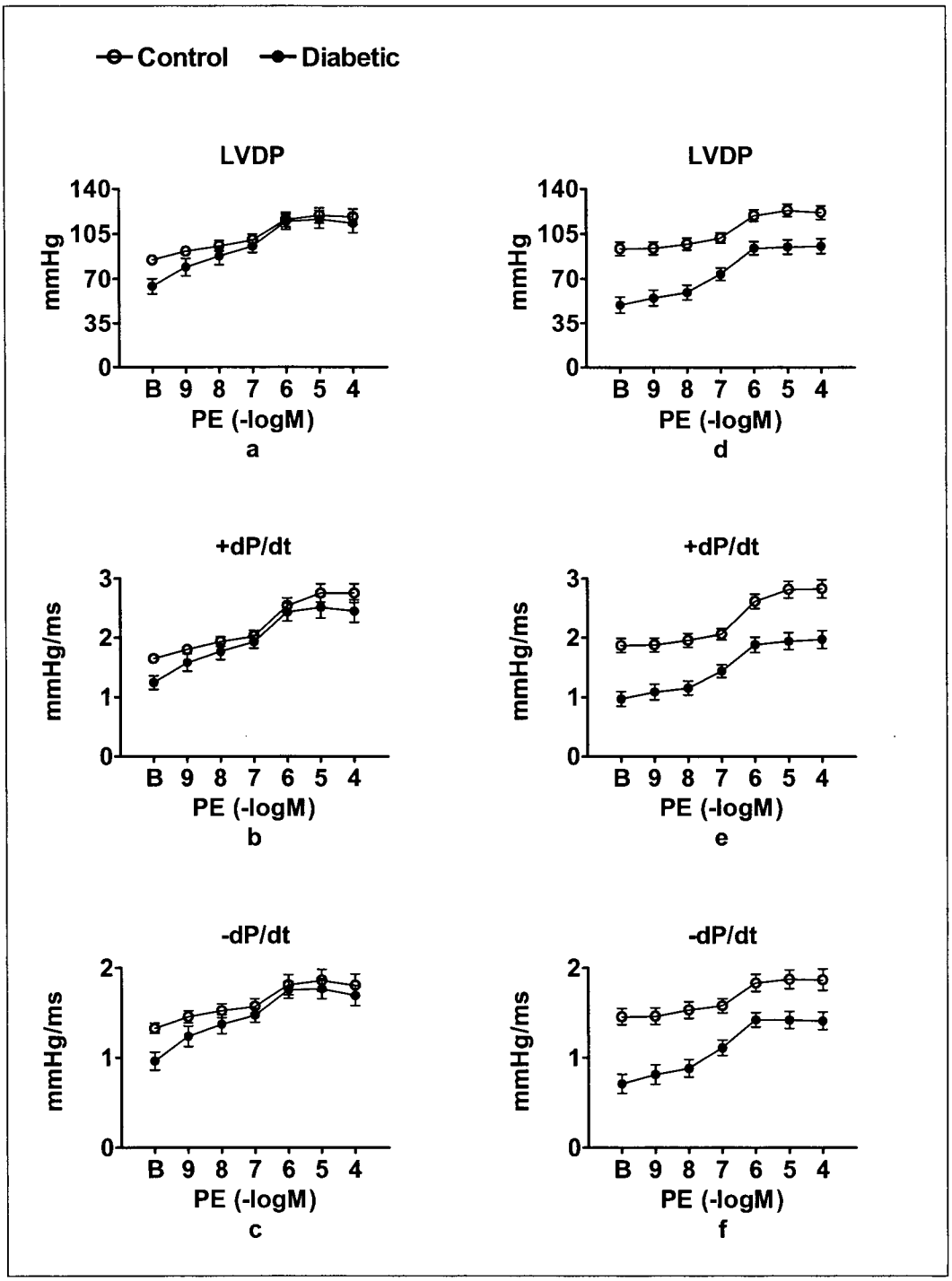

Figure 7 LVDP, +dP/dt and -dP/dt produced by cumulative addition of PE (10^{-9}~10^{-4} M) in 6~7 week (a, b, c) and 12~15 week (d, e, f) diabetic and age-matched control hearts. "B" in the x-axes stands for basal contractile performance before the addition of PE. n = 9~10 hearts in each group.

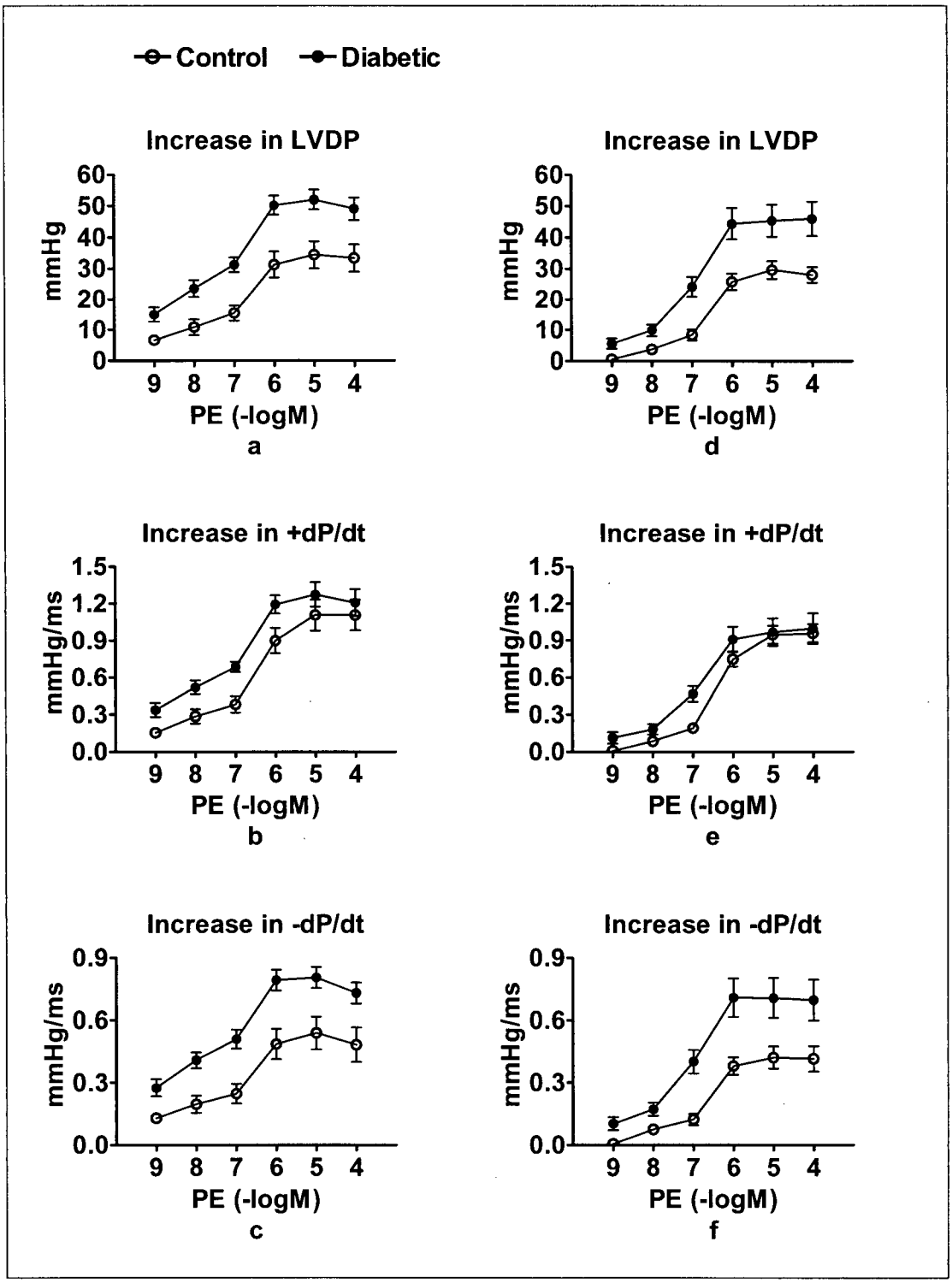

Figure 8 Increase in LVDP, +dP/dt and -dP/dt produced by cumulative addition of PE (10^{-9}~10^{-4} M) in 6~7 week (a, b, c) and 12~15 week (d, e, f) diabetic and age-matched control hearts. n = 9~10 hearts in each group.

		Rmax			pD_2		
		LVDP (mmHg)	+dP/dt (mmHg/ms)	-dP/dt (mmHg/ms)	LVDP (-logM)	+dP/dt (-logM)	-dP/dt (-logM)
6~7 week	Control	34.6±4.3	1.13±0.13	0.53±0.08	6.72±0.21	6.46±0.08	6.73±0.24
	Diabetic	51.6±3.4*	1.26±0.11	0.79±0.05*	7.02±0.19	6.94±0.18*	7.09±0.21
12~15 week	Control	29.9±2.6	0.99±0.07	0.44±0.05	6.61±0.09	6.42±0.09	6.61±0.13
	Diabetic	46.7±5.3*	1.00±0.12	0.72±0.10*	6.95±0.07*	6.87±0.09*	7.02±0.09*

Table 1 Rmax (maximal response) and pD_2 (-log[EC_{50}]) values for the PE-induced PIE in 6~7 week and 12~15 week diabetic and age-matched control hearts. n = 9~10 hearts in each group.

* Significantly different from age-matched controls. $P<0.05$

3.1.5 Cardiac Function Study #2: Investigation of the role for PKC in the α_1-AR-induced PIE

3.1.5.1 Choice of PKC inhibitor

Figure 9 shows the basal LVDP, +dP/dt, -dP/dt and the increase in these parameters in response to PE in control hearts, BIM I-treated hearts, RO-treated hearts and CE-treated hearts. The basal contractile performance was slightly attenuated in BIM I- and RO-treated hearts, suggesting non-specific inhibitory effects of these two inhibitors, but was not changed in CE-treated hearts compared to control. The increase in the three contractile parameters in response to PE was not changed in BIM I- and RO-treated hearts. However, the increase in these parameters in response to PE was attenuated in CE-treated hearts. Higher concentrations of BIM I ($5*10^{-6}$ M), RO ($5*10^{-6}$ M) and CE ($5*10^{-5}$ M) were tried in a few normal hearts, but all of them failed before the administration of PE. Therefore, 10^{-5} M CE was chosen for further investigations.

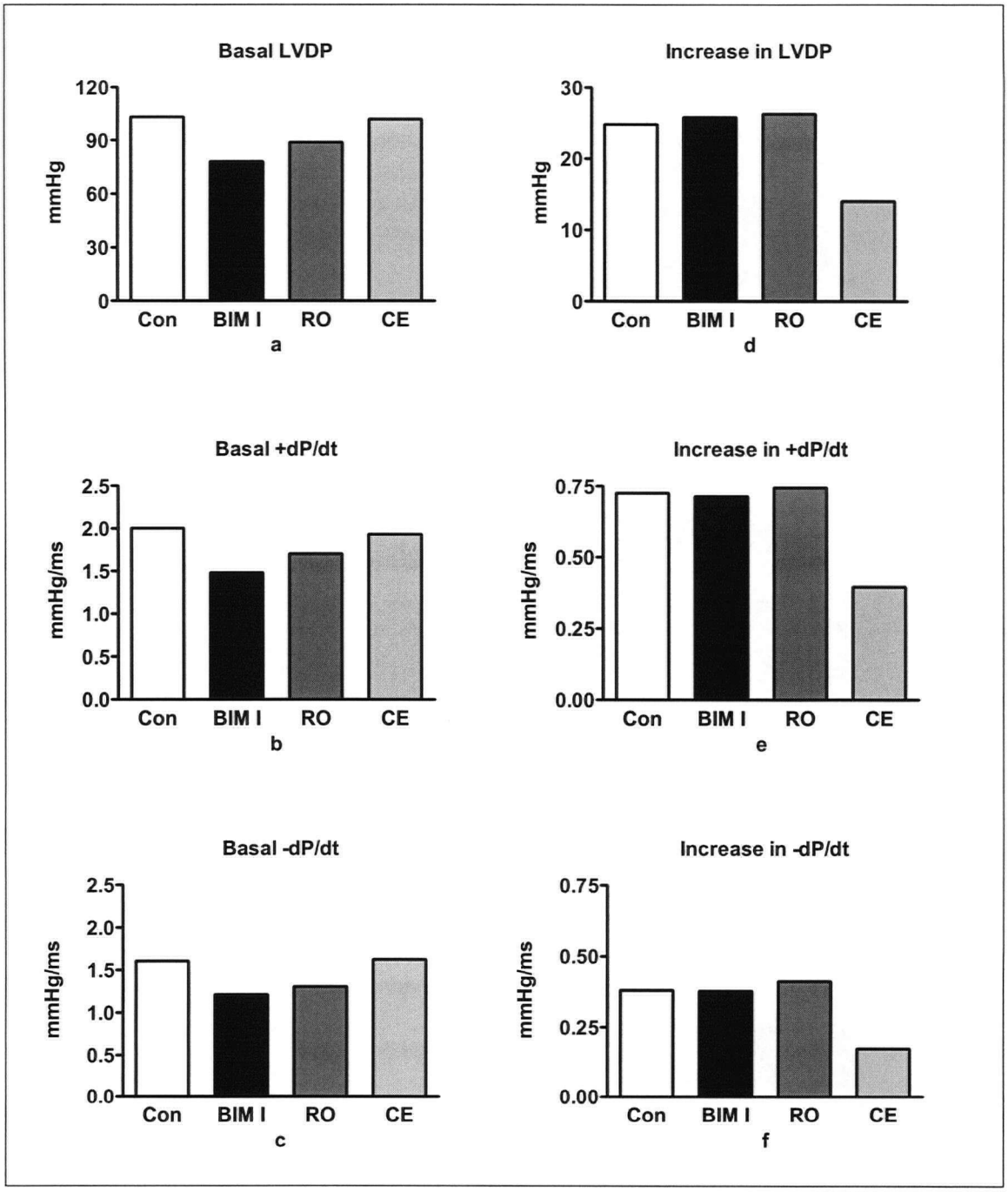

Figure 9 Basal LVDP (a), +dP/dt (b), -dP/dt (c) and the increase in these parameters (d, e, f) in response to PE (10^{-5} M; 2min) in the control, BIM I-treated ($3*10^{-6}$ M; 20min), RO-treated ($2*10^{-6}$ M; 20min) and CE-treated (10^{-5} M; 20min) normal hearts. n = 2 hearts in each group. All values are presented as means.

3.1.5.2 Effect of chelerythrine on the PIE induced by β-AR stimulation in normal hearts

In this experiment, because the hearts were perfused at a lower basal CPP and paced at a faster rate as compared to previous experiments (section 2.2.3.4.2), a lower basal contractile performance was attained (Figure 10 a, b, c). As was found previously (Figure 9 a, b, c), the basal LVDP, +dP/dt and -dP/dt values were not affected by CE. Similarily, the PIE induced by isoproterenol was not changed in the CE-treated hearts compared to control (Figure 10 d, e, f). In summary, incubation of the hearts with 10^{-5} M CE for 20 minutes did not affect basal contractile function, nor did it inhibit the β-AR-induced PIE. In the next experiment, the effect of CE (10^{-5} M; 20min) on the PIE to PE in diabetic and age-matched control hearts was determined.

