MECHANISM OF CHANGES IN CARDIAC MYOFILAMENT ACTIVATION IN OVARIECTOMIZED RATS WITH DIABETIC COMPLICATION

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MECHANISM OF CHANGES IN CARDIAC MYOFILAMENT ACTIVATION IN OVARIECTOMIZED RATS WITH DIABETIC COMPLICATION

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ABSTRACT

The cardio-protective effect of estrogen (E_2) on the myofilament activation has been reported from our laboratory in a series of experiments. However, the underlying mechanisms are still unclear. Based on the possible interactive action of E_2 and insulin (INS) on cardiac myofilament activation, diabetes (DM) was then used as an approach in this study to challenge the regulatory effect of E_2 on myofilament response to Ca^{2+}. The relationships of pCa-myofilament ATPase activity were compared among sham, ovariectomized (OVX) rats with and without DM complication, and DM-OVX rats with E_2 and/or INS supplementation. The same magnitude of suppression in maximum myofilament ATPase activity was confirmed in OVX, DM and DM-OVX rats, and was restored by supplementation with E_2, INS and coadministration of E_2 and INS, respectively. Specific induction in myofilament Ca^{2+} hypersensitivity after E_2 deficiency was further confirmed in DM-OVX rats in which could be reversed by E_2 supplementation. Additionally, upregulation of β_1-adrenoceptors (β_1-AR) was found to be associated with the myofilament Ca^{2+} hypersensitivity in E_2-deficient groups. Conversely, changes in the heat shock protein 72 (HSP72) paralleled that of maximum myofibrillar ATPase activity.

The cardio-regulatory effect of E_2 on crossbridge (CB) cycling kinetics of the contractile activation was further analyzed. Relations of maximum myofilament ATPase activity and the % α-myosin heavy chain (MHC) were compared among OVX, DM and DM-OVX groups using thyroid hormone or propyl thiouracil (PTU) in inducing varying range of α-MHC. While linear relations with the same slope were detected in DM and DM-OVX groups, a concave relation was observed in OVX groups. These results indicated that maximum myofilament ATPase activity was solely determined by α-MHC in DM rats but also other contractile modifications in E_2-deficient group. Moreover, the effect of E_2 on the contractile modifications may, in part, underlie the induction of cardiac myofilament Ca^{2+} sensitivity. The mechanistic role of E_2 in affecting myofilament Ca^{2+} sensitivity was further studied by evaluating effects of E_2 deficiency with DM complication on the intracellular Ca^{2+} handling by sarcoplasmic reticulum (SR). A similar magnitude of suppression in maximum SR Ca^{2+} uptake and SR Ca^{2+}-ATPase (SERCA2a) activities with increased sensitivity was clearly demonstrated in OVX, DM, and DM-OVX rats. Immunoblot analysis also showed the same degree of reduction in SERCA proteins in these rats. All changes were abolished by E_2 and/or INS supplementation. Thus, there was no or less contribution of the SR Ca^{2+} uptake function in affecting myofilament Ca^{2+} sensitivity.

The whole series of results indicated that E_2 affects the cardiac myofilament Ca^{2+} activation through changes in protein expressions of β_1-AR, HSP72, MHC and modifications of the contractile proteins. It is the contractile proteins modification that contributes to an induction in the myofilament Ca^{2+} hypersensitivity with less or no contribution of SR Ca^{2+} uptake function.

KEY WORDS: CARDIAC CONTRACTILE ACTIVATION/ ESTROGEN/ INSULIN/ β_1-ADRENOCEPTORS/ HEAT SHOCK PROTEIN 72/ MYOSIN HEAVY CHAIN ISOFORM/ SERCA2a

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กลไกการเปลี่ยนแปลงการทำงานของเส้นใยกล้ามเนื้อหัวใจในหนูตัดรังไข่ที่เป็นเบาหวาน
(MECHANISM OF CHANGES IN CARDIAC MYOFILAMENT ACTIVATION IN OVARIECTOMIZED RATS WITH DIABETIC COMPLICATION)

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บทคัดย่อ

การศึกษาของห้องปฏิบัติการเราได้พบแล้วว่าเอสโตรเจนมีผลควบคุมการทำงานของกล้ามเนื้อหัวใจ แต่กลไกยังไม่เป็นที่ทราบชัด จากผลกระทบร่วมของเอสโตรเจนและอินซูลินต่อหัวใจ จึงใช้ภาวะเบาหวานเพื่อรบกวนผลของเอสโตรเจนในการควบคุมการทำงานของเส้นใยกล้ามเนื้อ เมื่อเปรียบเทียบความสัมพันธ์ของค่าปฏิกิริยาการหดตัวที่ปริมาณแคลเซียมระดับต่างๆ ของหนูกลุ่มควบคุม กลุ่มตัดรังไข่ที่เป็นหรือไม่เป็นเบาหวาน และกลุ่มตัดรังไข่เป็นเบาหวานร่วมแล้วให้ฮอร์โมนทดแทน พบการลดลงที่เท่ากันของค่าปฏิกิริยาการหดตัวสูงสุดของเส้นใยกล้ามเนื้อในหนูตัดรังไข่ หนูเบาหวาน และหนูตัดรังไข่เป็นเบาหวานร่วม ส่วนความไวต่อการกระตุ้นของแคลเซียมพบสูงขึ้นจำเพาะในหนูตัดรังไข่แม้เมื่อเป็นเบาหวานร่วม และมีการเพิ่มจำนวนเบต้าอะดรีเนอร์จิกรีเซพเตอร์ที่หัวใจในลักษณะจำเพาะทำนองเดียวกัน แต่การเปลี่ยนแปลงค่าปฏิกิริยาการหดตัวสูงสุดพบสอดคล้องกับการเปลี่ยนแปลงปริมาณของฮีทช็อคโปรตีน72

เมื่อศึกษาผลของเอสโตรเจนต่อจลนศาสตร์การหดตัวของเส้นใยกล้ามเนื้อหัวใจโดยการเปรียบเทียบความสัมพันธ์ระหว่างค่าปฏิกิริยาการหดตัวสูงสุดกับปริมาณแอลฟาไมโอซินเฮฟวีเชนในหนูกลุ่มตัดรังไข่ หนูเบาหวาน และหนูตัดรังไข่เป็นเบาหวานร่วม ที่มีปริมาณแอลฟาไมโอซินเฮฟวีเชนแตกต่างกันจากการเพิ่ม/ลดปริมาณไทรอยด์ฮอร์โมน พบความสัมพันธ์แบบเส้นตรงในหนูกลุ่มที่เป็นเบาหวาน แต่ความสัมพันธ์แบบเส้นโค้งในหนูตัดรังไข่ ซึ่งแสดงถึงอิทธิพลของแอลฟาไมโอซินเฮฟวีเชนเพียงอย่างเดียวสำหรับหนูเบาหวานในการควบคุมปฏิกิริยาการหดตัวสูงสุดของเส้นใยกล้ามเนื้อ แต่มีผลกระทบเพิ่มจากการเปลี่ยนแปลงของเส้นใยกล้ามเนื้อสำหรับหนูตัดรังไข่ ผลกระทบเพิ่มของเอสโตรเจนที่เส้นใยกล้ามเนื้อนี้น่าจะเป็นกลไกหนึ่งที่รองรับการปรับเปลี่ยนความไวของเส้นใยกล้ามเนื้อต่อการกระตุ้นของแคลเซียม เมื่อศึกษาความเกี่ยวข้องของการเก็บกลับแคลเซียมโดยซาร์โคพลาสมิกเรติคูลัม พบว่าหนูตัดรังไข่ หนูเบาหวาน และหนูตัดรังไข่เป็นเบาหวานร่วม ล้วนมีค่าปฏิกิริยาสูงสุดในการดึงเก็บแคลเซียมเข้าซาร์โคพลาสมิกเรติคูลัม และจำนวนของโปรตีนซาร์โคพลาสมิกเรทิคูลัมเอทีพีเอสลดลงแต่ความไวเพิ่มขึ้นเท่ากันในทุกกลุ่ม ดังนั้นการเก็บกลับแคลเซียมโดยซาร์โคพลาสมิกเรติคูลัมมีส่วนเกี่ยวข้องน้อยหรือแทบไม่มีต่อการปรับเปลี่ยนความไวของเส้นใยกล้ามเนื้อต่อการกระตุ้นของแคลเซียม

ผลการศึกษาสรุปได้ว่าเอสโตเจนควบคุมการทำงานของกล้ามเนื้อหัวใจ ที่ปริมาณของเบต้าอะดรีเนอจิกรีเซพเตอร์ ฮีตช็อคโปรตีน72 ไมโอซินเฮฟวีเชน และโปรตีนเส้นใยกล้ามเนื้อ ซึ่งอิทธิพลต่อเส้นใยกล้ามเนื้อเป็นส่วนหนึ่งของกลไกการปรับเปลี่ยนความไวของเส้นใยกล้ามเนื้อต่อแคลเซียมโดยไม่มีหรือมีส่วนร่วมน้อยจากการเก็บกลับแคลเซียมโดยซาร์โคพลาสมิกเรติคูลัม

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LIST OF ABBREVIATIONS

µg	microgram
µM	micromole per liter
ADP	adenosine diphosphate
ANOVA	analysis of variance
AR	adrenergic receptor
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
B_{max}	maximum ligand binding
BSA	bovine serum albumin
BW	body weight
Ca, Ca^{2+}	calcium, calcium ion
^{45}Ca	calcium-45 radioisotope
$[Ca^{2+}]_i$	intracellular calcium concentration
$CaCl_2$	calcium chloride
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	adenosine 3', 5'-cyclic monophosphate
CB	crossbridge
Ci	Curi
CICR	calcium-induced calcium-released
Cl, Cl^-	chloride, chloride ion
CSQ	calsequestrin
cpm	count per minute
cTnC	cardiac isoform of troponin C
cTnI	cardiac isoform of troponin I
cTnT	cardiac isoform of troponin T
DMSO	dimethyl sulphoxide

LIST OF ABBREVIATIONS (Continued)

DM	diabetic mellitus
DM-OVX	diabetic-ovariectomized
DTT	DL-dithiothreitol
E_2	17β-estradiol
EC_{50}	effective concentration
ECC	excitation-contraction coupling
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethylene glycol-bis(b-aminoethyl ether)-N,N, N', N'-tetraacetic acid
ELC	essential light chain
ER	estrogen receptor
F-actin	fibrous actin
fmole	femtomole
fsTnI	fast skeletal isoform of troponin I
g	gram
G-actin	globular actin
G_s	stimulating G-protein
GPCR	G protein-coupled receptor
hr	hour
H^+	Hydrogen ion, proton
[^{3}H]-DHA	tritriated dihydroalprenolol
HEPES	N-[2-Hydroxylethy] piperazine-N'-[2-ethane-sulfonic acid]
HSP	heat shock protein
HW	heart weight
%HW/BW	the percentage of heart-to-body weight
INS	Insulin
ip	intraperitoneal injection

LIST OF ABBREVIATIONS (Continued)

K, K^+	potassium, potassium ion
KCL	potassium chloride
k_d	dissociation constant
kDa	kilodalton
kg	kilogram
LTCC	L-type Ca^{2+} channel
M	molar, mole per liter
Mg, Mg^{2+}	magnesium, magnesium ion
Mg^{2+}-ATPase, MgATPase	magnesium adenosine triphosphatase
MHC	myosin heavy chain
min	minute
ml	milliliter
MLC	myosin light chain
MLCK	myosin light chain kinase
MOPS	3-[N-morpholino]propanesulfonic acid
mM	millimole per liter
mRNA	messenger ribonucleic acid
MW	molecular weight
MyBP-C	myosin binding protein C
n	Hill coefficient
Na, Na^+	sodium, sodium ion
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NCX	sodium-calcium exchanger
nM	nanomole per liter

LIST OF ABBREVIATIONS (Continued)

OVX	ovariectomized
pCa	-log molar free calcium
pCa_{50}	half-maximally activating calcium concentration
ΔpCa_{50}	changes in half-maximally activating calcium concentration
Pi	inorganic phosphate
PKA	cAMP-dependent protein kinase
PKC	calcium-phospholipids-dependent protein kinase
PK/LDH	pyruvate kinase/lactate dehydrogenase enzyme
PLB	phospholamban
PMSF	phenylmethysulfonyl fluoride
POPOP	1,4 bis[4-methyl-5-phenyl-2-oxazolyl]benzene
PP	phosphatase
PPO	2,5-diphenyl oxazole
PTU	6-n-propyl-2-thiouracil
rpm	round per minute
RyR	ryanodine receptor
RLC	regulatory light chain
S_1	subfragment 1 of heavy meromyosin
S_2	subfragment 2 of heavy meromyosin
SDS-PAGE	sodium dodecyle sulfate polyacrylamide gel electrophoresis
SE	standard error of mean
Ser	serine

LIST OF ABBREVIATIONS (Continued)

SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SHAM	sham-operated rat
SR	sarcoplasmic reticulum
ssTnI	slow skeletal troponin I
STZ	streptozotocin
T_3	triiodothyronine
TCA	trichloroacetic acid
Thr	threonin
Tm	tropomyosin
Tn	troponin complex
TnC	troponin C
TnI	troponin I
TnT	troponin T
TR	thyroid hormone receptor
TRE	thyroid response element
Tris	(2-hydroxymethyl) imido-tris(hydroxymethyl)methane
UW	uterine weight
V_1	the highest Ca^{2+}-ATPase activity isoform of myosin heavy chain
V_2	the intermediate Ca^{2+}-ATPase activity isoform of myosin heavy chain
V_3	the slowest Ca^{2+}-ATPase activity isoform of myosin heavy chain
V_{max}	the maximal velocity
wk	week

CHAPTER I
THEME OF THESIS

The well-recognized gender difference in the incidence of cardiovascular diseases has raised the cardioprotective role of female sex hormones in the cardiac contractile function. Data from epidemiological study has demonstrated a lower incidence of cardiovascular diseases in premenopausal women than that in age-matched men. However, the incidence rate was markedly increased after menopause to reach the same rate as that in men (Kuhn and Rackley, 1993). Many studies from both clinical and animal models have reported the significant role of female sex hormones in the cardiac contractile activation. Decreases in ejection fraction and rate were clearly detected in post-menopausal women using echocardiographic study (Schillaci et al., 1998). In ovariectomized (OVX) rat hearts reductions in cardiac output, ejection fraction, and prolonged relaxation period have also been reported (Scheuer et al., 1987). In addition, a shift in myosin isoenzymes from a predominant V_1, the highest ATPase activity isoenzyme, to a predominant V_3, the lowest ATPase activity isoenzyme, was also found in OVX rat hearts (Malhotra et al., 1990; Schaible et al., 1984). Our laboratory has further demonstrated a suppressed maximum myofibrillar ATPase activity, myofibrillar Ca^{2+} hypersensitivity, and a significant shift in myosin heavy chain (MHC) towards β-MHC isoform in OVX rat hearts (Wattanapermpool, 1998; Wattanapermpool and Reiser 1999). Using isolated skinned fiber preparations, we found that the Ca^{2+} hypersensitivity of the myofilament is a specific effect induced in cardiac tissue after ovariectomy (Wattanapermpool and Reiser, 1999). Furthermore, upregulation of β_1-adrenergic receptors, which may underlie changes in the myofilament Ca^{2+} activation, was clearly demonstrated in OVX rat hearts (Thawornkaiwong et al., 2003). All changes could be reversed by either estrogen (E_2) supplementation or exercise training (Wattanapermpool et al.,

2000; Thawornkaiwong et al., 2003; Bupha-Intr and Wattanapermpool, 2004). The similarity of cardiac Ca^{2+} hypersensitivity detected in ovariectomized rats to that in cardiomyopathic condition (Heyder et al., 1995; Wolff et al., 1995; 1996) strongly supports the beneficial role of estrogen in the myocardial activation.