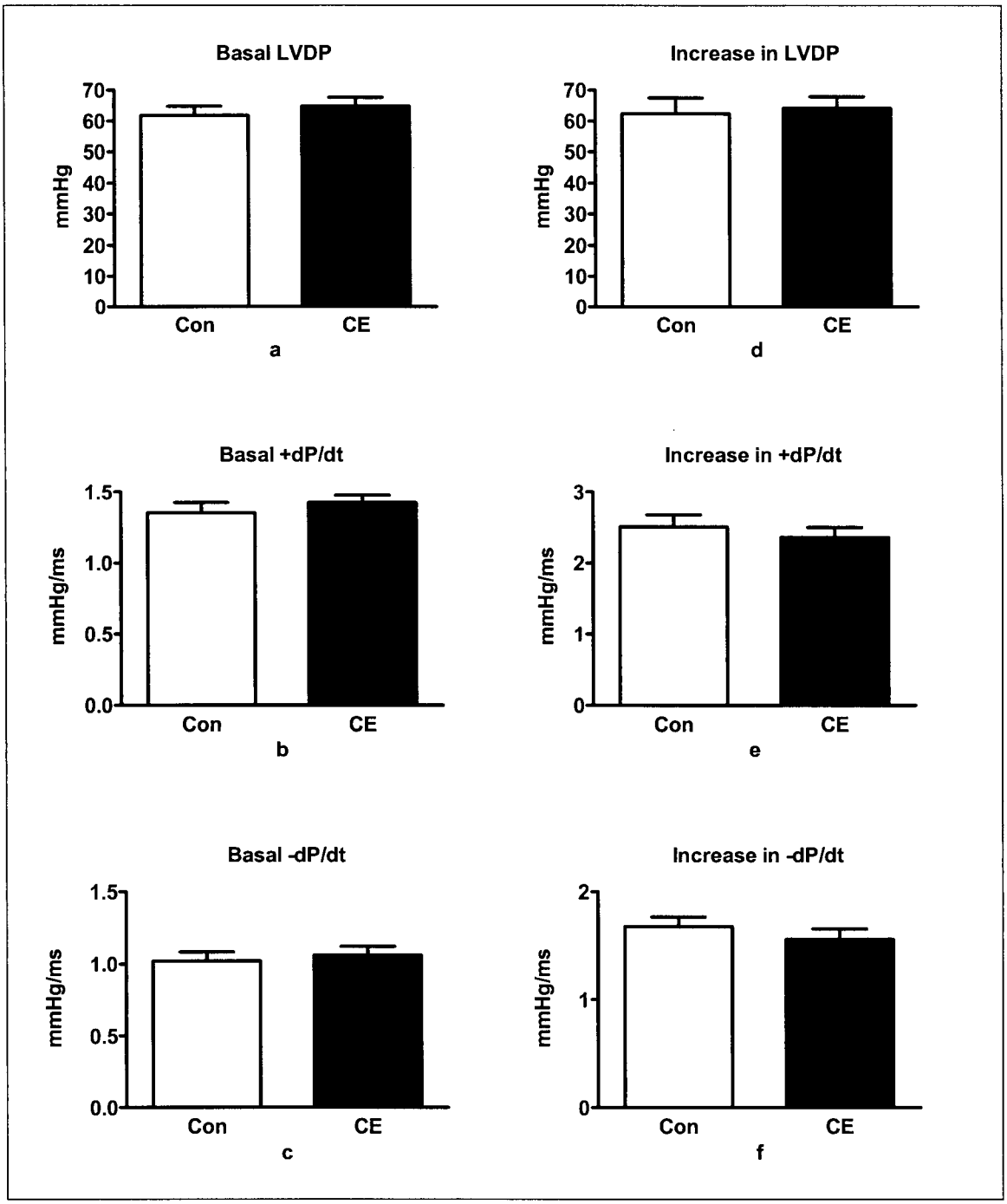

Figure 10 Basal LVDP (a), +dP/dt (b), -dP/dt (c) and the increase in these parameters (d, e, f) in response to isoproterenol (10^{-6} M; 1min) in the control and CE-treated (10^{-5} M; 20min) normal hearts. n = 4~5 hearts in each group.

3.1.5.3 Effect of chelerythrine on basal contractile performance and the α_1-AR-induced PIE in hearts from 12~15 week diabetic and age-matched control rats

Figure 11 shows the basal LVDP, +dP/dt, -dP/dt and the PIE to PE in 12~15 week diabetic and age-matched control hearts, in the absence or presence of CE. As was found previously in normal hearts (Figure 9 a, b, c; Figure 10 a, b, c), the basal LVDP, +dP/dt and -dP/dt were not affected by CE. Consistent with the results obtained from the PE CRCs (Figure 8 d, e, f; Table 1), 10^{-5} M PE produced a significantly greater increase in LVDP and -dP/dt, but no difference in +dP/dt in diabetic hearts compared to control. CE significantly attenuated the PE-induced increase in all three parameters in both diabetic and control hearts (Figure 11 d, e, f). Moreover, in the presence of CE, the increases in LVDP and -dP/dt to PE in diabetic hearts were no longer significantly greater than control (Figure 11 d, f).

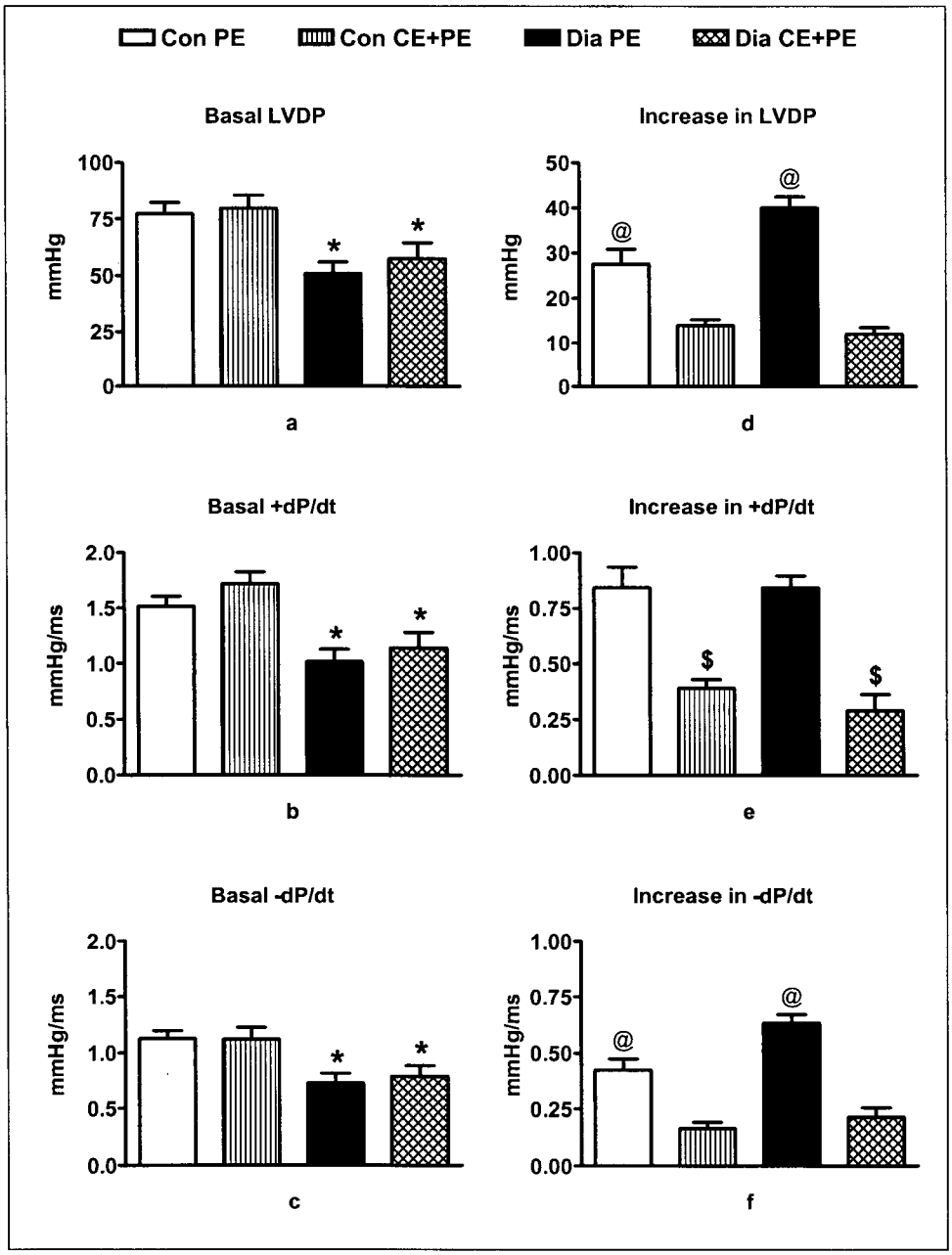

Figure 11 Basal LVDP (a), +dP/dt (b), -dP/dt (c) and the increase in these parameters (d, e, f) in response to PE (10^{-5} M; 2min) in 12~15 week diabetic (Dia) and control (Con) hearts, in the absence (PE) or presence (CE+PE) of CE (10^{-5} M; 20min). n = 5 hearts in each group.

* Significantly different from Con PE and Con CE+PE groups. $P<0.05$

@ Significantly different from all other three groups. $P<0.05$

$ Significantly different from Con PE and Dia PE groups. $P<0.05$

3.1.6 Cardiac Function Study #3: Investigation of the role for Rho kinase in the α_1-AR-induced PIE

3.1.6.1 Effect of Y-27632 on basal contractile performance and the α_1-AR-induced PIE in normal hearts

PE CRCs (10^{-8}~10^{-4} M) and the increase in LVDP, +dP/dt and -dP/dt produced by cumulative addition of PE in the absence or presence of Y-27632, are shown in Figure 12. Table 2 shows the Rmax and pD_2 values calculated from the PE CRCs. The basal LVDP, +dP/dt and -dP/dt were not affected by Y-27632 (Figure 12 a, b, c). In all three contractile parameters, there was no change in the maximal increase or rightward shift in the PE CRCs of the Y-27632-treated hearts compared to control (Figure 12 d, e, f). Correspondingly, none of the Rmax and pD_2 values were changed in the Y-27632-treated hearts compared to control (Table 2). Therefore, treatment with Y-27632 (10^{-6} M; 20min) did not affect the basal contractile performance or the PE-induced PIE in normal hearts.

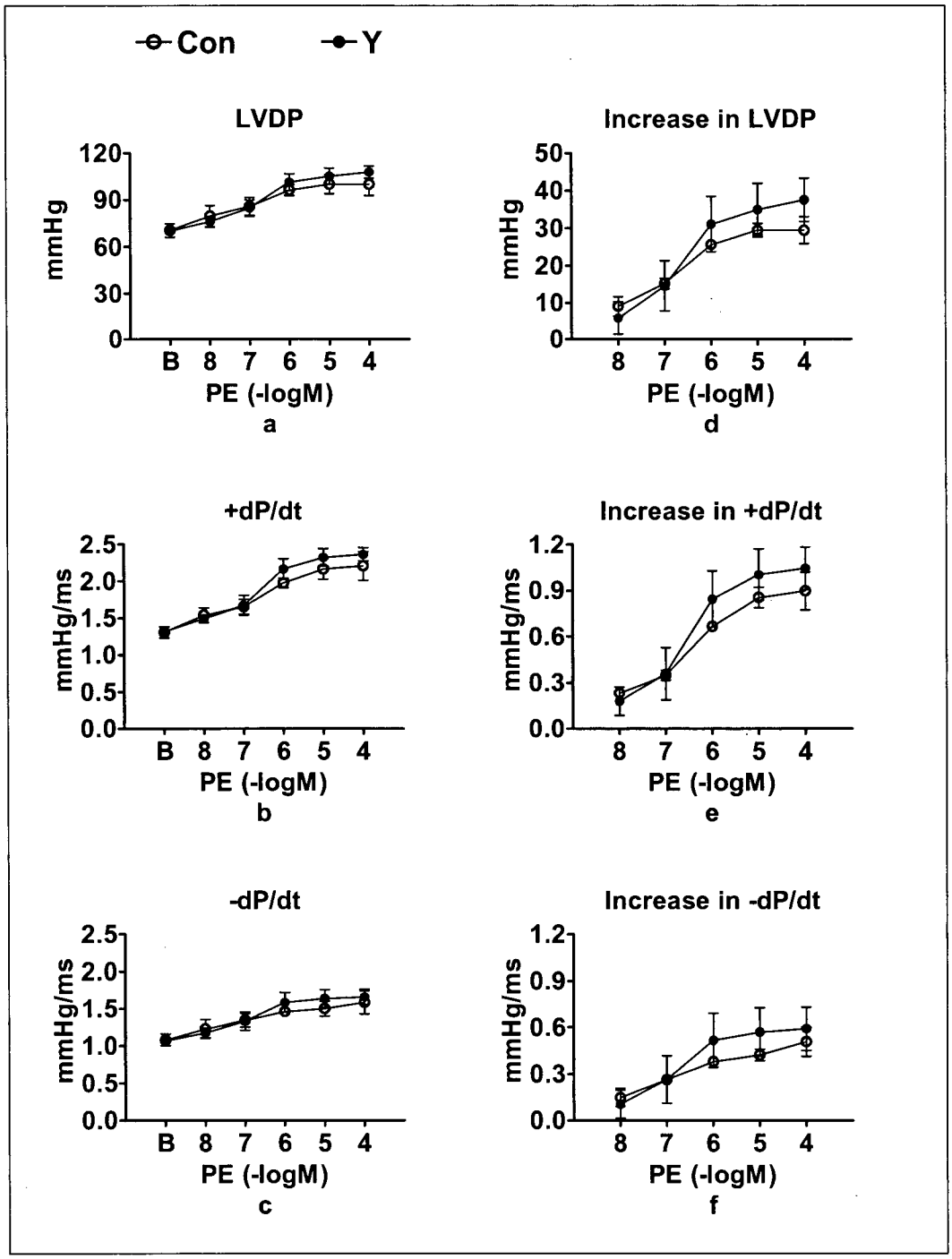

Figure 12 LVDP (a), +dP/dt (b), -dP/dt (c) and the increase in these parameters (d, e, f) produced by cumulative addition of PE (10^{-8}~10^{-4} M) in control (Con) and Y-27632-treated (Y; 10^{-6} M; 20min) normal hearts. "B" in the x-axes (a, b, c) stands for basal contractile performance before the addition of PE. n = 3 hearts in each group.

	Rmax			pD_2		
	LVDP (mmHg)	+dP/dt (mmHg/ms)	-dP/dt (mmHg/ms)	LVDP (-logM)	+dP/dt (-logM)	-dP/dt (-logM)
Control	30.2±2.2	0.91±0.10	0.52±0.08	6.52±0.26	6.28±0.24	6.10±0.66
Y-27632	36.9±6.1	1.04±0.15	0.59±0.15	6.61±0.18	6.51±0.16	6.64±0.29

Table 2 Rmax and pD_2 values for the PE-induced PIE in the control and Y-27632-treated normal hearts. n = 3 hearts in each group.

3.1.6.2 Effect of H1152 on basal contractile performance and the α_1-AR-induced PIE in hearts from normal and 12-week diabetic rats

Due to the ineffectiveness of 10^{-6} M Y-27632, and the expense associated with the perfusion with a higher concentration of this inhibitor, H1152, which is a more potent non-isoform-selective Rho kinase inhibitor than Y-27632, was used in this experiment.

Unpublished data from our lab showed that 10^{-7} M H1152 significantly attenuated the contraction induced by PE in vascular smooth muscles. H1152 at the same concentration also improved the function of isolated working hearts from diabetic rats, while having no effect on the control heart function. H1152 at $5*10^{-7}$ M produced a further improvement in the function of isolated working hearts from diabetic rats; however, this concentration slightly attenuated the function of control hearts. In Langendorff-perfused hearts, perfusion with $5*10^{-7}$ M H1152 for 20 minutes before treatment with a single dose of 10^{-5} M PE for 2 minutes had no effect on the PE-induced PIE. As a result, a higher concentration (10^{-6} M) of H1152 was used in the current experiment.

Before the addition of PE, 10^{-6} M H1152 did not significantly affect the basal contractile performance of either normal or 12-week diabetic hearts (Figure 13 a, b, c). On the other hand, the PE-induced PIE was not significantly affected by H1152 in either normal or 12-week diabetic hearts (Figure 13 d, e, f). The concentration of H1152 was not further increased, because the cost of the inhibitor is high, and the basal contractile function of normal hearts was already slightly (though not significantly) impaired at the concentration of 10^{-6} M (Figure 13 a, b, c).

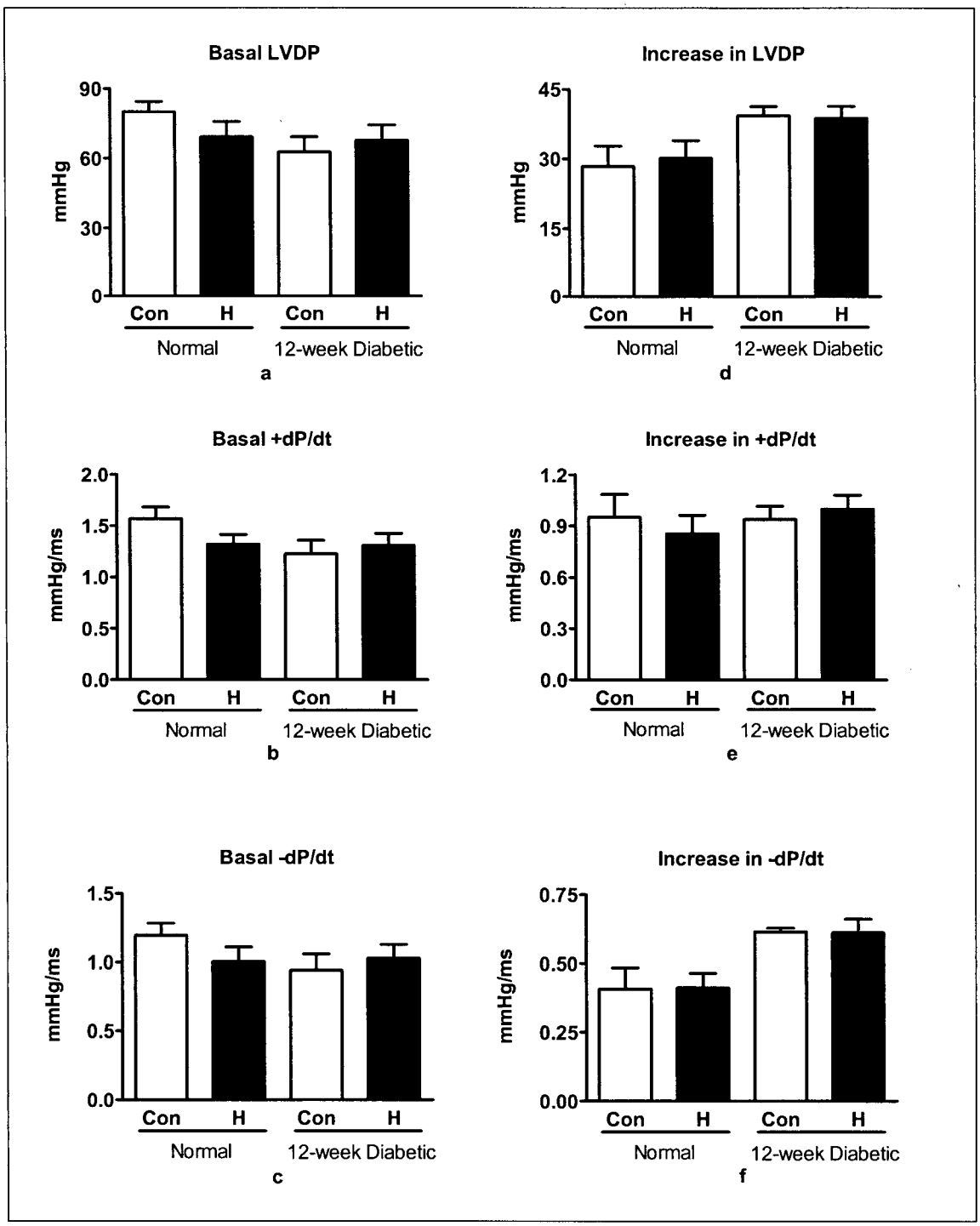

Figure 13 Basal LVDP (a), +dP/dt (b), -dP/dt (c) and the increase in these parameters (d, e, f) in response to PE (10^{-5} M; 2min) in the control hearts and H1152-treated (10^{-6} M; 20min) hearts from normal and 12-week diabetic rats. n = 4~5 hearts in each group.

3.1.7 Cardiac Function Study #4: Investigation of effects of AT_1 receptor blockade on basal contractile performance and the α_1-AR-induced PIE in hearts from 12-week diabetic and age-matched control rats

Figure 14 shows the basal LVDP, +dP/dt, -dP/dt, and the PIE to PE in hearts from 12-week diabetic and age-matched control rats, with or without L-158,809 treatment. Consistent with previous results, hearts from untreated diabetic rats exhibited attenuated basal contractile performance compared to untreated controls. However, the basal cardiac function of hearts from L-158,809-treated diabetic rats was significantly improved compared to that of hearts from untreated diabetic rats, and was no longer significantly different than control. Treatment of control rats with L-158,809 had no effect on the basal cardiac function. Consistent with results obtained from the PE CRCs (Figure 8 d, e, f, Table 1), and the results from the chelerythrine study (Figure 11 d, e, f), 10^{-5} M PE produced a significant greater increase in LVDP and -dP/dt, but no difference in +dP/dt in untreated diabetic hearts compared to untreated controls. However, L-158,809 treatment had no significant influence on the PE-induced PIE in either diabetic or control hearts, and the PE-induced increase in LVDP and -dP/dt in L-158,809-treated diabetic hearts remained significantly elevated compared to control (Figure 14 d, f).

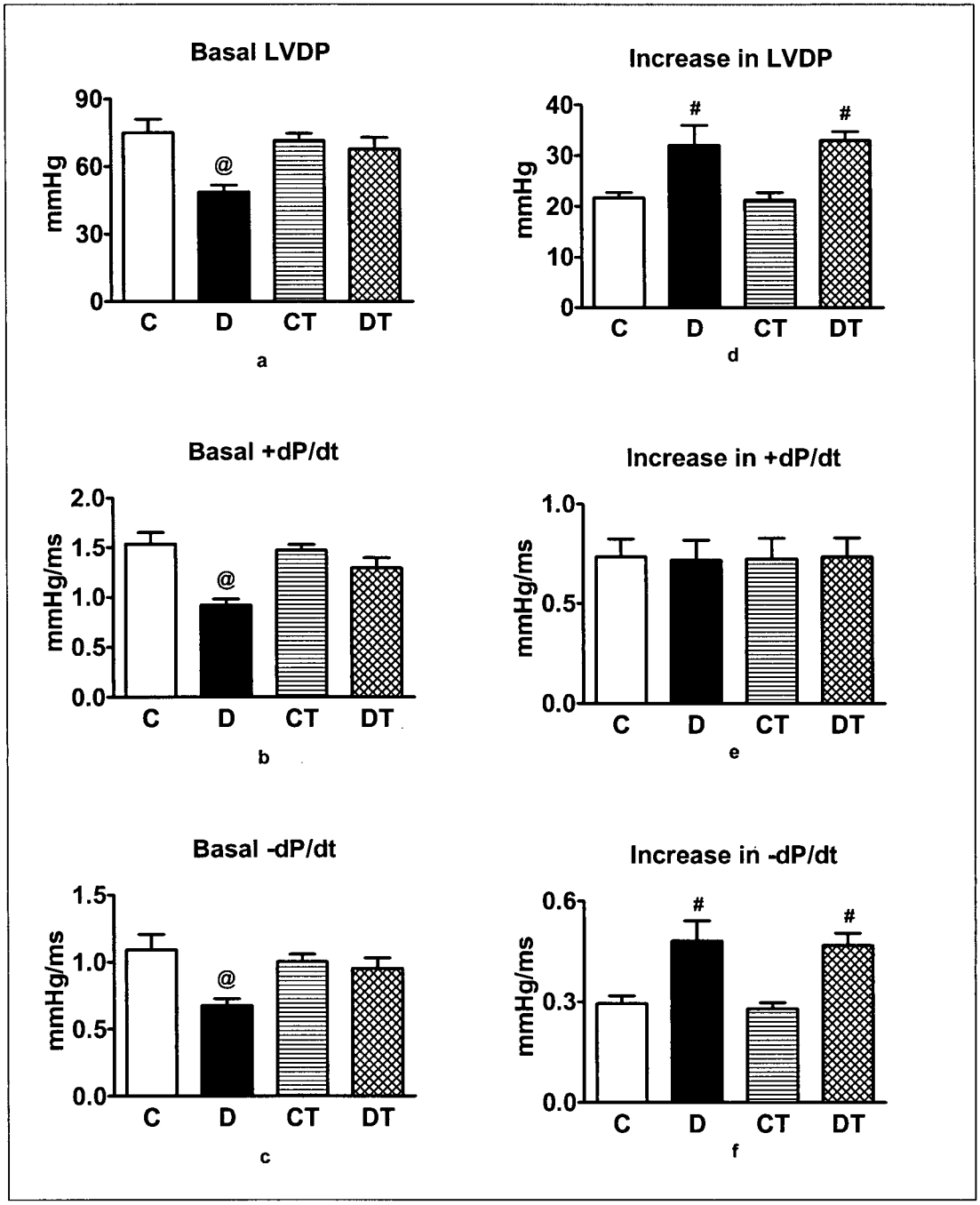

Figure 14 Basal LVDP (a), +dP/dt (b), -dP/dt (c) and the increase in these parameters (d, e, f) in response to PE (10^{-5} M; 2min) in hearts from 12-week untreated control (C), untreated diabetic (D), L-158,809-treated control (CT) and L-158,809-treated diabetic (DT) rats. n = 7~13 hearts in each group.

@ Significantly different from all other three groups. $P<0.05$

Significantly different from C and CT groups. $P<0.05$

3.2 Enhanced chemiluminescence Western blot studies

3.2.1 Preliminary experiment to determine the appropriate amount of protein that should be loaded

The plots of "Densitometric reading vs. Amount of protein" are shown in Figure 15. The points were subjected to linear regression analysis. No points deviated from the linear regression trend lines, and all the R^2 values were higher than 0.99. These data suggest that 50 μg protein of PKC or Rho kinase in the soluble or particulate fration fell within the linear range of densitomertric detection. As a result, in all the following Western blot experiments, 50 μg of soluble or particulate protein was loaded into lanes of polyacrylamide gels.

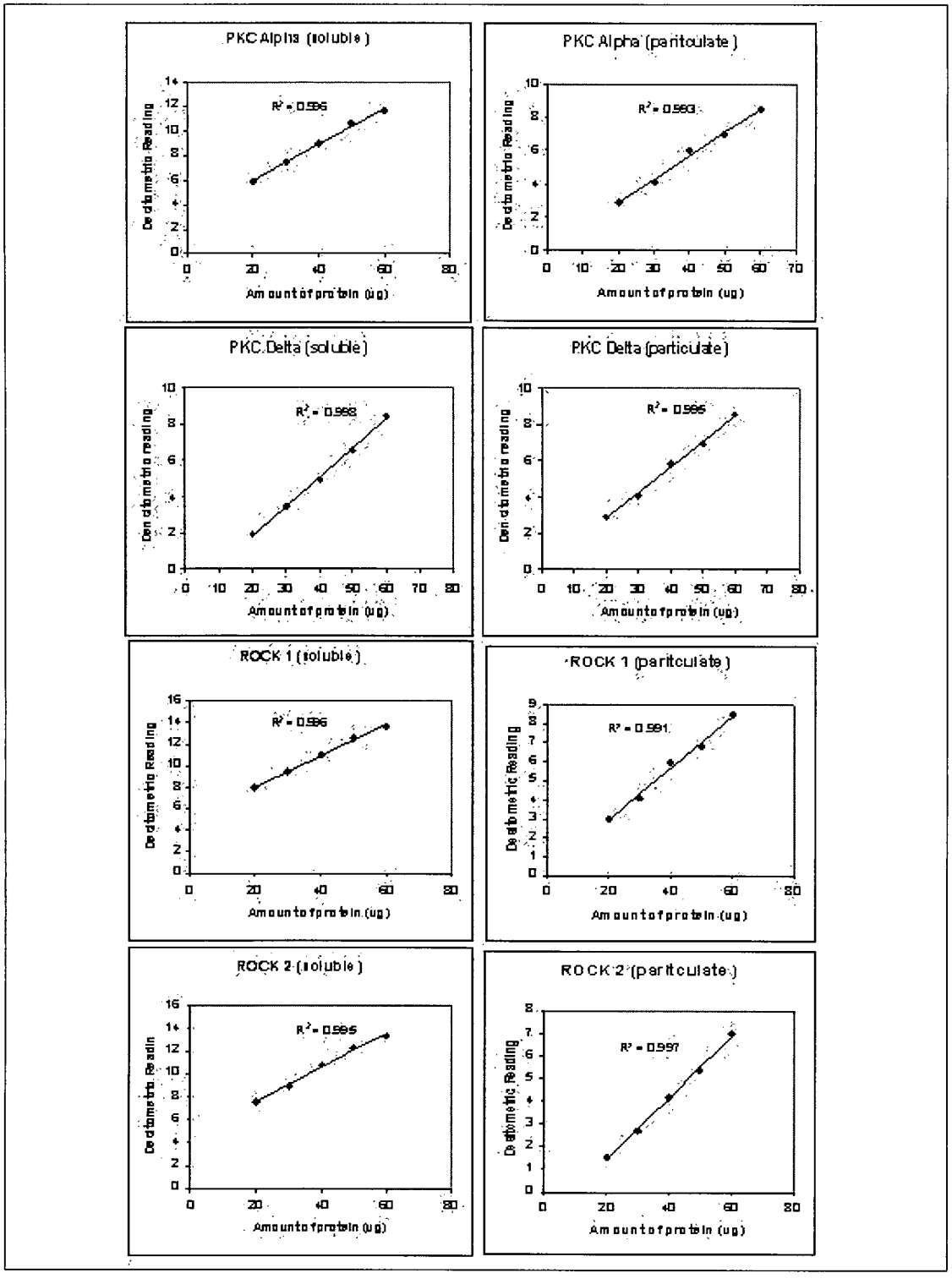

Figure 15 Densitometric reading vs. amount of soluble and particulate protein loaded, for incubation with antibodies to PKCδ, PKCα, ROCK 1 or ROCK 2. Trend lines for linear regression and R^2 values are also shown in the figure.

3.2.2 Effect of diabetes on the protein levels of actin in the soluble and the particulate fractions

Actin is used as an internal control to minimize the errors that could occur during protein loading and/or transfer in Western blot assays (see section 2.2.4 for detailed correction methods). A representative blot of soluble and particulate actin in unstimulated and PE-stimulated hearts from 12~15 week diabetic and age-matched control rats is shown in Figure 16. Diabetes did not have distinguishable influence on the protein levels of actin in either the soluble or the particulate fractions.