Surprisingly, the cardioprotective effect of estrogen on myocardial function seems to be overcome by diabetes (DM) (Gustafsson et al., 2004; Kannel et al., 1974; Sowers, 1998). The morbidity and mortality of cardiovascular diseases in diabetic patients appear to be increased in female compared to age-matched male (Gustafsson, 2004). The reversed gender differences in heart disease incidence suggest a reasonable hypothesis that deprivation of E_2 and insulin (INS) induces interactive effects on cardiac myofilament response to Ca^{2+}. Our laboratory has investigated the interactive actions of E_2 and INS on the cardiac myofilament activation using streptozotocin-induced diabetes in OVX rats as the model of study (Pantharanontaga, 2000). We found a significant reduction in maximum ATPase activity of cardiac myofilaments in diabetic ovariectomized (DM-OVX) rats with the same magnitude of suppression as that detected in OVX or DM rats. In contrast, an increased cardiac myofilament Ca^{2+} sensitivity detected in OVX rats was observed only in DM-OVX but not DM rats. These results partially imply a similar final common pathway of the hormone action on the contractile activation. The adaptive response of the cardiac myofilament to become more sensitive to Ca^{2+} even under diabetic complication implies a dominant effect of E_2 deficiency in inducing cardiac myofilament Ca^{2+} hypersensitivity. The potential interactive action of E_2 and INS on cardiac contractile activation and the possible underlying mechanism of E_2 in affecting the cardiac myofilament response to Ca^{2+} were therefore focused in this study.

E_2 and insulin may interactively affect the response of cardiac myofilament to Ca^{2+} through changes in the regulatory effect of β_1-adrenergic stimulation and/or the protective effect of heat shock proteins (HSP). Physiologically, stimulation of β_1-adrenergic receptors plays a significant role in enhancing cardiac contractility through modifications of Ca^{2+} mobilization in the excitation contraction process (Bers, 2002). However, over- or chronic adrenergic stimulation to the heart was found to induce harmful effects on the contractile function (Bristow et al., 1992; Chakraborti et al., 2000; Kaye et al., 1995; Lefkowitz et al., 2000; Post et al., 1999). Our laboratory has

reported an upregulation of β_1-adrenergic receptors in cardiac membrane of OVX rats which could be restored by either E_2 supplementation (Thawornkaiwong et al., 2003) or exercise training (Bupha-Intr and Wattanapermpool, 2004). Despite controversial data in the expression of β_1-adrenoceptors, an elevated norepinephrine spillover chronic stimulation of the receptors has been demonstrated in DM hearts (Ganguly et al., 1987). There is also evidence of sex hormone-related loss of cardiac protection through a reduction in HSP72 expression in OVX rats, which could be restored by E_2 supplementation or exercise training (Bupha-Intr and Wattanapermpool, 2004; Voss et al., 2003). Similarly, downregulation of HSP72 has been documented in diabetic hearts in which the impaired HSP could also be offset by endurance exercise (Atalay et al., 2004). It is therefore interesting to investigate whether deficiency of E_2 and INS could interactively induce a synergistic effect associated with increased β_1-adrenergic stimulation and/or loss of protective effect via reduced HSP72 expression on cardiac contractile response to Ca^{2+}.

The interactive effect of E_2 and insulin on the maximum myofibrillar ATPase activity reported from our previous studies (Pantharanontaga, 2000) indicates a final common pathway of the E_2 and insulin in regulating the cardiac contractile activity. A shift in the isoform population of myosin heavy chain (MHC) is a well recognizable mechanism underlying the suppressed maximum myofilament ATPase activity in OVX and DM rat hearts (Wattanapermpool, 1998; Pierce et al., 1985). Furthermore, direct effects of E_2 and insulin on MHC expression have clearly been demonstrated (Bupha-Intr and Wattanapermpool, 2004; Danzi et al., 2003). However, while the same degree of suppression in maximum myofibrillar ATPase activity was demonstrated, a lesser extent of shift in MHC toward β-MHC isoform was detected in OVX rat hearts (unpublished data). The information thus suggests an additional mechanism besides the shift in MHC isoforms being responsible for the regulatory effects of E_2 on cardiac contractile activity.

Alterations in cardiac contractile activity could be induced by the deterioration in kinetic properties of crossbridge (CB) cycling, indicated by the isoform expression of MHC and the modification of cardiac contractile proteins (Rundell et al., 2005; Andruchov et al., 2006). A direct relation of CB cycling kinetics to the expression of β-MHC has been demonstrated in diabetic rat myocardium (Rundell et al., 2004).

Despite the known effect of E_2 deficiency on the MHC expression, the impact of altered MHC on the CB cycling kinetics under the condition has not been studied. The evidence of cardiac myofilament Ca^{2+} hypersensitivity in OVX rats (Wattanapermpool, 1998) suggests a possible modification of cardiac myofilament proteins, could be by phosphorylation, which then accounts, in part, for the suppression of the cardiac contractile activity. The implication from the evidence of a higher α-MHC content but the same level of maximum myofibrillar ATPase activity to those of DM rat hearts does support possible differential phosphorylation states of cardiac myofilament proteins in OVX rats. It is then interesting to evaluate whether E_2 deficiency induces changes in cardiac contractile responses through shifts in MHC isoform and/or phosphorylation of cardiac contractile proteins.

The dominant effect of E_2 deprivation in inducing an enhanced Ca^{2+} sensitivity of cardiac myofilament even under diabetic complication (Pantharanontaga, 2000) also supports the significant role of E_2 in cardiac myocyte activation. It is, however, still not clear how cardiac myofilament Ca^{2+} hypersensitivity is induced in the E_2 deficient rats. Generally, sensitivity of myofilaments to Ca^{2+} can be affected by two possible mechanisms, including changes in the myofilament machinery in response to Ca^{2+} and changes in the intracellular mobilization of Ca^{2+} around the myofilaments. Our laboratory had first proved the hypothesis that it is the decrease in the intracellular Ca^{2+} mobilization induced after E_2 deficiency that induces adaptation of the myofilaments to be hypersensitive to Ca^{2+} activation. A significant reduction in the peak Ca^{2+} transient with a prolonged decay was clearly demonstrated in cardiomyocytes isolated from OVX hearts (Ren et al., 2003; Bupha-Intr et al., 2007). These results support our hypothesis that the decrease in intracellular Ca^{2+} mobilization after E_2 deficiency induces a hypersensitive adaptation of the cardiac myofilament to the Ca^{2+} activation. We then further proved that the decrease in intracellular Ca^{2+} mobilization causes an increase in cardiac Ca^{2+} hypersensitivity after E_2 deprivation.

Alterations in dynamic transports of Ca^{2+} between the sarcoplasmic reticulum (SR) storage and the cytosol could have an impact on the Ca^{2+} transient profile during the cardiac cycle of contraction and relaxation. Suppression in the $[Ca^{2+}]_i$ transient magnitude with a prolongation of $[Ca^{2+}]_i$ decay in cardiomyocytes of OVX rats points

to a possible adaptive response in the SR Ca^{2+} handling activity. Our laboratory has recently reported a significant decrease in the maximum SR Ca^{2+}-uptake rate with an increase in the SR Ca^{2+}-uptake sensitivity in both the left ventricle homogenate and the SR vesicle preparations from 10-wk OVX rat hearts (Bupha-Intr and Wattanapermpool, 2006). This suppression of SR Ca^{2+}-uptake activity appears to be related to significant decreases in the activity and expression of the SR Ca^{2+}-ATPase pump (SERCA) protein and mRNA levels (Bupha-Intr and Wattanapermpool, 2006), which could be restored by E_2 supplementation (Bupha-Intr and Wattanapermpool, 2006) and by exercise training (Laosiripisan, 2006). Despite controversial data in the myofilament Ca^{2+} sensitivity, reductions in intracellular Ca^{2+} transient and SR Ca^{2+} activity have also been demonstrated in DM rats either with or without alteration in the expression of SR Ca^{2+}-associated proteins (Choi et al., 2002; Ligeti et al., 2006). It is therefore interesting to evaluate the significant role of E_2 in the cardiac SR Ca^{2+} uptake function using the approach of DM complication in OVX rats.