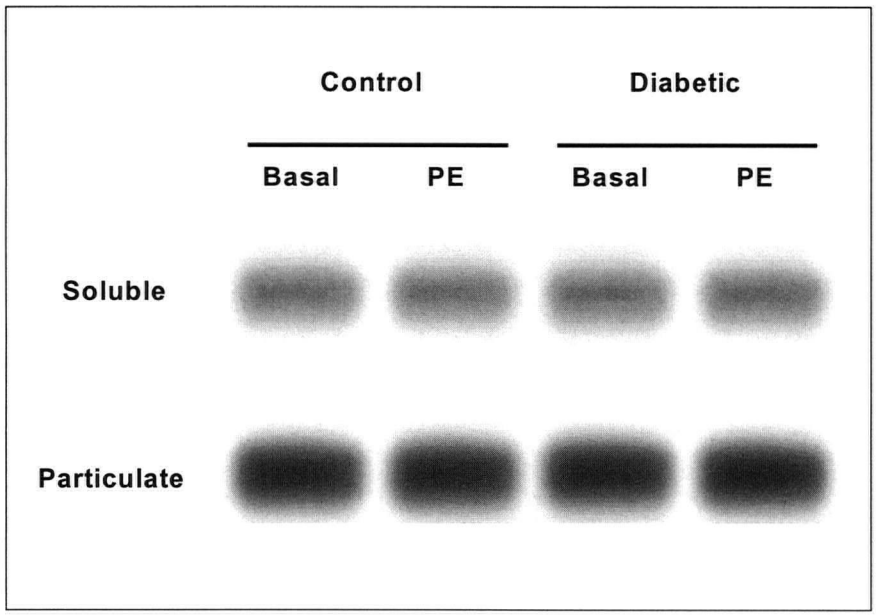

Figure 16 Representative blot of soluble and particulate actin in unstimulated and PE-stimulated hearts from 12~15 week diabetic and age-matched control rats.

3.2.3 Effect of diabetes, α_1-AR stimulation and PKC inhibition on the subcellular distribution of PKC and Rho kinase isoforms

In this set of experiments (Figure 17~22), all the soluble and particulate values were expressed relative to the mean value in the soluble fraction of the Control Basal group, which was set at 1.

Four isoforms of PKC (α, β_2, δ and ε) were investigated. No significant changes in the levels of PKCα and β_2 in the soluble or particulate fractions were detected in untreated 12~15 week diabetic hearts, and PE produced no significant change in the soluble or particulate levels of either isoform in either control or diabetic hearts (Figure 17; Figure 18).

In unstimulated diabetic hearts, levels of both PKCδ and PKCϵ in the particulate fraction were significantly increased, but no significant change in their levels in the soluble fraction was detected (Figure 19; Figure 20). Exposure of both control and diabetic hearts to PE for 2 minutes, the time required for the peak PIE to this agonist, resulted in a significant increase in the particulate levels of PKCδ, but without a corresponding decrease in the soluble levels of this isoform (Figure 19). The increase in PKCδ over its own basal levels in diabetic hearts (0.74±0.07) was significantly greater than the PE-induced increase in levels of this isoform in control hearts (0.45±0.10). PE also produced a significant increase in the levels of PKCϵ in the particulate fraction of both control and diabetic hearts, again without a corresponding decrease in levels in the soluble fraction (Figure 20). Although in the presence of PE, total levels of PKCϵ in the particulate fraction of diabetic hearts were significantly greater than control, the

magnitude of the PE-induced increase in PKCε over the corresponding basal levels was not significantly different in diabetic (0.72±0.17) and control (0.63±0.08) hearts. Although CE was without effect on basal levels of PKCα, β_2, δ and ϵ (data not shown), it completely prevented the PE-induced increases in the particulate levels of PKCδ and ϵ in both control and diabetic hearts (Figure 19; Figure 20).

No significant changes in the levels of ROCK 1 and ROCK 2 in the soluble or particulate fractions were detected in unstimulated 12~15 week diabetic hearts, and PE produced no significant change in the soluble or particulate levels of either isoform in either control or diabetic hearts (Figure 21; Figure 22).

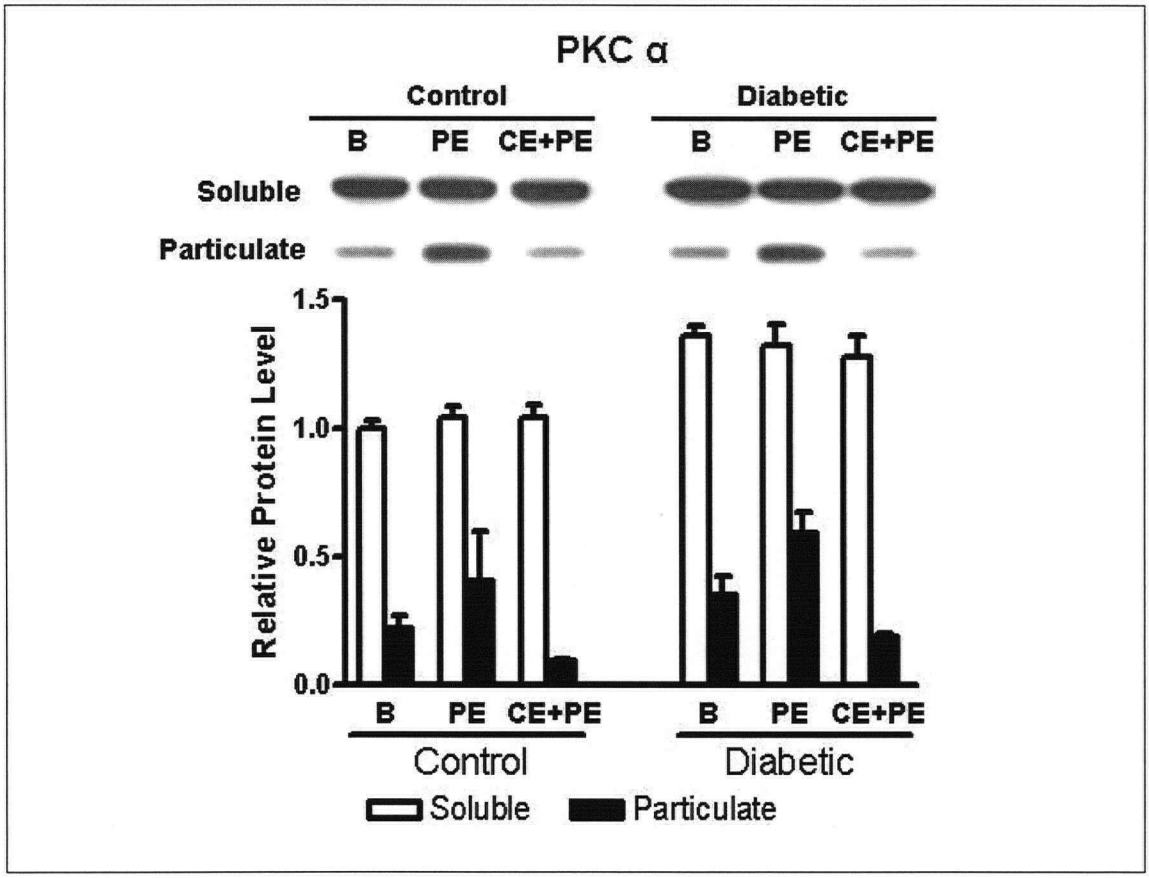

Figure 17 Relative protein levels and a representative blot of PKCα in the soluble and particulate fractions of basal (B), PE-treated (PE) and CE plus PE-treated (CE+PE) hearts from 12~15 week diabetic and age-matched control rats. All the soluble and particulate values are expressed relative to the mean value in the soluble fraction of the Control B group, which is set at 1. n = 10 in B groups; n = 5 in PE and CE+PE groups.

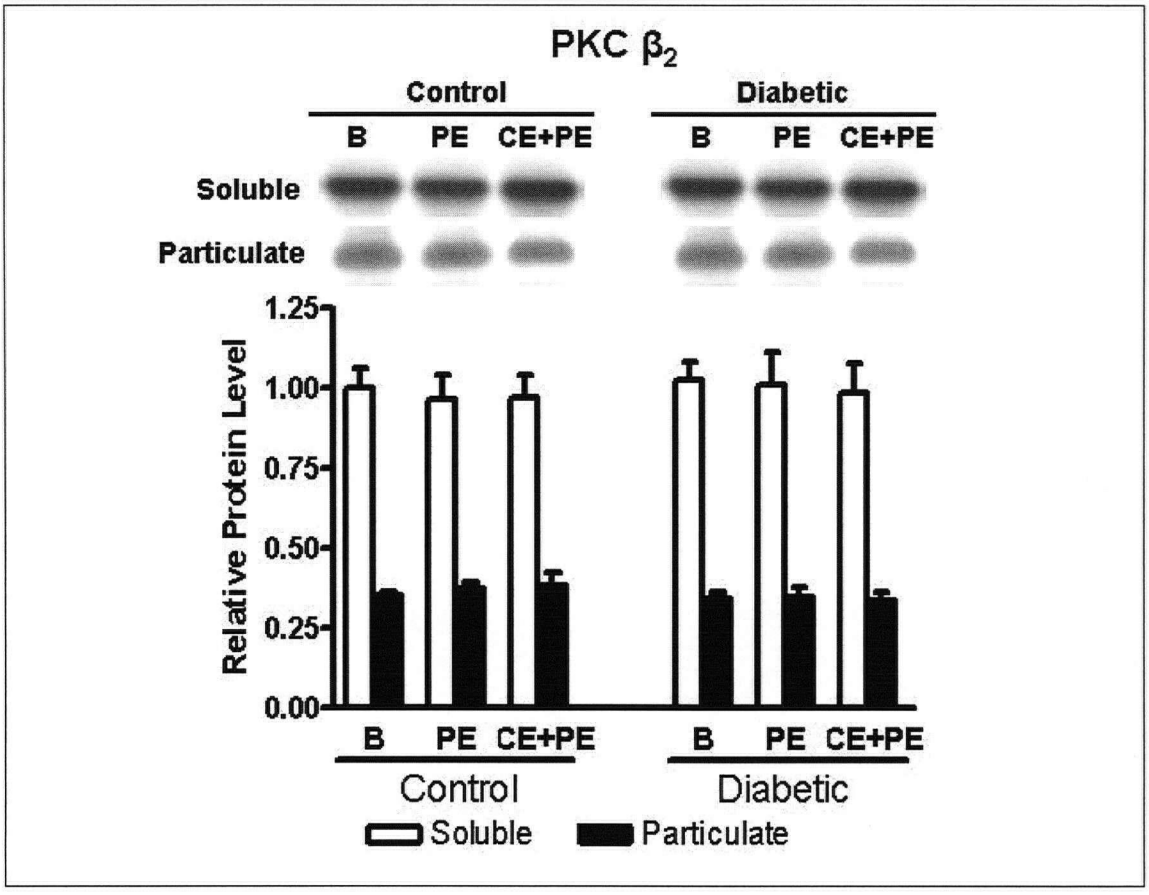

Figure 18 Relative protein levels and a representative blot of PKCβ_2 in the soluble and particulate fractions of basal (B), PE-treated (PE) and CE plus PE-treated (CE+PE) hearts from 12~15 week diabetic and age-matched control rats. All the soluble and particulate values are expressed relative to the mean value in the soluble fraction of the Control B group, which is set at 1. n = 10 in B groups; n = 5 in PE and CE+PE groups.

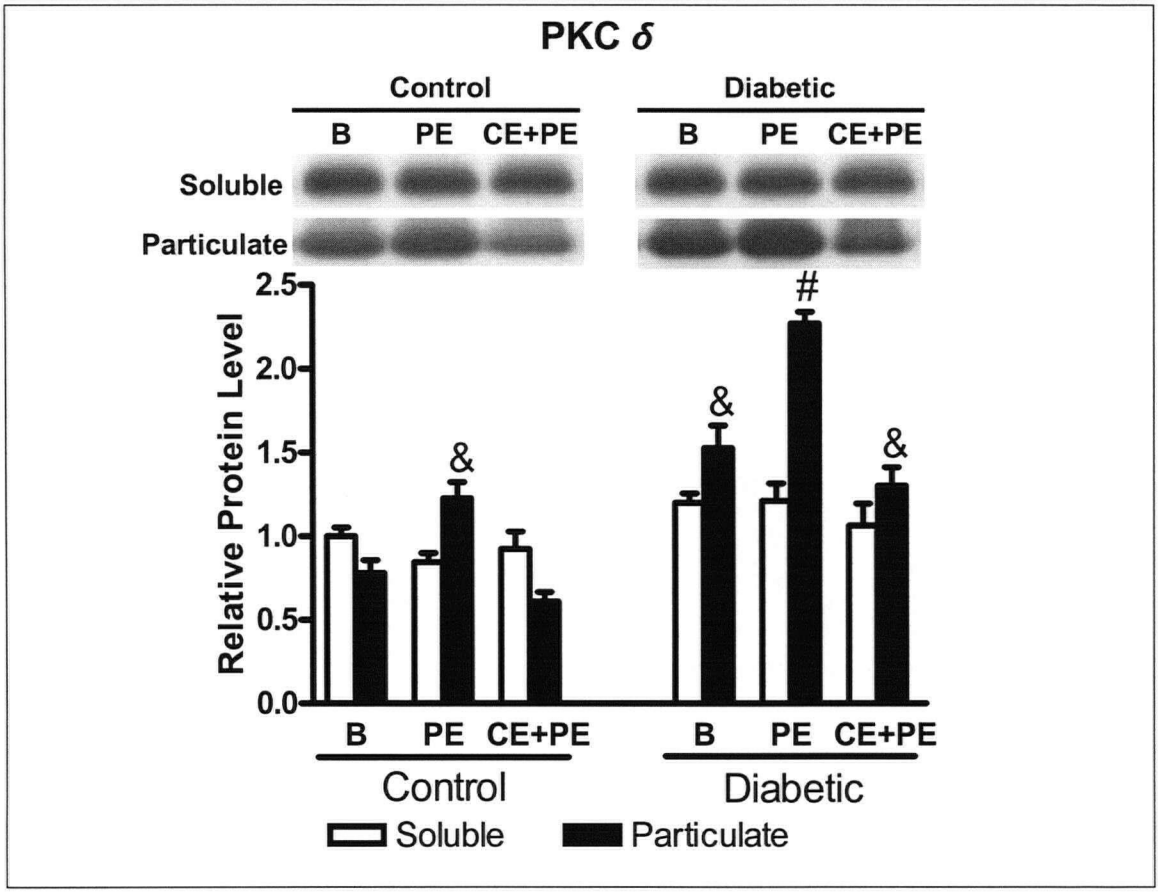

Figure 19 Relative protein levels and a representative blot of PKCδ in the soluble and particulate fractions of basal (B), PE-treated (PE) and CE plus PE-treated (CE+PE) hearts from 12~15 week diabetic and age-matched control rats. All the soluble and particulate values are expressed relative to the mean value in the soluble fraction of the Control B group, which is set at 1. n = 10 in B groups; n = 5 in PE and CE+PE groups.