The goal of this thesis project was set to understand the cellular, subcellular, and molecular adaptation of cardiac myocytes after E_2 deficiency with diabetic complication to draw the possible interactive effect of E_2 and insulin on cardiac myofilament response to Ca^{2+} and the underlying mechanism. Four specific objectives were raised in this study.

1. To evaluate the influence of diabetes on changes in response to Ca^{2+} of cardiac myofilament from OVX rats
2. To elucidate whether deficiency of E_2 and INS interactively affects the cardiac contractile response to Ca^{2+} through alterations in either β_1-adrenergic stimulation and/or HSP72
3. To investigate the regulatory effect of E_2 on the crossbridge cycling kinetics of the contractile filament
4. To test the significant role of E_2 in the regulation of intracellular Ca^{2+} handling in cardiac myocytes by the sarcoplasmic reticulum under diabetic complication

Relationships of –log free Ca^{2+} concentration (pCa)-myofilament ATPase activity were plotted to evaluate the influence of DM on changes in the cardiac myofilament response to Ca^{2+} in OVX rats using isolated myofibrillar preparations

from sham, OVX, DM, and DM-OVX with and without supplementations with E_2, INS, or E_2 plus INS. To test the interactive effect of E_2 and INS deprivation on β_1-adrenergic receptor and HSP72 expression, the radioligand receptor binding assay of β_1-adrenoceptors and immunoblot analysis using specific antibodies of β_1-adrenergic receptor and HSP72 were performed in various experimental groups, including sham, OVX, DM, DM-OVX, with and without E_2, INS, or E_2 plus INS. The regulatory effect of E_2 on the crossbridge cycling kinetics of the cardiac myofilament was investigated by plotting the relationships of % α-MHC isoform and maximum myofibrillar ATPase in various experimental groups, including OVX with and without E_2 supplementation, DM with and without INS supplementation, DM-OVX with and without E_2, INS, or E_2 plus INS together with sham group. To further probe for changes in the crossbridge kinetics, the level of cardiac α-MHC expression in the heart of OVX, DM and DM-OVX rats was varied by manipulating the thyroid status. Lastly, the significant role of E_2 in the SR function under DM complication was tested using left ventricular homogenate and SR vesicles for measurements of the SR Ca^{2+} uptake and the SR Ca^{2+}-ATPase activity, respectively, in various groups including sham, OVX, DM with and without INS supplementation, DM-OVX with and without supplementations with E_2, INS, or E_2 plus INS. Immunoblot of The SR Ca^{2+}-ATPase and its associated protein were also analyzed.

CHAPTER II
LITERATURE REVIEW

A. Impact of female sex hormones on cardiac contraction

Estrogen (E_2) and progesterone are two important female sex hormones in reproductive physiology. Their receptors belong to the steroid receptor family, which activates both genomic and non-genomic effects (Babiker et al., 2002; Li and O'Malley, 2003). The genomic effect of estrogen on the cardiovascular system mediates via two isoforms of E_2 receptor (ER), ER_α and ER_β. Interaction of the ligand-receptor complex to the specific hormone responsive element on the promoter region of the target gene underlies the classical genomic pathway. The genomic effect of these receptors activation gives rise to the suppression or activation of the target gene transcription. For example, a decreased expression of atrial natriuretic factor in the myocardium is observed by the genomic effect of E_2 (Horio et al., 2000). In contrast to the genomic action, the non-genomic action of E_2 in the myocardium is activated in both a ligand-dependent and a ligand-independent manner. The ligand-dependent activation by E_2 has been suggested to act via a membrane-bound receptor through G protein α_i (Wyckoff et al., 2001). On the other hand, the ligand-independent activation involves the alteration of phophorylation levels of the receptors (Kazmi et al., 1993; El-Tanani and Green, 1997). In the cardiac myocyte, direct effects of female sex hormones on cardiac contractile functions are supported by the presence of both E_2 and progesterone receptors (Ingegno et al., 1988; Grohe et al., 1997; 1998; Taylor and Al-Azzawi, 2000). However, the lack of E_2 and progesterone responsive elements on the genes of cardiac myofilament and Ca^{2+} handling proteins (Bourdeau et al., 2004; Richer et al., 2002) indicate that female sex hormones may regulate the cardiac contractile function through the other mechanisms.

The well-recognized gender difference in the cardiovascular disease has brought to a number of studies on the influence of female sex hormones on the cardiac

contractile activation. A reduction in the midwall fractional shortening with a greater relative wall thickness in early menopause has been demonstrated using echocardiographic study (Schillaci et al., 1998). Studies in both pre-pubertal and post-pubertal ovariectomized (OVX) rats have also found reductions in stroke volume and ejection fraction (Schaible et al., 1984; Scheuer et al., 1987). Moreover, significant decreases in the myosin Ca^{2+}-ATPase activity and the percentage content of V_1 myosin isoenzyme were also detected. These changes could be prevented by E_2 supplementation. Our laboratory has further reported a series of studies on the effects of chronic ovarian sex hormone deficiency on the cardiac myofilament Ca^{2+} activation. A suppression of maximum Ca^{2+}-dependent actomyosin MgATPase activity and a significant shift in myosin heavy chain (MHC) isoforms toward β-MHC isoform have been detected in the hearts of OVX rats (Wattanapermpool, 1998; Wattanapermpool and Reiser, 1999). Strikingly, an increase in the Ca^{2+} responsiveness of myofilament activation in OVX rat hearts was also shown in both biochemical and mechanical studies (Wattanapermpool, 1998; Wattanapermpool and Reiser, 1999). Supplementation of E_2 could prevent these changes (Wattanapermpool, 2000). The similarity of Ca^{2+} hypersensitivity detected in OVX rats to that in cardiomyopathic hearts (Heyder et al., 1995; Wolff et al., 1995; 1996) previously reported supports the beneficial role of E_2 in the myocardial activation. However, it is not known how myofilament Ca^{2+} hypersensitivity was induced in the E_2-deficient hearts. A significant reduction in the magnitude and a prolonged decay of Ca^{2+} transient has been recently reported in cardiomyocyte isolated from OVX rat hearts, and was completely restored by E_2 supplementation, which may, in part, underlie the induction of myofilament respose to Ca^{2+} after E_2 deprivation (Ren et al., 2003; Bupha-Intr et al., 2007). A significant reduction in maximum sarcoplasmic reticulum (SR) Ca^{2+}-uptake rate with an increase in the SR Ca^{2+}-uptake sensitivity paralleled to significant decreases in the activity and expression of the SR Ca^{2+}-ATPase pump (SERCA) protein and mRNA levels have also been demonstrated in OVX rat hearts. These changes could be restored by E_2 supplementation. Moreover, an upregulation of β_1-adrenergic receptors, which implies a harmful effect on the contractile function, was also detected in OVX rat hearts (Thawornkaiwong et al., 2003). Taken together, these data indicated a maladaptive response of cardiac

myofilament response to Ca^{2+} after E_2 deprivation. Further studies concerning the mechanism underlying changes induced after ovarian sex hormone deficiency certainly provide a better view of potential targets for therapeutic and preventive approaches for cardiovascular disease especially in postmenopausal women.