Significantly different from all control groups and other diabetic groups. $P<0.05$

& Significantly different from control B, control CE+PE and diabetic PE groups. $P<0.05$

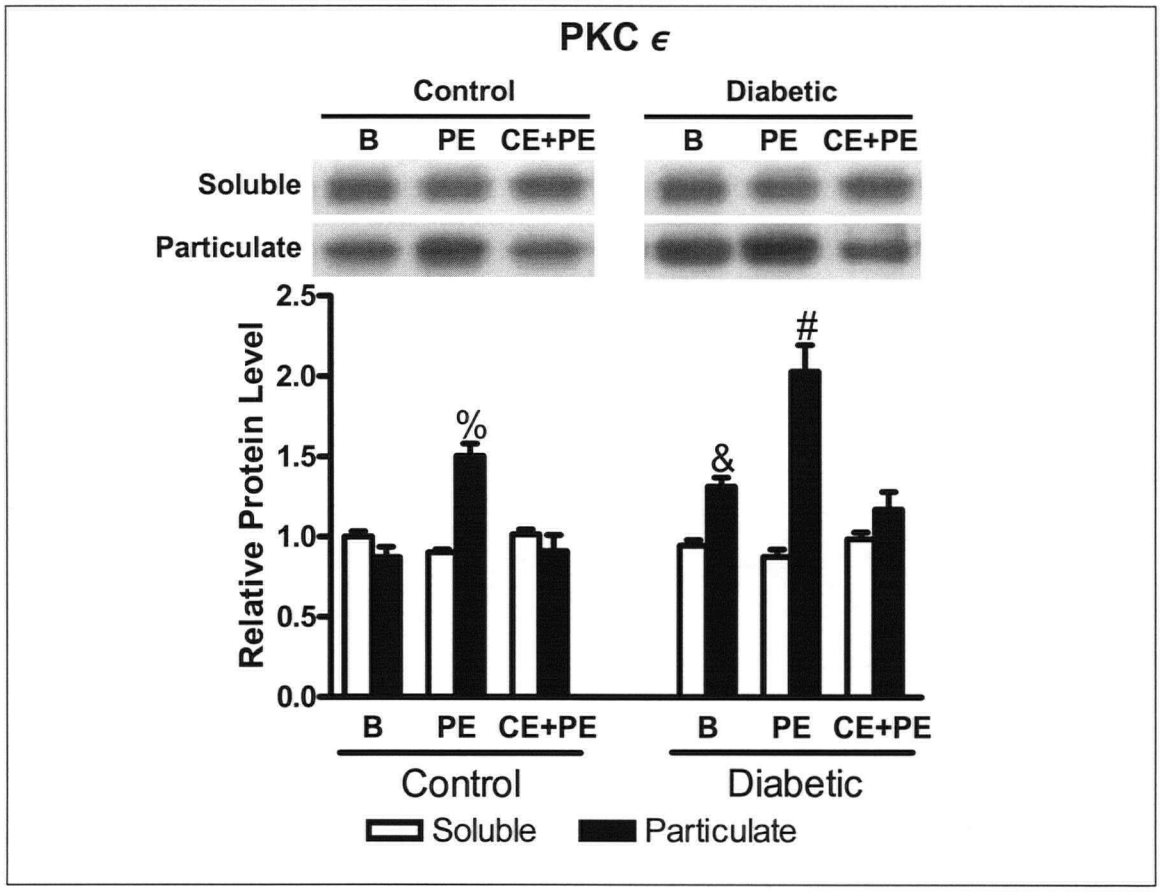

Figure 20 Relative protein levels and a representative blot of PKCε in the soluble and particulate fractions of basal (B), PE-treated (PE) and CE plus PE-treated (CE+PE) hearts from 12~15 week diabetic and age-matched control rats. All the soluble and particulate values are expressed relative to the mean value in the soluble fraction of the Control B group, which is set at 1. n = 10 in B groups; n = 5 in PE and CE+PE groups.

Significantly different from all control groups and other diabetic groups. $P<0.05$

& Significantly different from control B, control CE+PE and diabetic PE groups. $P<0.05$

% Significantly different from control B, control CE+PE, diabetic CE+PE and diabetic PE groups. $P<0.05$

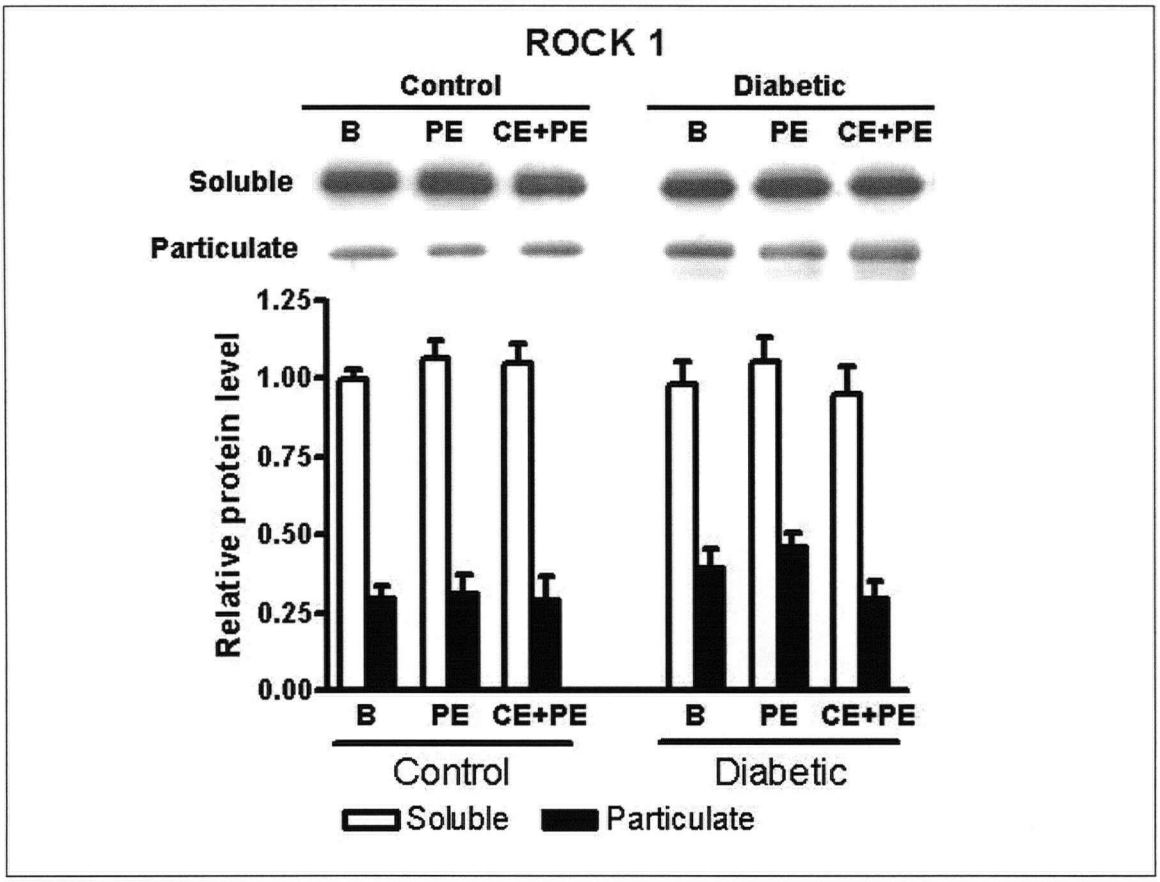

Figure 21 Relative protein levels and a representative blot of ROCK 1 in the soluble and particulate fractions of basal (B), PE-treated (PE) and CE plus PE-treated (CE+PE) hearts from 12~15 week diabetic and age-matched control rats. All the soluble and particulate values are expressed relative to the mean value in the soluble fraction of the Control B group, which is set at 1. n = 10 in B groups; n = 5 in PE and CE+PE groups.

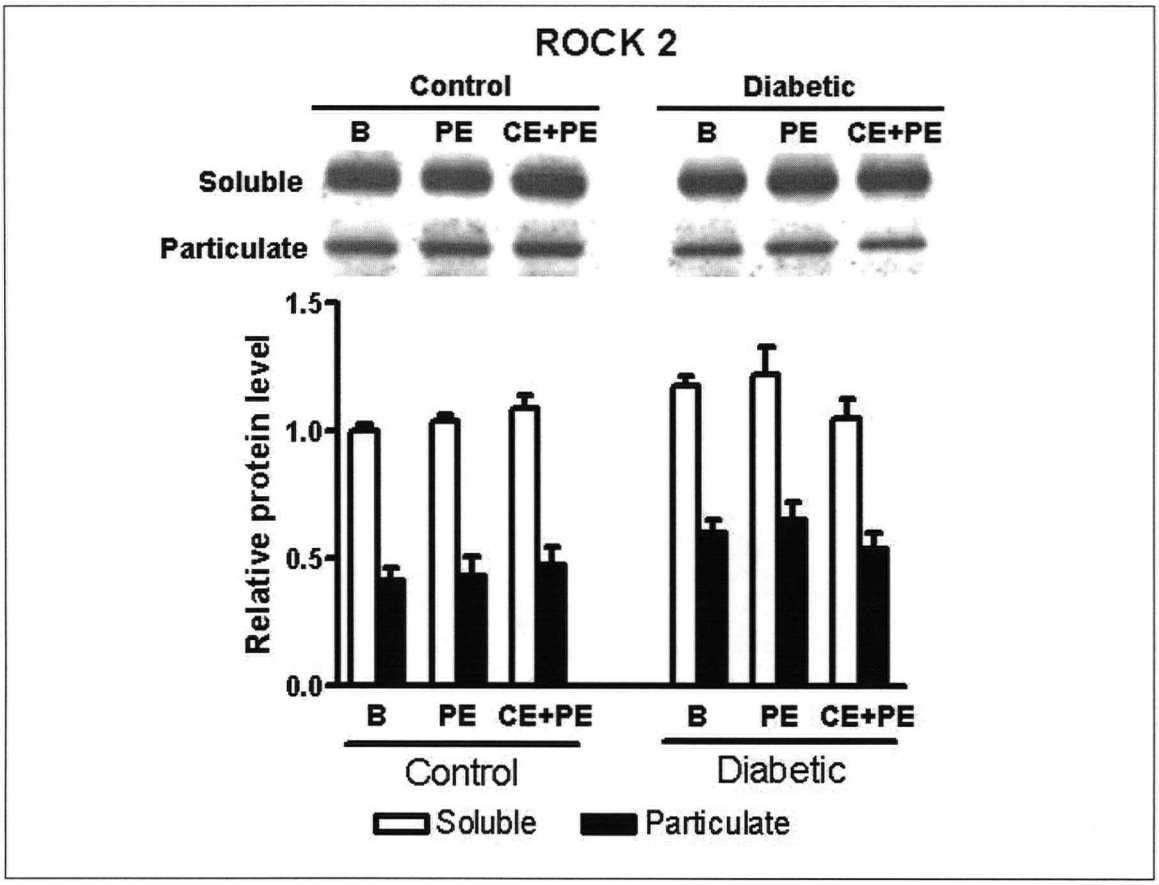

Figure 22 Relative protein levels and a representative blot of ROCK 2 in the soluble and particulate fractions of basal (B), PE-treated (PE) and CE plus PE-treated (CE+PE) hearts from 12~15 week diabetic and age-matched control rats. All the soluble and particulate values are expressed relative to the mean value in the soluble fraction of the Control B group, which is set at 1. n = 10 in B groups; n = 5 in PE and CE+PE groups.

3.2.4 Effect of L-158,809 treatment on the levels of PKCδ, PKCε, ROCK 1 and ROCK 2 in the particulate fraction in unstimulated and PE-stimulated hearts from 12-week diabetic and age-matched control rats

In this set of experiments (Figure 23~26), only changes in the particulate levels of PKCδ, PKCε, ROCK 1 and ROCK 2 were investigated, as no changes in the soluble fractions or other PKC isoforms were detected in previous experiments. All particulate values were expressed relative to the mean value of the Control Basal group, which was set at 1.

In the absence of PE treatment, the particulate levels of both PKCδ and PKCε were significantly increased in diabetic hearts. L-158,809 treatment did not significantly change the particulate levels of either isoform in either diabetic or control hearts. Exposure of both control and diabetic hearts to PE for 2 minutes resulted in a significant increase in the particulate levels of PKCδ (Figure 23). As was found in the previous experiment, the increase in PKCδ over its own basal levels in diabetic hearts (0.86±0.11) was also significantly greater than the PE-induced increase of this isoform in control hearts (0.32±0.13) this time. PE also produced a significant increase in the levels of PKCϵ in the particulate fraction of both control and diabetic hearts (Figure 24). Similar to what had been found in the previous experiment, in the presence of PE, total levels of PKCϵ in the particulate fraction of diabetic hearts were significantly greater than control, while the magnitude of the PE-induced increase in PKCε over its corresponding basal level was also not significantly different in diabetic (0.49±0.07) and control (0.47±0.14)

hearts this time. L-158,809 treatment did not significantly change the particulate levels of either isoform in either PE-treated diabetic or PE-treated control hearts. However, in hearts from L-158,809-treated rats, particulate levels of PKCε in the presence of PE were not significantly different from those in the absence of PE.

No significant changes in the particulate levels of ROCK 1 and ROCK 2 were detected in diabetic hearts. Neither L-158,809 nor PE treatment had any significant effect on the particulate levels of either isoform in either control or diabetic hearts (Figure 25; Figure 26).

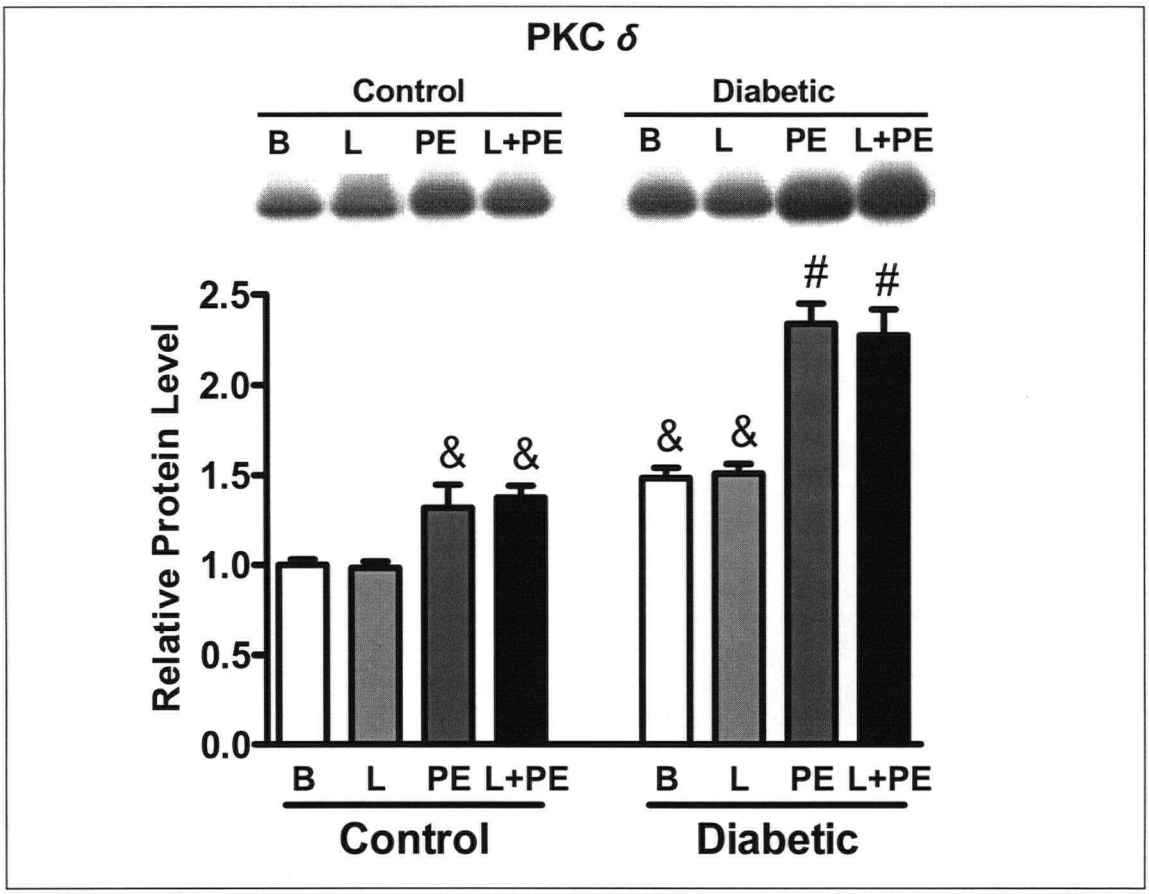

Figure 23 Relative protein levels and a representative blot of PKCδ in the particulate fraction of basal (B) and PE-treated (PE) hearts from 12-week diabetic and age-matched control rats with (L) or without L-158,809 treatment. All particulate values are expressed relative to the mean value of the Control B group, which is set at 1. n = 5 hearts in each group.