B. Impact of diabetes on cardiac contraction

Diabetes is a well-known risk factor for development of cardiovascular abnormalities and impaired myocardial performance (Rubler et al., 1972; Dhalla et al., 1985). The myocardial defect in diabetes is associated with a specific cardiomyopathy, which can progress toward heart failure. Diabetic cardiomyopathy is a condition of ventricular dysfunction developed in diabetic status in the absence of coronary atherosclerosis and hypertension (Ahmed et al., 1975; Fein et al., 1985). Cardiac dysfunction in diabetic cardiomyopathy is characterized by depressions in the contractile force generation, cardiac relaxation, and pumping activity. Impaired diastolic filling is one of the earliest signs of the disease (Ruddy et al., 1988). Changes in the cardiac contractile function in diabetes include a decrease in ATPase activity of contractile proteins, changes in sarcolemmal channels, SERCA, and Ca^{2+} sensitivity of myofilaments (Dillmann, 1990; Liu et al., 1996; 1997; Malhotra and Sanghi, 1997; Depre et al., 2000). A shift in the cardiac myosin isozyme from V_1, αα-homodimer, to V_3, ββ-homodimer, has been reported in the diabetes (Golfman et al., 1999; Depre et al., 2000). The α-MHC protein content as well as mRNA level were decreased, whereas β-MHC content and mRNA expression were increased in the left ventricle of diabetic animals. Insulin treatment could reverse the depressed myofibrillar ATPase activity and the shift in MHC isoforms in diabetic animal models (Dillmann, 1980; Pierce and Dhalla, 1981; 1985; Dhalla et al., 1985; MacLean et al., 1987). Hypothyroidism (i.e., variable degrees of decreased plasma T_3 levels) with neither changes in the number nor the affinity of thyroid receptors (TRs) has also been demonstrated in streptozocin-induced diabetic rats (Haddad et al., 1997*a*). The pattern of changes in cardiac myosin mRNA levels in the diabetic rat is similar to that reported in hypothyroidism (Dillmann, 1989).

Alterations in the composition and/or phosphorylation level of the contractile and regulatory proteins have been reported to be the cause of cardiac dysfunction in

diabetic rats. A significant increase in cardiac troponin I (cTnI) phosphorylation (by 40%) in the diabetic hearts which could be reversed by insulin administration has been reported (Liu et al., 1996). PKC-mediated cTnI phosphorylation is postulated to possibly happen because it mainly blunts the maximal myofibrillar ATPase activity (Noland et al., 1996). This postulation is supported by the increased PKC protein content and activity in diabetic rat heart (Liu et al., 1999). Although some investigators have found no significant changes in cTnI content and gene expression in the right and left ventricles of diabetic rats (Liu et al., 1996), others have demonstrated a downregulation of the total TnI (Dhalla et al., 1998). In addition, significant reductions of protein contents of myosin light chain (MLC), myosin light chain kinase (MLCK) (40% to 45%), and MLC phsophorylation (30% to 45%) in diabetic hearts which alter the myofilament ATPase activity were also detected and were reversible upon treatment with insulin. Changes in the cTnT isoform pattern represented by a shift from $cTnT_1$ to $cTnT_3$ are also reported in diabetic heart (Akella et al., 1995). Not only diabetes-related alterations are restricted to myofibrils (Takeda et al., 1996; Pierce and Russell, 1997), changes in the cellular Ca^{2+} homeostasis are also demonstrated in association to SERCA2a content and activity (Ganguly et al., 1983; Takeda et al., 1996; Golfman et al., 1999; Trost et al., 2002; Zhong et al., 2003), and to phospholamban content and phosphorylation (Trost et al., 2002; Zhong et al., 2003). Although myofibrillar changes are considered as an early sign of cardiac remodeling in diabetic cardiomyopathy (Schaffer, 1991), alterations in SR function was found in the late phase of diabetes.

C. Impact of thyroid hormone on cardiac contraction

Thyroid hormone is an important regulator of cardiac contractile function (Dillmann, 1990; Klein and Ojamaa, 2001). Many of the cardiac effects of triiodothyronine (T_3), the biologically active form of the hormone, involve the expression of several important cardiac genes. After transport to the myocyte nucleus, T_3 binds to thyroid hormone nuclear receptors (TRs), which in turn bind to T_3 response elements located within the 5′ flanking regions of T_3-responsive genes (Brent et al., 1994). TRs act in a bimodal fashion to activate the transcription in the presence of T_3 by recruiting coactivator complexes and to repress the transcription in

the absence of ligand by recruiting corepressor complexes. Two distinct genes encode the family of TRs, which are two splice variants, TRα-1 and TRα-2, and one primarily splice variant of the TRβ gene, TRβ1 (Dillmann et al., 1990; Lazar, 1993; Ribeiro et al., 1998; Mansen et al., 2001). There are multiple T_3-regulated cardiac-specific genes including α-MHC, β-MHC, phospholamban, and SERCA2 (Klein and Ojamaa, 2001; Moolman, 2002).

Alterations in thyroid hormone level affect T_3-responsive cardiac genes resulting in pathogenesis of cardiac disease. Hypothyroidism causes a decreased expression of positively regulated T_3-responsive cardiac genes such as α-MHC and SERCA2, whereas the expression of negatively regulated T_3-responsive genes such as β-MHC and phospholamban is increased (Dillmann et al. 1990; Morkin, 2000; Danzi and Klein, 2002). A shift in the cardiac phenotype of rodents with hypothyroidism results in decreases in both systolic and diastolic contractile function (Dillmann, 1990). Treatment of thyroid hormone in hypothyroid animals could restore the expression of these genes, increase cardiac mass, and improves contractile function (Danzi and Klein, 2002; Danzi et al., 2003; Haddad et al., 2003). In contrast, hyperthyroidism promotes α-MHC and SERCA2 expression in the myocardium resulting in faster rates of isotonic shortening and isometric contractions (Jakab et al., 1994; Wolska et al., 1997; Yagi et al., 2001). Moreover, excessive administration of thyroid hormone or hyperthyroidism induces pronounced cardiac hypertrophy, which is attributed to hypertrophic cardiomyopathy and heart failure.

D. Myofilament Proteins

Cardiac muscle contraction is generated by the interaction of myofilament protein under the regulation of Ca^{2+}using energy powered by ATP hydrolysis. The contractile machinery unit is composed of many proteins arranged into thick and thin sliding filaments. Thick filaments are made up of myosin heavy chain, myosin light chain, and myosin binding protein C. Thin filaments contain actin, tropomyosin, and troponin complex, which function as regulators of the crossbridge interactions.

1. <u>Troponin Complex</u>

The troponin complex (Tn) includes three protein subunits, troponin T (TnT), troponin C (TnC) and troponin I (TnI) with 1:1:1 stoichiometry, forming a complex molecule on the thin filament (Filatov et al., 1999). These three subunits include an 18.4 kDa calcium binding subunit TnC, a 24 kDa inhibitory subunit TnI, that inhibits contraction in an absence of Ca^{2+}, and a 34 kDa tropomyosin binding subunit TnT, that holds other troponin subunits to tropomyosin (Tm). Each troponin complex is associated with one Tm molecule and seven actins. At the blocked stage in the crossbridge interaction, highly binding affinity of TnI to actin prevents myosin heads in interacting with actin on their binding sites. When Ca^{2+} bind to TnC, the affinity of TnI and TnT to TnC will be strengthened which then opens up the crossbridge binding site of actin for myosin head. Movement of troponin complex simultaneously rearranges the Tm to move out of the groove of actin and therefore enhances the strong actin-crossbridge interaction. The importance of troponin complex in regulating cardiac contraction has been focused on the modification of myofilament Ca^{2+} sensitivity by two possibilities, including protein mutation or isoform switching and protein phosphorylation (Solaro and Van Eyk, 1996).

Troponin C: TnC belongs to the Ca^{2+}-binding protein family and consists of two globular domains, which look like a dumbbell structure. Each globular domain contains a pair of Ca^{2+}-binding site in which binding affinities are different depending on location and isoforms. TnC has two isoforms derived from different gene coding (Kawasaki and Kretsinger, 1994). One isoform is found in fast skeletal muscle and the other one is expressed in both slow skeletal and cardiac muscles. Two Ca^{2+} molecules can bind at each domain of fast skeletal muscle TnC, whereas one Ca^{2+} molecule can bind at N-terminal domain of cardiac TnC. Moreover, the N-terminal globular domain of both fast skeletal and cardiac TnC has a lower affinity to Ca^{2+} than that of the C-terminal domain. However, the N-terminal domain of TnC is an important site in regulating myofilament contraction, while the C-terminal domain and the central α-helix of TnC play an important role in transducing a conformational signal among troponin subunits (Tobacman, 1996).

Troponin I: TnI is responsible for inhibiting the actin-activated myosin ATPase activity or actomyosin interaction (Filatov et al., 1999). Three isoforms of TnI are derived from three different genes including fast skeletal TnI (fsTnI), slow skeletal

TnI (ssTnI) and cardiac TnI (cTnI). ssTnI predominantly expressed in fetal heart of human and is gradually replaced by cardiac TnI soon after birth (Sasse et al. 1993). cTnI has a specific 31-amino acid sequence at the N-terminal of the molecule which contains two cyclic AMP-dependent protein kinase (PKA) phosphorylation sites, serine 23 and 24, (Chandra et al., 1997). The carboxyl-terminus of TnI contains binding sites for TnC and TnT. The amino-terminal of TnI contains inhibitory (at amino acid 115-131) and regulatory (at amino acid 147-163) regions, which will bind to Ca^{2+}-saturated TnC but to actin and Tm in the absence of Ca^{2+}. In the heart, cardiac TnI can be phosphorylated by three protein kinases, including PKA, PKC, and cGMP-dependent protein kinase (PKG) (Noland et al., 1995; Chandra et al., 1997). Phosphorylation of cTnI induces conformational changes in the troponin complex, leading to altered Ca^{2+} affinity of TnC and relaxation (Zhang et al., 1995). PKA and PKG act on the same phosphorylation sites on the cTnI resulting in a decreased contractile sensitivity to Ca^{2+} and an enhanced crossbridge cycling rate. PKC could phosphorylate many residues including Thr^{144}, $Ser^{43, 45, 23, 24}$ of cTnI predominantly depending on isoforms of PKC. Phosphorylation of cTnI by PKC-specific sites results in a reduction in the Ca^{2+}-dependent actomyosin Mg-ATPase activity (Noland and Kuo, 1993b; Solaro and Rarick 1998) leading to a depressed crossbridge cycling rate. A decreased phosphorylation of cTnI was demonstrated in heart failure in which the hypersensitivity of the contractile response to Ca^{2+} was observed (Wolff et al., 1996; van der Velden et al., 2003).

Troponin T: TnT regulates Ca^{2+} sensitivity for tension development via allosteric mechanism (Perry, 1999). Ca^{2+}-dependent binding sites of TnT form a triple-coiled binding to Tm and TnC. Binding of Ca^{2+} to TnC increases the probability of TnT-Tm cross-linking. In addition, one part of TnT interacts with actin and plays a crucial role in fixing the whole troponin complex on the thin filament during the weaken contact of TnI to actin in the presence of Ca^{2+}. There are multiple isoforms of TnT either from different genes or from alternative splicing (Solaro and Van Eyk, 1996). At least four isoforms have been reported in human cardiac muscle including cTnT1, cTNT2, cTNT3, and cTnT4. The expression of TnT isoforms in the heart is developmentally regulated with the predominance of cTnT1, cTnT2 and cTnT4 in the fetal life, and cTnT3 in adults (Anderson et al., 1995; Saba et al., 1996).

TnT isoforms differ in the N-terminal hypervariable region, have different effects on maximal force development and on Ca^{2+} sensitivity of force and ATPase activity (Mesnard et al. 1995; Schiaffino and Reggiani, 1996). Therefore, shifts in cTnT isoforms, which may happen in the postnatal period and heart failure condition, can induce functional consequences. Morover, phosphorylation of TnT by PKC at Thr ^{190,199,280} and Ser^{194} can modify the function of TnT and provide different contraction results depending on sites of TnT phosphorylation (Solaro and Van Eyk, 1996). As a result, phosphorylation of TnT gives rise to an additional mechanism for the regulation of crossbridge kinetics and may be important for Ca^{2+}-sensitive interactions of TnT with TnC and Tm (Farah and Reinach, 1995).

2. Tropomyosin

Tropomyosin is a coiled-coil dimmer that coils around the actin spans over seven actin monomer to block its interaction with myosin. There is one tropomyosin for every Tn complex (Solaro and Rarick, 1998: Wolska and Wieczorek, 2003). Tm regulates interactions of actin to myosin in a Ca^{2+}-dependent manner under the control of Tn complex. Two isoforms of Tm, α and β isoform, have been reported in cardiac muscle, and form to be αα-, ββ- homodimers and αβ-heterodimer. The ratios among them in the heart are dependent on species and growth stages, and are critical in determining myocardial relaxation time as well as maximum rate of relaxation (Muthuchamy et al. 1995; Gaffin et al. 2004). Moreover, Tm is a regulatory protein that is necessary and sufficient for cooperativity of acto-myosin interactions (Moraczewska, 2002).

3. Actin

Actin is a ubiquitous cytoskeletal protein and the most conserved contractile proteins. It is a globular molecule (G-actin) with a molecular weight of ~42 kDa polymerized to form fiber (F-actin). Two sarcomeric actins, α-cardiac and α-skeletal, are co-expressed in the normal adult heart. Although these two isoforms have four different amino acids among the 375 residues, there is no significantly different effect on crossbridge interactions. There are two important biological properties of actin including 1) activation of myosin ATPase and reversible binding to myosin, and 2)

reconstitution of actomyosin which liberates chemical energy from ATP for producing physiochemical change in the myosin crossbridges

4. Myosin heavy chain

Myosin heavy chain is the molecular structure of myosin that interacts with actin in forming crossbridges and is responsible for the conversion of chemical energy to mechanical energy (Spudich, 1994). Two isoforms of MHC, α- and β-MHC, are expressed in cardiac muscle which is transcripted from two distinct genes. α-MHC isoform is larger (MW~220 kDa depends on the species) and has a higher ATPase activity than that of β-MHC isoform (Moss and Buck, 2002). The α- and β-MHC isoforms form three isoenzymes termed V_1-V_3. The V_1 and V_3 forms are comprised of αα-homodimer and ββ-homodimer, respectively, whereas the V_2 form is made up of heterodimer of α- and β-MHC. The expression of cardiac MHC composition varies among species, with a predominantly α-MHC isoform in small rodent and a predominantly β-MHC isoform in large mammals including human. Their relative expression levels in cardiac tissue are also changed by developmental stage, heart diseases, and hormones such as thyroid hormone. An increase in the expression of cardiac β-MHC is well accepted to be a pathological indicator of the heart. This characteristic has been found in human heart failure in a similar fashion to those in animal models (Miyata et al., 2000; Reiser et al., 2001).

5. Myosin light chain

Myosin light chains are small protein (18-27 kDa) associates with the neck region of the myosin-S1 segment, and are classified into essential light chain (ELC) and regulatory light chain (RLC) (Moss and Buck, 2002). Both types of light chains exert effect on crossbridge interactions, but only RLC can be phosphorylated by Ca^{2+}/ calmodulin-dependent myosin light chain kinase and protein kinase C (Noland and Kuo, 1993a) and dephosphorylated by light chain phosphatase (Morano, 1992). Phosphorylation of RLC has been found to increase the Ca^{2+}-responsiveness of force (van der Velden et al., 2003) and the rate of force development (Morano, 1992). In human heart, two ELC isoforms and three RLC isoforms were detected. Changes in

the isoform components induce changes in actin-crossbridge interactions. A decreased RLC phosphorylation has been reported in end-stage failing human hearts (van der Velden et al., 2001) which may be a potential compensatory mechanism to reverse the detrimental effects of increased Ca^{2+} -sensitivity in human heart failure.

6. Myosin binding protein C

Besides myosin light chain, myosin-binding protein C (MyBP-C) is another thick filament protein that has three isofroms, including slow skeletal, fast skeletal and cardiac isoforms. This protein has a binding site for myosin, titin and actin (Squire et al., 2003). The exact physiological function of MyBP-C is not fully understood. However, there is a report demonstrating that the structure, charge and position of MyBP-C determine the cardiac function as a regulator of the actin-crossbridge interaction (Weisberg and Winegrad, 1998). The cardiac isoform contains four accessible phosphorylation sites (Gautel et al. 1995), which were specific sites for PKA and Ca^{2+}/calmodulin–regulated kinease (CaMK). Phosphorylation of MyBP-C by PKA promotes myosin head to move away from the thick filament backbone and reduces flexibility of crossbridge heads (Weisberg and Winegrad, 1998). Moreover, structural changes of thick filament were found to be accompanied with MyBP-C phosphorylation, which may modify the kinetics of crossbridge cycling (Weisberg and Winegrad, 1998), the activity and/or Ca^{2+} sensitivity of myosin ATPase (Winegrad, 1999; McClellan et al., 2001), and force development in skinned fiber (Witt et al., 2001).