Significantly different from all control groups and diabetic B, diabetic L group. $P<0.05$

& Significantly different from control B, control L, diabetic PE and diabetic L+PE group. $P<0.05$

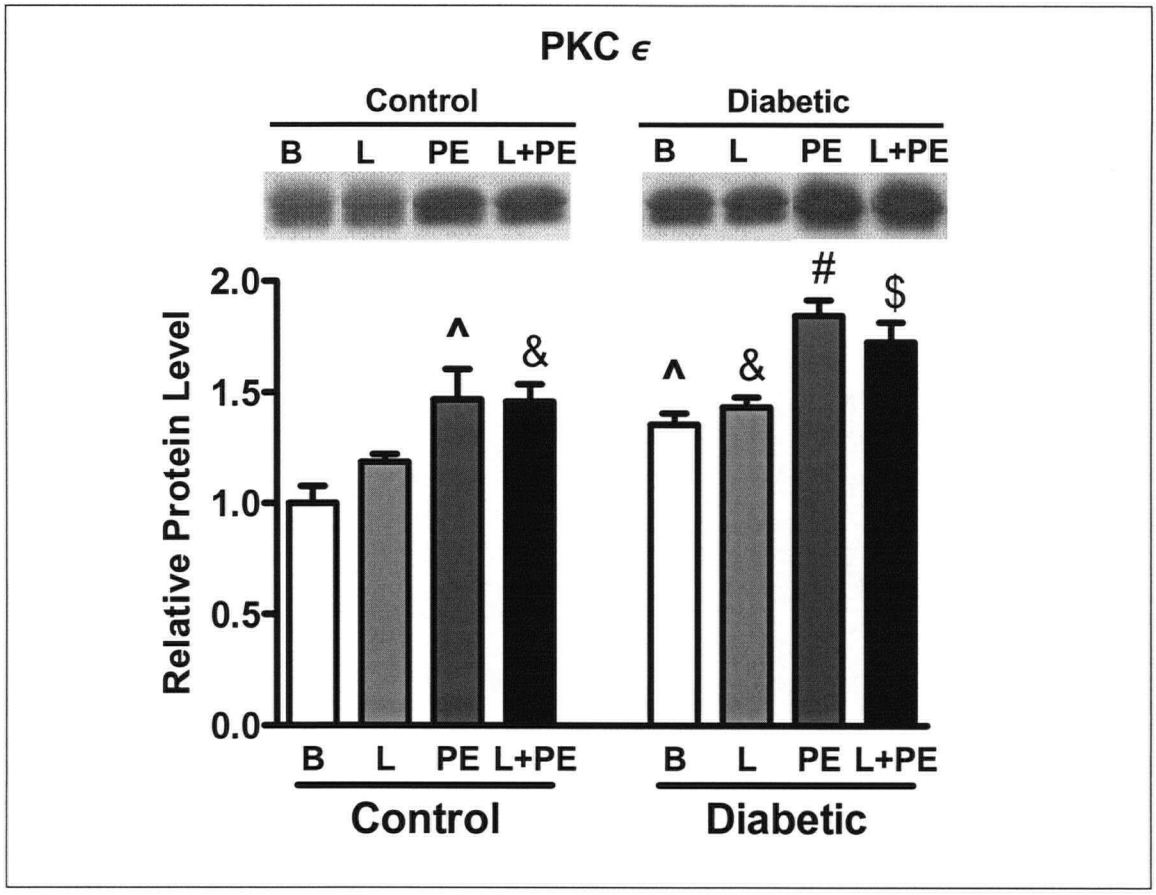

Figure 24 Relative protein levels and a representative blot of PKCε in the particulate fraction of basal (B) and PE-treated (PE) hearts from 12-week diabetic and age-matched control rats with (L) or without L-158,809 treatment. All particulate values are expressed relative to the mean value of the Control B group, which is set at 1. n = 5 hearts in each group.

Significantly different from all control groups and diabetic B, diabetic L group. P<0.05

$ Significantly different from control B, control L, control PE, and diabetic B group. P<0.05

^ Significantly different from control B, diabetic PE and diabetic L+PE group. P<0.05

& Significantly different from control B and diabetic PE group. P<0.05

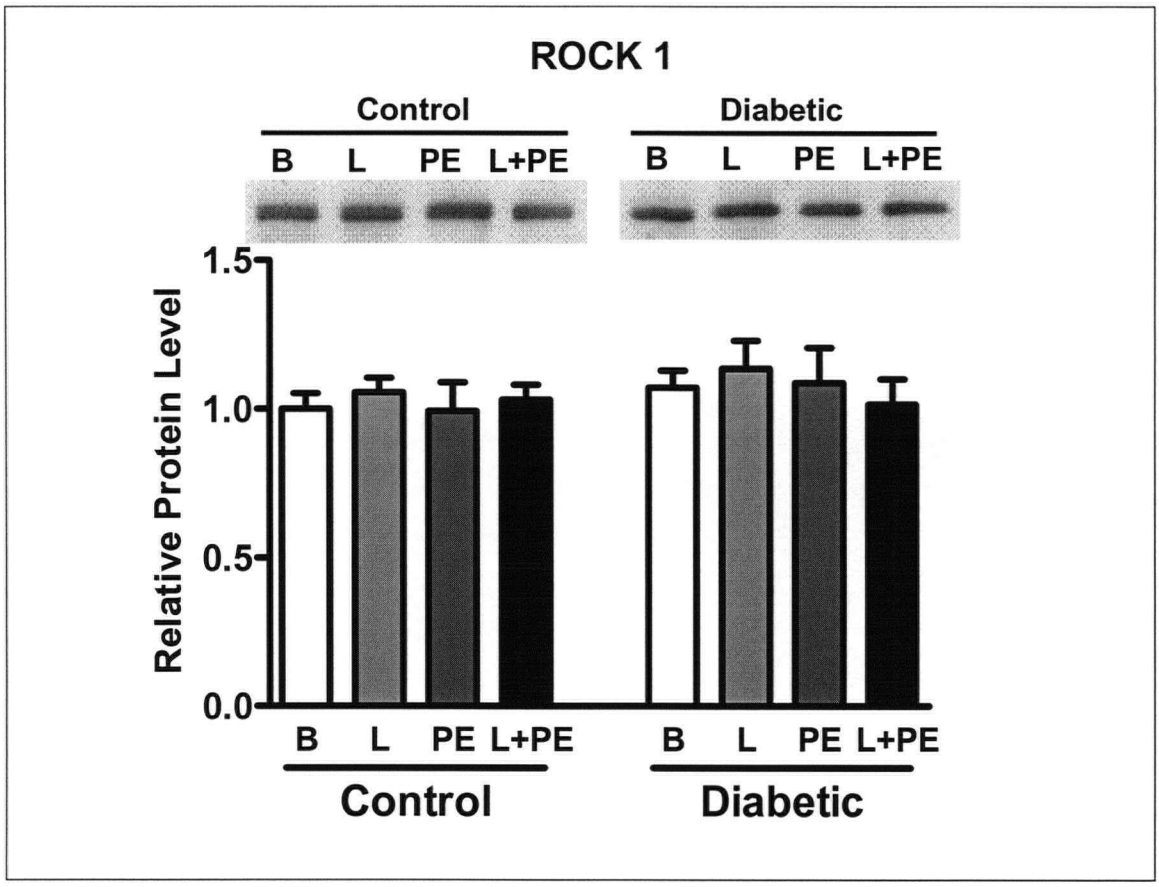

Figure 25 Relative protein levels and a representative blot of ROCK 1 in the particulate fraction of basal (B) and PE-treated (PE) hearts from 12-week diabetic and age-matched control rats with (L) or without L-158,809 treatment. All particulate values are expressed relative to the mean value of the Control B group, which is set at 1. n = 5 hearts in each group.

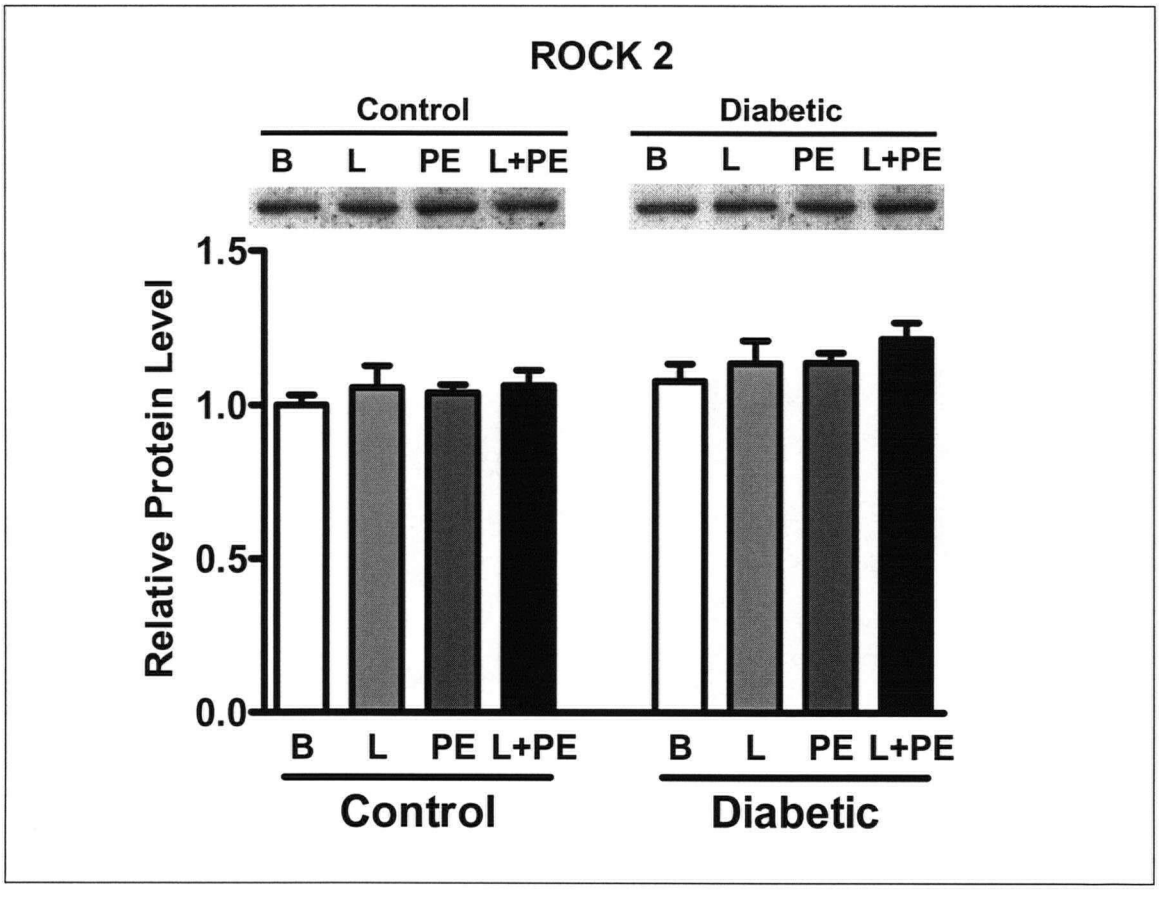

Figure 26 Relative protein levels and a representative blot of ROCK 2 in the particulate fraction of basal (B) and PE-treated (PE) hearts from 12-week diabetic and age-matched control rats with (L) or without L-158,809 treatment. All particulate values are expressed relative to the mean value of the Control B group, which is set at 1. n = 5 hearts in each group.

4 DISCUSSION

4.1 Summary of results

The present study showed that in the absence of adrenergic stimulation, the basal contractile performance was attenuated in diabetic hearts, whereas the PIE in response to PE was enhanced compared to control. In the presence of CE, the PE-induced PIE in both diabetic and control hearts was suppressed, and the PIE in diabetic hearts was no longer significantly different than control. Under basal conditions, the subcellular distribution of PKCα, PKCβ_2, ROCK 1 and ROCK 2 was not altered in diabetic hearts. However, the levels of PKCδ and ε in the particulate fraction of diabetic hearts were increased, without a corresponding decrease in the soluble fraction. PE produced a significant increase in the levels of PKCδ and ε in the particulate fraction of hearts from both diabetic and control rats, again without a corresponding decrease in the soluble fraction. The increase in particulate PKCδ over its own basal levels in diabetic hearts was significantly greater than control, whereas the increase in particulate PKCϵ over its own basal levels in diabetic and control hearts was not different. In the presence of CE, the PE-induced increase in the levels of PKCδ and ε in the particulate fraction of both diabetic and control hearts was completely blocked. PE had no detectable effect on the subcellular distribution of PKCα, PKCβ_2, ROCK 1 or ROCK 2. Treatment with the AT_1 receptor antagonist, L-158,809, significantly improved the basal contractile function of diabetic hearts. However, it did not normalize the enhanced α_1-AR-induced PIE. L-158,809 had no effect on the basal levels of PKCδ, PKCε, ROCK 1 or ROCK 2 in the

particulate fraction in either diabetic or control hearts, nor did it affect the PE-induced changes in these two PKC isozymes.

4.2 Choice of cardiac preparation

One purpose of the present study was to investigate the effect of long-term STZ-diabetes on the α_1-AR-induced contractile responses in the heart. Several functional studies have reported the α_1-AR-mediated PIE is enhanced in diabetic rat hearts. Most of these investigations used isolated cardiac muscle strip preparations, such as intact atria or atrial strips (Canga *et al.* 1986; Jackson *et al.* 1986; Durante *et al.* 1989; Brown *et al.* 1994), right ventricular strips (Wald *et al.* 1988; Yu *et al.* 1991) or left ventricular papillary muscles (Brown *et al.* 1994). Since it is the left ventricle that determines cardiac output and drives the systemic circulation, the studies using atrial or right ventricular preparations may not reflect the effects of diabetes on cardiac function. As a result, left ventricular preparations were used in the study. The left ventricular papillary muscle preparation is a relatively simple and easy technique compared to whole heart perfusion. However, since multiple biochemical assays had to be performed after functional measurements, left ventricular papillary muscles were not able to provide enough protein for Western blot assays. A second disadvantage of the left ventricular papillary muscle preparation is that due to its thickness, it is highly susceptible to hypoxia. The perfused whole heart preparations, in which the coronary vessels are perfused and the oxygenated buffer rapidly gains access to all cardiomyocytes, are less likely to be subject to ischemia. Whole heart preparations such as the working heart or

the Langendorff iosvolumic heart are ideal choices because the function of the whole left ventricle can be measured, and the amount of protein available for subsequent biochemical assays is relatively high. Heijnis *et al.* (1992), using the working heart model, showed the α_1-AR-mediated PIE was elevated in hearts from STZ-diabetic rats. So far there has been no investigation using the Langendorff isovolumic heart for the measurement of the α_1-AR-mediated PIE. Compared to working heart techniques, the Langendorff-perfused heart is simpler and less demanding in terms of equipment and the operator's skill. Secondly, in the working heart model, the coronary perfusion is dependent on left ventricular function, while in the Langendorff heart, the coronary perfusion is independent of ventricular function, leading to the suggestion that the Langendorff heart is a better model to study the concentration-dependent effects of inotropic agents on cardiac contractile function (Fawzi 1997). As a result, in the study, the Langendorff isovolumic heart model was chosen.

4.3 Setting of experimental conditions

One important issue of this study was to treat the diabetic and age-matched control hearts with the same basal conditions. Contractile parameters such as LVDP, +dP/dt and -dP/dt of the Langendorff heart are affected by CPP, as well as by other factors, such as the temperature, balloon size and heart rate (Fawzi 1997). In the Langendorff setup of this study, the temperature, balloon size and heart rate were controlled and maintained the same in diabetic and control hearts. In addition, a pressure transducer was connected to the aortic cannula to monitor CPP, and adjusted the

perfusion flow rate to give the same CPP (80 mmHg) in both diabetic and control hearts. Because diabetic hearts were smaller than age-matched control hearts, the coronary vessels were also smaller in diabetic hearts. When perfused at the same CPP, the coronary perfusion flow rate in diabetic hearts was lower than that in control hearts. However, when normalized for heart weight, the ratio of flow rate / heart weight in diabetic hearts was not different from that in control hearts (Figure 5).

4.4 Basal contractile function and the α_1-AR-mediated PIE are two independent processes

The present study demonstrated an attenuated basal contractile performance as well as an elevated α_1-AR-mediated PIE in diabetic hearts compared to control. While it might be argued that the greater maximal increase (though not sensitivity) to PE is simply the result of the lower basal contractile performance in the diabetic hearts, two observations from the study argue against this possibility. First of all, in preliminary experiments, two groups of normal hearts were perfused with a basal CPP of 50 mmHg and 70 mmHg, respectively. The group perfused at a CPP of 70 mmHg exhibited a higher basal contractile performance. If the above argument is true, this group would be expected to be associated with a smaller maximal increase to PE. In fact, this group showed a greater response to PE. Moreover, in Cardiac Function Study #4, the basal contractile function of hearts from L-158,809-treated diabetic rats was significantly improved compared to that of hearts from untreated diabetic rats, and was no longer significantly different than control. However, the hearts from L-158,809-treated diabetic

rats still exhibited a significantly greater maximal increase to PE compared to control. Based on these two findings, it could be ruled out that a lower basal contractile performance mechanically allowed the heart to produce a higher contractile response to PE. There is no mechanical correlation between the basal cardiac function and the α_1-AR-mediated PIE.

4.5 Changes in the subcellular distribution of PKC isoforms

The present study showed that both diabetes and α_1-AR stimulation elevated the particulate levels of PKCδ and ϵ, without a corresponding decrease in the soluble levels of these two isozymes. Similar observations have also been found in other investigations with diabetic animal models. For example, Inoguchi *et al.* (1992) showed a significant increase in particulate PKCβ_2 in hearts from 2-week STZ-diabetic rats compared to control, but the soluble levels of this isoform remained unchanged. Kang *et al.* (1999) found that the particulate levels of PKCα and ε were increased in renal tissues from 4-week STZ-diabetic rats, but the percentage of the soluble versus the particulate levels of these two isoforms was unaltered. In addition, some investigations also showed that PE as well as other G-protein-coupled receptor agonists induced a significant elevation in particulate PKC isozymes, but without a corresponding decrease in the soluble levels. For instance, Puceat *et al.* (1994) exposed isolated cardiomyocytes from adult rats to PE and produced 2~3 fold increases in particulate PKCε, but without a decrease in the soluble levels. Wang *et al.* (2003) also found an elevation in particulate PKCε produced by PE, associated with a much smaller decrease in the soluble levels of this isoform. In

another study (Henry *et al.* 1996), adenosine receptor agonists produced 2~3 fold increases in particulate PKCδ in isolated cardiomyocytes from adult rats, without an apparent concomitant decrease in the soluble fraction.

Two arguments may help to explain the above observations. Firstly, although the translocation of PKC isozymes from the soluble fraction to the particulate fraction has been recognized as a hallmark of their activation, some studies have suggested that additional changes in PKC isoforms may occur upon their activation. Recent studies have shown that stimulation of PKCδ and ϵ by neurohormones or phorbol 12-myristate 13-acetate (PMA, a non-isoform-selective PKC activator) not only produces a translocation of the isozymes from the soluble fraction to the particulate fraction, but also induces phosphorylation as well as conformational changes in the isoforms (Rybin *et al.* 2003; Rybin *et al.* 2004). Thus, the binding of the PKC isoform molecules with their corresponding antibodies may be affected, resulting in changes in their immunoreactivity. Therefore, in the present study, the changes in PKCδ and ϵ in diabetes or in response to PE stimulation could have resulted not only from a physical translocation, but also from a structural modification of the isozymes leading to altered immunoreactivity.

Secondly, the increase in levels of PKCδ and PKCε in the particulate fraction without a corresponding decrease in the soluble fraction could be due to the uneven distribution of the isoforms between the two fractions. This theory was uttered by Henry *et al.* (1996), who also detected a significant increase in particulate PKCδ in isolated rat cardiomyocytes after the treatment with an adenosine receptor agonist, but without a corresponding decrease in the soluble fraction. In the present study, if we take PKCε as an example, examination of Figure 20 suggests that there are approximately equal

amounts of PKCε in 50 μg of soluble and 50 μg of particulate protein. However, on fractionation of left ventricular preparations, approximately 80% of the total protein remains in the soluble fraction, while 20% is found in the particulate fraction. Therefore, there is about 4 times more PKCε in the soluble than that in the particulate fraction. Following stimulation with PE, there is an approximately 50% increase in PKCε in the particulate fraction, but this would correspond to only a 12.5% decrease in the soluble fraction, an amount that would be difficult to detect given the variability between hearts and assays.

4.6 Effect of diabetes on the subcellular distribution of cardiac PKC isoforms

A number of studies have suggested that in hearts from STZ-diabetic rats, the membrane-associated (particulate) or total PKC activity is increased (Inoguchi *et al.* 1992; Tanaka *et al.* 1992; Xiang *et al.* 1992; Liu *et al.* 1999). Although the mechanisms are not fully understood, hyperglycemia, which increases the DAG content in rat myocardium, has been suggested to play a causal role in the activation of PKC (Okumura *et al.* 1988; Inoguchi *et al.* 1992; Porte *et al.* 1996). On the other hand, changes in the subcellular distribution of PKC isoforms in STZ-diabetic hearts have been shown in some studies, but the results are far from consistent. Inoguchi *et al.* (1992) reported that in whole heart preparations from 2-week diabetic male Sprague-Dawley rats, there was an increase in particulate PKCβ_2 with no change in the soluble fraction, and the subcellular distribution of PKCα was not altered. Malhotra *et al.* (1997) showed that in isolated cardiomyocytes from 3~4 week diabetic female Wistar rats, PKCε translocated from the

soluble fraction to the particulate fraction, while no change in PKCδ was detected. Liu *et al.* (1999) demonstrated that in ventricular preparations from 8-week diabetic male Sprague-Dawley rats, levels of PKCα, β and ε were increased in the total protein and in the soluble fraction, but there was no change in the particulate fraction. Kang *et al.* (1999) showed that in whole heart preparations from 4-week diabetic male Sprague-Dawley rats, the total protein levels of PKCα were increased, with no changes in $PKC\beta_2$ and δ, and a decrease in PKCε. In the present study, a significant increase in the levels of PKCδ and ε in the particulate fraction was detected, without a corresponding decrease in the soluble fraction, and the subcellular distribution of PKCα and β_2 was not altered. Note that in the present study, the duration of STZ-diabetes was much longer (12~15 week), only left ventricular preparations (left ventricular walls and ventricular septa) were used, and the hearts were perfused with K-H buffer for some time (22 minutes) before being frozen for Western blot assays. Therefore, the discrepancies between the present study and the investigations mentioned above could be due to difference in the types of rats, the duration of diabetes, the cardiac preparations and/or the experimental conditions.

4.7 PKC isozymes and diabetic cardiomyopathy

PKC has been implicated in the pathological progress of myocardial diseases, including diabetic cardiomyopathy and other disease-induced cardiac dysfunctions. As mentioned above, diabetes appears to induce the activation of specific PKC isozymes in

the heart. The involvement of PKCδ, ε and β_2 in the pathogenesis of diabetic cardiomyopathy has been suggested by a variety of studies.

Activation of PKCδ and ε has been suggested to contribute to the over-expression of β-myosin heavy chain (β-MHC) and to the increased secretion of atrial natriuretic peptide (ANP) and angiotensin-converting enzyme (ACE) in the heart; on the other hand, the levels of these proteins in the hypertrophic heart have been shown to be increased (Zarich *et al.* 1989; Uusitupa *et al.* 1990). Thus the activation of PKCδ and ε, and the subsequent elevation in β-MHC, ANP and ACE, may be an important component in the development of diabetic cardiomyopathy (Steinberg *et al.* 1995). More recent studies have suggested PKCδ and ε might have distinct effects. Over-expression of a constitutively-active PKCϵ mutant in cardiac culture has been reported to induce cellular remodeling and elongation (Strait *et al.* 2001), which are fundamental processes for cardiac hypertrophy. Similarly, transgenic over-expression of PKCϵ has been shown to cause concentric cardiac hypertrophy (Takeishi *et al.* 2000). On the other hand, PKCδ over-expression has been shown to result in cell detachment and cardiomyocyte apoptosis, which are different outcomes than the hypertrophic effect of PKCϵ (Heidkamp *et al.* 2001). Though the exact signaling mechanisms underlying the apoptotic effect of PKCδ and the hypertrophic effect of PKCϵ are not fully clear, some reports have suggested that the activation of the two PKC isoforms leads to the selective activation of specific terminal kinases of the mitogen-activated protein kinase (MAPK) cascade, a signaling cascade for cardiac hypertrophic and apoptotic gene expression (Bueno *et al.* 2002). PKCδ preferentially activates two terminal kinases, c-Jun N-terminal kinase (JNK) and p38-MAPK, resulting in apoptosis, while PKCϵ selectively activates

extracellular-regulated kinase (ERK), a terminal kinase of the MAPK cascade generally implicated in growth responses and hypertrophy (Heidkamp *et al.* 2001). Malhotra *et al.* (1997) showed a tranlocation of PKCϵ and an increased phosphorylation of TnI in cardiomyocytes from diabetic rats, and they also suggested this isoform might participate in diabetic cardiomyopathy. In all, both PKCδ and ε may be activated in the diabetic heart and could contribute to the development of diabetic cardiomyopathy.

PKCβ_2 has been suggested to participate in several chronic pathological processes in the heart, such as cardiac hypertrophy, heart failure and diabetic cardiomyopathy (Koya *et al.* 1998; Sabri *et al.* 2003). In short-term (2-week) STZ-diabetic rat hearts, particulate levels of PKC β_2 were increased (Inoguchi *et al.* 1992). In pressure-overload cardiac hypertrophic rats, an increase in particulate levels of PKCβ_2 was also observed (Gu *et al.* 1994). Ventricles from patients with end-stage heart failure showed increased expression of PKCβ_2 (Bowling *et al.* 1999). Transgenic mice over-expressing PKCβ_2 specifically in myocardium developed cardiac hypertrophy, cardiomyocyte injuries and fibrosis at 8~12 weeks of life. Later, cardiac atrophy and severe fibrosis were observed. Treatment with a selective PKCβ inhibitor, LY333531, prevented most of the functional and pathological changes in hearts from these transgenic mice (Wakasaki *et al.* 1997). This study, along with two other investigations (Bowman *et al.* 1997; Takeishi *et al.* 1998), in which similar findings were observed, has implicated the role for this PKC isoform in cardiomyopathy and cardiac contractile dysfunction. Moreover, in a number of phase 2 clinical trials, LY333531 has been shown to be efficacious in diabetes-induced cardiac dysfunction (Hayat *et al.* 2004). Transforming growth factor β and connective tissue growth factor can induce production of collagen and fibronectin in cardiac

fibroblasts and cardiomyocytes, resulting in myocardial stiffness and attenuated contractility (Ohnishi *et al.* 1998; Chen *et al.* 2000). A correlation between the activation of PKCβ_2 and the expression of these two growth factors in hearts from STZ-diabetic mice has been suggested (Way *et al.* 2002). In all, PKCβ_2 has been implicated in the development of diabetic cardiomyopathy. In the present study, no significant change was detected in the subcellular distribution of PKCβ_2 in the diabetic heart. However, there are a number of differences in the animal models, duration of diabetes and experimental conditions between the present study and the ones mentioned above. Thus at this point, it cannot be ruled out that this PKC isozyme is involved in diabetic cardiomyopathy.

4.8 Diabetes may not have effects on the subcellular distribution of Rho kinase in the heart

The role for RhoA – Rho kinase in myocardial contractile function is poorly understood. There have been several investigations suggesting that RhoA – Rho kinase may be involved in myocardial hypertrophy and heart failure (Kuwahara *et al.* 1999; Suematsu *et al.* 2001; Yanazume *et al.* 2002). On the other hand, the mRNA levels of RhoA in hearts from transgenic diabetic mice have been shown to be elevated (Duan *et al.* 2003). However, there have been no studies reporting diabetes-induced changes in cardiac Rho kinase. The present study did not observe any significant effect of diabetes on the subcellular distribution of Rho kinase. Therefore it does not support a role for this kinase in diabetic cardiomyopathy.

4.9 Contribution of PKC isozymes to the α_1-AR-mediated PIE

In the early 1990's, several groups investigated the role for PKC in the α-AR-mediated PIE (Otani *et al.* 1992; Talosi *et al.* 1992; Endoh *et al.* 1993). In these studies, the PKC inhibitors staurosporine and H-7 were shown to inhibit the α-AR-mediated PIE in the heart. However, both of these agents lack specificity and are equally potent inhibitors of both PKC and protein kinase A (Toullec *et al.* 1991). Later on, a more selective PKC inhibitor, BIM (Toullec *et al.* 1991), was used in a study investigating the α_1-AR-mediated PIE in neonatal rat hearts (Deng *et al.* 1997). BIM, a non-isoform-selective PKC inhibitor, significantly blocked the positive inotropic response to PE and the PE-induced increase in PKC activity in the particulate fraction. Thymeleatoxin, a selective activator of conventional PKC isoforms (Ryves *et al.* 1991), produced small inhibition in myocardial contraction, while Go-6976, a selective inhibitor of conventional PKC isoforms (Martiny-Baron *et al.* 1993), did not inhibit the PE-induced PIE. The Western blot results from this study showed that PE produced a translocation of PKCδ and ϵ from the soluble to the particulate fraction but had no effect on PKCα. Thus it was concluded in this study that the activation of novel PKCδ and ϵ by α_1-AR agonists plays a key role in the α_1-AR-mediated PIE in neonatal rat hearts. In studies using ventricular myocytes isolated from adult rat hearts, it was also shown that upon the stimulation with PE, the levels of PKCδ and ε in the particulate fraction were elevated, but PKCα was not changed (Puceat *et al.* 1994; Wang *et al.* 2003). In the current study, among the conventional PKC isoforms (PKCα and β_2) and the novel PKC isoforms (PKCδ and ϵ) found in the heart, only PKCδ and ϵ responded to PE stimulation and were inhibited by

CE. These results, along with the findings from the above studies, suggest that PKCδ and/or PKCϵ play a role in the α_1-AR-mediated PIE in both normal and diabetic hearts. However, due to a lack of selective PKCδ and PKCϵ inhibitors, the individual role for these two isozymes in the α_1-AR-mediated PIE could not be further clarified.