E. Role of Ca^{2+} in myofilament activation

Cardiac excitation-contraction coupling (ECC) is the process that converts the electrical depolarization of the myocytes to the contraction of the cardiac myofilaments. This process requires the ubiquitous second messenger Ca^{2+} as a direct activator of myofilament contraction (Bers, 1991). Many cardiac sarcolemmal and cellular components of the Ca^{2+} handling protein involve in controlling the movement of Ca^{2+} around the myofilaments leading to the beat-to-beat fluctuation of the cytosolic Ca^{2+} concentration. During the depolarization of cardiac myocytes, small amounts of extracellular Ca^{2+} enter the cell through voltage-dependent L-type Ca^{2+}

channel (LTCC). These Ca^{2+} entries trigger Ca^{2+} release from the intracellular Ca^{2+} storage called sarcoplasmic reticulum via the ryanodine Ca^{2+} release channels (RyR). This process is known as Ca^{2+}-induced-Ca^{2+} release (CICR). As a result, there is an increase in the intracellular Ca^{2+} concentration [Ca^{2+}] from 0.1-0.15 μM in the resting cell to about 1 μM. The increase in the intracellular Ca^{2+} activates myofilament contraction by binding of Ca^{2+} to troponin C, which then switches on the contractile machinery. After contraction, initiation of muscle relaxation occurs when intracellular Ca^{2+} concentration decline, allowing Ca^{2+} to dissociate from troponin C. This process requires Ca^{2+} transport out of the cytosol by three pathways involving SERCA, sarcolemmal Na^{+}/Ca^{2+} exchange (NCX), and sarcolemmal Ca^{2+}-pump. The depletion of cytosolic Ca^{2+} during relaxation is mostly accomplished by the function of SERCA which is responsible for about 70-90 % of reduction in $[Ca^{2+}]_i$ during relaxation period depending on species. After reuptake into the SR, Ca^{2+} then binds to a Ca^{2+}-binding protein called calsequestrin (CSQ) and is stored near to the Ca^{2+} release channels. In addition, Na^{+}/Ca^{2+} exchange and sarcolemmal Ca^{2+} pump also involve in removing Ca^{2+} for about 10-30 % and 2-3 % of Ca^{2+}, respectively, out to the extracellular side as shown in Figure 1. The alteration of Ca^{2+} handling process in myocyte is a central cause of both contractile dysfunction and arrhythmias in pathophysiologic

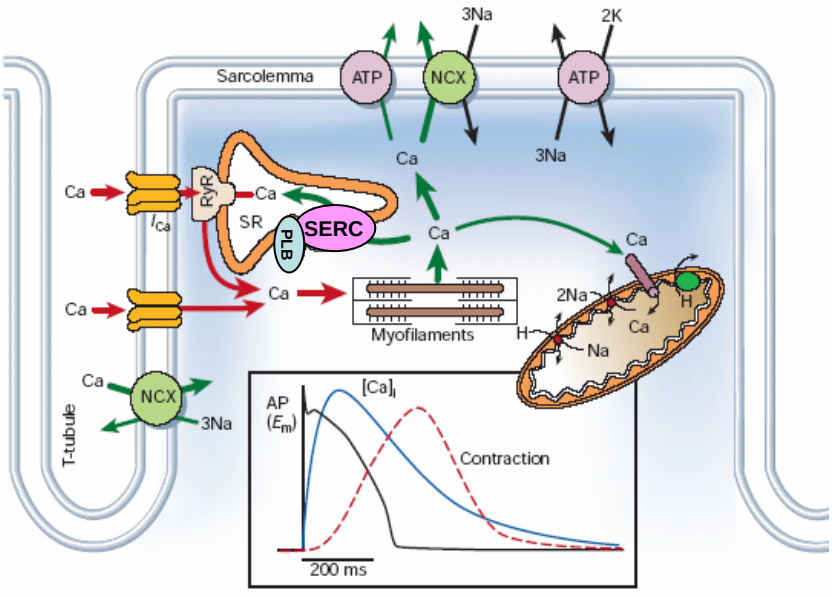


Figure1 Diagram of Ca^{2+} transport in cardiac myocyte (Bers, 2002)

F. Cardiac myofilament Ca^{2+} sensitivity

The cardiac contractile response to Ca^{2+} activation can be modified by mechanical, physiological, and pathological impacts. Muscle contraction is activated by the binding of Ca^{2+} ions to TnC. The binding of Ca^{2+} to TnC results in the enhancement of its binding affinity to TnI which then weakens the TnI-actin interaction and swings off the tropomyosin binding subunit, TnT. Strong binding of TnI to TnC leads to the opening of the myosin binding site on the actin. Consequencely, the movement of the troponin complex in the presence of Ca^{2+} rearranges the Tm to move out of the groove of actin and then allows the strong actin-crossbridge interaction (Moss and Buck, 2002). As a result, the thin filament slides toward the center of sarcomere resulting in force generation of the muscle. The ATPase activity used during this process is the so called Ca^{2+}-dependent actomyosin MgATPase activity, which indicates numbers and rates of crossbridge cycling. After that, detachment of crossbridges from actin occurs by the binding of ATP to myosin which allows the cycling of actomyosin interaction to be repeated if the Ca^{2+} is still bound to TnC (Solaro, 1982). Ca^{2+} dissociation from TnC can reverse the crossbridge interaction into blocking state until the next Ca^{2+} stimulation. This molecular mechanism clearly indicates the impact of Ca^{2+} binding affinity to TnC in determining the contraction and relaxation performance of the heart. Modification of TnC-Ca^{2+} binding interaction will induce changes in cardiac contractile status as represented by alterations in the Ca^{2+} responsiveness of myofilament activation or the sensitivity of myofilament to Ca^{2+} activation. The myofilament Ca^{2+} sensitivity, however, can be changed by other cardiac modifications without affecting TnC-Ca^{2+} binding affinity such as increased MLC or MyBP-C phosphorylation (van der Velden et al., 2003; Oakley et al., 2007).

The myofilament Ca^{2+} sensitivity can be calculated from the relation between Ca^{2+} concentrations to either force development or ATPase activity. A non-linear relation between Ca^{2+} concentration and cardiac contraction reflects the cooperative function of the contractile proteins in such a way that binding of Ca^{2+} to TnC provides higher Ca^{2+} binding affinity of another TnC on the neighboring contractile unit. Changes in this cooperative function thus affect the Ca^{2+} responsiveness of myofilament activation. Moreover, molecular alteration in generating force per cross-

bridge imparts the change in the sensitivity of myofilament. An increase in the Ca^{2+} sensitivity results in a greater force generation for a given Ca^{2+} concentration, whereas a reduction in Ca^{2+} responsiveness results in less force (Lee and Allen, 1993). On the other hand, a decrease in Ca^{2+} sensitivity of myofilament activation associated with an increase in the off rate of Ca^{2+} exchange to TnC as occurred during β-adrenergic activation causes a decrease in the duration of muscle relaxation (Solaro, 2002).

Changes in myofilament Ca^{2+} responsiveness can be modified by many factors including mechanical factors, covalent and non-covalent factors, and molecular mutation. Phosphorylations of TnI and MyBP-C by PKA, acidosis, and low temperature all induce a rightward shift in pCa-force relations of muscle fibers (Solaro, 2002; Blanchard and Solaro, 1984; Harrison and Bers, 1990). On the other hand, phosphorylation of MLC2, Ca^{2+} sensitizing agents, cardiomyopathy, female sex hormone deficiency, and heart failure induce leftwards shift in pCa-force relations which indicates the Ca^{2+} hypersensitivity of myofilament activation (Heyder et al., 1995; Wolff et al., 1995; 1996; Solaro, 2002). Differences in myofibrillar protein isoforms and gene mutations also alter the Ca^{2+} sensitivity of myofilament activation (Westfall et al., 2002). In addition, changes in the intracellular Ca^{2+} may involve in changes of the cardiac Ca^{2+} sensitivity (Kinugawa et al., 1999; Brixius et al., 2002).