4.10 Contribution of PKC to the enhanced α_1-AR-mediated PIE in the diabetic heart

Although a number of studies have shown that in the diabetic rat heart, the α_1-AR-mediated PIE is elevated (Canga *et al.* 1986; Jackson *et al.* 1986; Wald *et al.* 1988; Durante *et al.* 1989; Yu *et al.* 1991; Heijnis *et al.* 1992; Brown *et al.* 1994; Ha *et al.* 1999), the underlying molecular mechanisms are poorly understood. Binding studies have consistently found that in diabetic cardiomyocytes, the number of α_1-AR-binding sites is reduced (Heyliger *et al.* 1982; Wald *et al.* 1988; Tanaka *et al.* 1992; Kamata *et al.* 1997). This is associated with no change (Heyliger *et al.* 1982; Tanaka *et al.* 1992; Kamata *et al.* 1997) or an increase (Wald *et al.* 1988) in their affinity constants. As a result, the enhanced α_1-AR-mediated PIE may be due to post-receptor mechanisms. It has been shown that the enhanced α_1-AR-mediated PIE is associated with increased IP_3 production in diabetic ventricular preparations compared to control (Xiang *et al.* 1991), suggesting that α_1-AR-mediated stimulation of PLC is enhanced in diabetes. Although there have been no studies directly measuring the effect of diabetes on the activation of PLC or the levels of DAG content upon the stimulation of α_1-AR, Wald *et al.* (1988) obtained indirect evidence suggesting there is enhanced activation of the PLC-DAG

pathway upon α_1-AR stimulation in hearts from acutely diabetic rats compared to control. They found an enhanced α_1-AR-mediated PIE in diabetic ventricular preparations. In the presence of a PLC inhibitor, the α_1-AR-mediated PIE in diabetic ventricular preparations was attenuated, reaching a level similar to that in control preparations without the presence of the PLC inhibitor. Moreover, in the presence of synthetic DAG, the α_1-AR-mediated PIE in control preparations was increased, reaching a level similar to that in diabetic preparations without the presence of synthetic DAG. The present study suggests a role for PKC in the enhanced α_1-AR-mediated PIE in hearts from long-term diabetic rats, providing further evidence to support the hypothesis that there is enhanced activation of the PLC-DAG-PKC pathway upon α_1-AR stimulation in the diabetic heart, and this may be one molecular mechanism underlying the enhanced α_1-AR-mediated PIE in diabetes.

As mentioned above, at the present stage it cannot be concluded whether PKCδ, or PKCε, or both, play a role in the α_1-AR-mediated PIE. However, the present study suggests a role for PKCδ in the greater PIE in response to α_1-AR stimulation in diabetic hearts. With PE stimulation, the levels of PKCδ in the particulate fraction of both diabetic and control hearts were increased, and the increase in particulate levels over its own basal level in diabetic hearts was significantly greater than control. In the presence of CE, the PE-induced increase in particulate levels of this isoform in both diabetic and control hearts was completely surpressed. Compared to PKCδ, PKCϵ may be less important in the enhanced α_1-AR-mediated PIE in the diabetic heart, since the data from the current study showed that with PE stimulation, the increase in particulate levels of this isoform over its own basal level was not significantly different in diabetic and control

hearts. However, it cannot be ruled out that PKCϵ may also contribute to the enhanced α_1-AR-mediated PIE in the diabetic heart, since there is a lack of selective PKCδ and PKCϵ inhibitors, and the elevation in levels of PKCϵ the particulate fraction upon stimulation with PE is not as sustainable as that of PKCδ. Puceat *et al.* (1994) showed that in cardiomyocytes from normal adult rats, the increase in particulate PKCε induced by PE was fast and transient. The peak elevation occurred about one minute after the addition of PE, and the levels of particulate PKCε rapidly went back to its baseline level in five minutes. However, the elevation of PKCδ was much longer. Even after fifteen minutes of PE stimulation, the levels of particulate PKCδ remained high compared to its baseline levels. In the present study, the time for PE treatment was two minutes (the minimal time length required to reach the maximal PIE to PE). It is possible that for PKCε, two minutes of PE treatment was too long in terms of the measurement of peak elevation, whereas, because the elevation of particulate PKCδ was more sustainable, a significantly higher increase in this isoform in the diabetic heart was able to be shown.

In all, the data from this study suggest a role for PKCδ in the greater PIE to PE in the diabetic heart, but it cannot be excluded that PKCϵ may also contribute to this alteration in diabetes.

4.11 Activation of PKC: good or bad?

Due to defects in the β-AR signaling cascade, the increase in contractile performance in response to endogenous noradrenaline is depressed in the diabetic heart (Gotzsche 1983a; Gotzsche 1983b; Smith *et al.* 1984). Meanwhile, the α_1-AR-mediated

PIE is up-regulated, which has been suggested to be a compensatory mechanism to maintain cardiac contractile response to noradrenaline (Corr *et al.* 1981; Milligan *et al.* 1994; Beaulieu *et al.* 1997; Skomedal *et al.* 1997). The present study suggests a role for the novel PKC isoforms, PKCδ, or both PKCδ and PKCϵ, in the enhanced α_1-AR-mediated PIE in the diabetic heart. However, this may be only a transient compensatory mechanism, since long and sustained activation of these PKC isozymes may "turn on" the expression of a number of hypertrophic and apoptosis genes in the heart, including β-MHC, ANP, ACE (Zarich *et al.* 1989; Uusitupa *et al.* 1990), JNK, p38-MAPK and ERK (Heidkamp *et al.* 2001), contributing to the development of diabetic cardiomyopathy. In diabetes, DAG levels in cardiomyocytes are increased, possibly due to hyperglycemia (Okumura *et al.* 1988; Inoguchi *et al.* 1992; Porte *et al.* 1996). This leads to the activation of not only PKCδ and PKCϵ, but also PKCβ, which has been implicated in many studies to contribute to the development of diabetic cardiomyopathy. In all, the overall activation of PKC in the diabetic heart may lead to the destruction and reconstitution of myocardial structure and impaired contractile performance. This may be an important component in the pathogenesis of diabetic cardiomyopathy.

4.12 Role of Rho kinase in the α_1-AR-mediated PIE

A role for Rho kinase in the α_1-AR-mediated PIE in the heart has been suggested in several studies using the non-isoform-selective Rho kinase inhibitor, Y-27632. This inhibitor, at the concentration of $5*10^{-5}$ M and 10^{-5} M, respectively, was shown to effectively block the PE-induced PIE in rat left ventricular papillary muscles (Andersen

et al. 2002) and human atrium (Grimm *et al.* 2005). At a concentration of 10^{-5} M, it also blocked the PE-induced Ca^{2+} sensitization in isolated cardiomyocytes from failing hearts (Suematsu *et al.* 2001). So far there has been no study showing translocation or activation of Rho kinase in the heart or isolated cardiac preparations upon α_1-AR stimulation. In the present study, at a concentration of 10^{-6} M, Y-27632 did not inhibit PE-induced PIE in normal hearts. Another non-isoform-selective Rho kinase inhibitor, H1152, which is more potent and selective for Rho kinase than Y-27632 (Sasaki 2003) was used. However, even at a concentration of 10^{-6} M, H1152 had no effect on the PE-induced PIE in normal or 12-week diabetic hearts. Furthermore, stimulation of α_1-AR with PE did not alter the subcellular distribution of Rho kinase in either diabetic or control hearts. Therefore, a role for Rho kinase in the α_1-AR-mediated PIE could not be observed in this study. The conflicting results from the above studies and from the present investigation may be due to differences in cardiac preparations. At the present stage, the existence of Rho kinase in the proposed signaling pathways underlying the α_1-AR-mediated PIE is still poorly understood.

4.13 PKC may not be involved in the improvement of the impaired basal contractile function of the diabetic heart by the inhibition of RAS

There is strong evidence that activation of the RAS contributes to diabetic cardiomyopathy (Hoenack *et al.* 1996; Goyal *et al.* 1998; Al-Shafei *et al.* 2002; Privratsky *et al.* 2003; Shekelle *et al.* 2003), but the molecular signaling mechanisms are not clear. Malhotra *et al.* (1997) has suggested a PKC-dependent pathway. These

investigators found a translocation of PKCϵ as well as an increased phosphorylation of TnI in isolated cardiomyocytes from 4-week STZ-diabetic rats. Treatment of diabetic rats with L-158,809 completely prevented the changes in PKCε and TnI in diabetes. However, in the present study, although treatment with L-158,809 improved the attenuated basal contractile performance in diabetic hearts, it did not normalize the increase in basal particulate PKCδ and ϵ in diabetic hearts, in contrast with the results from Malhotra's group. There are a number of discrepancies between the current study and that of Malhotra's that might account for this inconsistence. In the former, the AT_1 blocker was dissolved in drinking water and fed to the animals daily (1 mg/kg/day), while in Malhotra's study the rats received subcutaneous injection of the drug twice a week (10 mg/kg). The duration of diabetes (12 weeks vs 4 weeks), cardiac preparations (left ventricles vs. isolated cardiomyocytes) and experimental protocols were also different.

The normalizing effect of L-158,809 on diabetic cardiac function in the absence of normalization of the subcellular distribution of PKCδ and ϵ, suggests that the AT_1 blocker is acting via some signaling mechanism other than PKC-dependent pathways. Some potential mechanisms have been suggested in other studies. Activation of AT_1 receptors leads to the activation of NADPH oxidase and consequently enhances the production of reactive oxygen species, such as superoxide anion, which can react with nitric oxide, leading to its inactivation by producing peroxynitrite. The latter can directly oxidize membrane components such as arachidonic acid, thus altering membrane integrity and cardiac function (Griendling *et al.* 2000; Sowers 2002). Activation of AT_1 receptors can also lead to the activation of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) (Mascareno *et al.* 2000). This pathway was initially

discovered as a major signal transduction pathway of the cytokine superfamilies (Ihle 1995). The JAK/STAT proteins are involved in the production of various kinds of cytokines and growth factors that contribute to cardiac hypertrophy (Schindler *et al.* 1995). Although these molecular mechanisms have been proposed to contribute to cardiomyopathy, the exact role for angiotensin II, activation of AT_1 receptors and their downstream signaling mechanisms in the onset of cardiac dysfunction, especially during diabetes, is still poorly understood.

4.14 The attenuated basal contractile performance and the enhanced α_1-AR-mediated PIE may be two relatively independent alterations in the diabetic heart

Although the present study showed that treatment of diabetic rats with L-158,809 significantly improved the basal contractile function of diabetic hearts, this AT_1 receptor antagonist did not normalize the enhanced α_1-AR-mediated PIE in diabetes, suggesting that the attenuated basal contractile function and the enhanced α_1-AR-mediated PIE may be two relatively independent alterations in diabetes. This phenomenon was also shown in several studies investigating the contribution of experimental diabetes-induced hypothyroidism to the enhanced α-AR-induced PIE and the attenuated basal cardiac function. It has been known for a long time that STZ-diabetes is associated with hypothyroidism, in which plasma triiodothyronine (T_3) levels are significantly low (Boado *et al.* 1978). In animals with hypothyroidism, an increased α-AR-mediated PIE has been observed (Simpson *et al.* 1981). A correlation between the enhanced α_1-AR-

mediated PIE and hypothyroidism in STZ-diabetes was suggested (Goyal *et al.* 1987). These investigators showed that left atrial preparations from 6-week STZ-diabetic rats exhibited greater inotropic responses to α-AR agonists, while treatment of the diabetic animals with T_3 prevented this change. In another study (Lafci-Erol *et al.* 1994), it was also shown that the α_1-AR-mediated PIE was enhanced in atrial preparations from alloxan-induced diabetic rats. Treatment with insulin normalized this change, while thyroidectomy prevented the effect of insulin. The atrial preparations from diabetic rats with insulin treatment and thyroidectomy still exhibited enhanced inotropic responses to α_1-AR stimulation. However, a study (Tahiliani *et al.* 1984) reported that the depression of the basal contractile performance of the diabetic heart was not normalized by T_3 treatment. In all, these studies showed that treatment of experimental diabetic rats with T_3 normalized the greater α-AR-mediated PIE but had no effect on the impaired basal cardiac function, suggesting diabetes-induced hypothyroidism may contribute to the former but not to the latter. Similarily, the results from the present study suggest activation of the RAS may contribute to the impaired basal cardiac function but not to the enhanced α_1-AR-mediated PIE in the diabetic heart. Although the exact molecular mechanisms are poorly understood, these two processes may be independent.

4.15 Summary and future directions

The results from the present study showed that in the absence of adrenergic stimulation, the basal contractile performance of the diabetic heart was attenuated. Since the basal levels of PKCδ and ϵ in the particulate fraction were increased in the diabetic

heart, activation of these two isozymes may contribute to the development of diabetic cardiomyopathy. Upon α_1-AR stimulation, a PIE was seen in both diabetic and control hearts, and the diabetic heart exhibited a greater PIE compared to control. In the presence of a PKC inhibitor, the PIE was suppressed and no longer different in diabetic and control hearts. Particulate levels of PKCδ and ϵ increased in response to α_1-AR stimulation and the increase in PKCδ over its own basal levels in the diabetic heart was significantly greater than that in control. In the presence of a PKC inhibitor, the α_1-AR-induced increase in particulate PKCδ and ϵ was totally suppressed. These results suggest that PKCδ and/or PKCϵ may play a role in the α_1-AR-induced PIE, and PKCδ may contribute to the enhanced α_1-AR-induced PIE in the diabetic heart. PKCα, PKCβ_2, ROCK 1 and ROCK 2 were detected in both diabetic and control hearts, but no significant influence of diabetes or α_1-AR stimulation on these isozymes was observed. Treatment of diabetic rats with an AT_1 receptor antagonist improved the impaired basal cardiac performance, but it did not normalize the enhanced PIE to PE, nor did it have significant effect on the associated changes in PKCδ and PKCϵ. This suggests that activation of the RAS contributes to diabetic cardiomyopathy, and PKC may not be involved in this process.

The present study has suggested a role for PKCδ and/or PKCϵ underlying the α_1-AR-induced PIE in the heart, and that PKCδ may contribute to the enhanced α_1-AR-mediated PIE in the diabetic heart. However, it was not able to be excluded that PKCε may also be involved in this process. The use of selective PKCδ and PKCε inhibitors would be helpful to clarify the individual role for these two PKC isozymes in the α_1-AR-mediated PIE, as well as in the elevated PIE in diabetes. In the present study, only the

Western blot assay was used for the measure of the activation of PKC isozymes. More supportive evidence for the activation of PKC could be provided. For example, since the activation of PKCδ and PKCε by neurohormones is associated with their phosphorylation, and antibodies specific for phosphorylated PKCδ and PKCε have been developed (Rybin *et al.* 2003; Rybin *et al.* 2004), investigating the levels of the phosphorylation of these isozymes would help to detect their activation. Moreover, although a direct measure of PKC activity using radioactive or non-radioactive methods cannot distinguish the activation of individual PKC isozymes, it could still be valuable, for it provides direct evidence for the activation of PKC. There are a variety of intracellular targets for PKC, among which MLC2 has been suggested to participate in the regulation of cardiac contractility following α_1-AR stimulation. Detecting the phosphorylation of MLC2 would also be important, since it provides a link between the activation of PKC and the α_1-AR-mediated contractile response.

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