G. Regulation of intracellular Ca^{2+}

Excitation-contraction (EC) coupling of the heart includes the process from a small inflow of Ca^{2+} through the L-type Ca^{2+} channels on the sarcolemma to a release of large amount of Ca^{2+} from SR storage via ryanodine receptors to induce muscle contraction. Subsequently, relaxation is mediated by the rapid removal of cytosolic Ca^{2+} predominantly by reuptaking into the SR storage using SERCA. Some of the Ca^{2+} will be extruded out of the cells through the functions of Na^{+}/Ca^{2+} exchanger and Ca^{2+}-ATPase on the sarcolemma. Some detailed information of these calcium cycling proteins will be briefly summarized here.

1. Cardiac Ca^{2+} Channel

Cardiac cells exhibit two classes of voltage-dependent Ca^{2+} channels named L-type and T-Type Ca^{2+} channels (Bers, 2002). The L-type Ca^{2+} channels are

responsible for the major influx of Ca^{2+} during the plateau phase of the action potential. In contrast, T-type Ca^{2+} channels are found in the sinus node, atrioventricular node, atrium, and in a specialized conducting system but are though to be absent in normal ventricles. L-type Ca^{2+} channels, which is called dihydropyridine receptors, are found to be located primarily at sarcolemmal-SR junctions where the SR Ca^{2+} release channels exist (Scriven et al., 2000). Sarcolemmal L-type Ca^{2+} channels are multimeric protein complexes comprised of five subunits termed α_1, α_2, β, γ, and δ. These subunits are encoded by separate gene (Hullin et al., 1993). The α_1-subunit contains the calcium conducting pore and the binding sites for calcium channel blockers. On the other hand, the α_2-, β-, γ-, and δ-subunits have regulatory properties.

2. <u>Ryanodine receptor</u>

Ca^{2+} is released from the SR via the calcium sensitive ryanodine receptors which are among the largest proteins identified to date. There are three isoforms of RyR receptors encoded by separate genes expressing in the mammalian tissue. RyR1 is the primarily dominant isoform in skeletal muscle, while RyR2 is dominant isoform in cardiac muscle. RyR3 is expressed at low levels in various tissues including cardiac muscle. RyR1 and RyR2 were functionally triggered by different signals. RyR1 in skeletal muscle can be activated in the absence of Ca^{2+} entry. Changes in the conformation of L-type Ca^{2+} channels after depolarization can trigger the opening of RyR1. In contrast, RyR2 in cardiac muscle is a ligand-gated Ca^{2+} channel, activated by Ca^{2+} influx through L-type Ca^{2+} channel. The RyR Ca^{2+} release channels form a homotetramers (565 kDa for each subunit). The N-terminal domain of RyR channels protrudes into the cytosol to act as a scaffolding protein that localized numerous key regulatory proteins to the junctional complex. These regulatory proteins include calmodulin (Fruen et al., 2000), FKBP 506 binding protein (FKBP 12.6 which also called calstabin2) (Brillantes et al., 1994; Marx et al., 2001), PKA (Marx et al., 2000), phosphatases1 and 2A (PP1 and PP2A) (Marx et al., 2000), and sorcin (Meyers et al., 1995). RyRs are also coupled to other proteins at the luminal SR surface including triadin, junctin, and CSQ (Zhang et al., 1997). Triadin and junctin take care of the

intra-SR anchoring of RyR and CSQ is a high-capacity intracellular calcium buffering protein.

The activity of RyR is regulated by several factors, including physiological substances (e.g. Ca^{2+}, Mg^{2+}, ATP), pharmacological agents (e.g. caffeine, ryanodine, ruthenium red, and the immunosuppressant drugs, FK506 and rapamycin), and intracellular processes (e.g. phosphorylation, oxidation) (Fill and Copello, 2002). Phosphorylation of RyR by PKA at Ser^{2809} results in an increased opening probability of the RyR channels by dissociation of calstabin2 (FKBP12.6), which stabilizes the closed state of RyR channels in the absence of PKA. In normal hearts, phosphorylation of RyR2 by PKA causes a reversible dissociation of FKBP12.6 and a transient increase in the SR calcium release, resulting in an enhanced contractility of the muscle cells (Valdivia et al., 1995). However, the defective regulation of RyR2 in failing hearts is associated with PKA hyperphosphorylation of the channels, resulting in dissociation of FKBP12.6 and an increased SR Ca^{2+} leak (Marx et al., 2000). Defective regulation of RyR2 leads to pathological consequences, including depletion of SR Ca^{2+} stores and aberrant release of SR Ca^{2+} during diastole, which may trigger fatal cardiac arrhythmias (Marx et al., 2000).

3. Sarcoplasmic reticulum Ca^{2+}-ATPase pump

The rate of Ca^{2+} sequestration by the SERCA is a major determinant of the muscle relaxation rate. There are three highly homologous genes, SERCA1, SERCA2 and SERCA3, encoding this pump. However, five different isoforms have been found. SERCA1 and SERCA1b are the isoform expressed in adult and neonatal fast-twitch skeletal muscle, respectively. SERCA2a is the cardiac and slow-twitch skeletal isoform. SERCA2b and SERCA3 are expressed in a broad variety of muscle and nonmuscle tissue. The cardiac isoform, SERCA2a, is abundantly expressed in the ventricular and atrial compartments of the mammalian heart. The Ca^{2+} uptake activities of SERCA2a involves transportation of two Ca^{2+} per molecule of ATP against a high ion gradient from a free intracellular Ca^{2+} concentration between 100 nM - 10 μM to a free calcium concentration of ~1 mM in the SR (Lytton et al., 1992; Bers, 1991). This pump is crucial for Ca^{2+} accumulation within the SR and thus this pump together with the Na^{+}/ Ca^{2+} exchanger and the sarcolemmal Ca^{2+}-ATPase

eliminates calcium from the cytosol in order to facilitate relaxation of the myocardium for the availability of Ca^{2+} release through the RyR during systolic.

The activity of SERCA2a can be modulated by both direct and indirect mechanisms. The most predominant mechanism is the indirect modulation by the 52 amino acids phosphoprotein called phospholamban (Koss and Kranias, 1996; Luo et al., 1994), located in the cardiac SR. Binding of phospholamban to SERCA2a reduced the Ca^{2+} binding affinity of SERCA protein. Phospholamban is present in two stoichiometric forms including monomeric, the active inhibitory form, and pentameric, an inactive or less active or reservoir form (Autry and Jones, 1997; Kimura et al., 1997). According to SDS-PAGE analysis, about 80-90 % of pentameric form and about 10-20 % monomeric form of phospholamban are present (Arkin et al., 1994; Simmerman et al., 1996). The phosphorylation of phospholamban by PKA at Ser^{16} or by CaMKII at Thr^{17} releases the inhibitory effect of phospholamban on SERCA2a. Consequently, the affinity of SERCA2a to Ca^{2+} is enhanced. In addition, phosphorylated phospholamban also favors the association of the monomeric phospholamban into pentameric phospholamban without affecting the maximal velocity (V_{max}) of SERCA2a (Cornea et al., 1997). Therefore, variations in the relative ratio of phospholamban to SERCA2a, the proportion of monomeric and pentameric forms of phospholamban, and the phosphorylation levels of phospholamban can all affect the SERCA2a binding affinity to Ca^{2+} and consequently alter the intracellular Ca^{2+} mobilization. Direct modulation of SERCA2a activity can be induced by the phosphorylation of SERCA2a protein by CaMKII at Ser^{38} (Toyofuku et al., 1994), resulting in an increase in the maximal activity (V_{max}) of the enzyme without affecting the Ca^{2+}-affinity (Narayanan and Xu, 1997; Toyofuku et al., 1994; Xu et al., 1993). Thyroid hormone and insulin are other direct factors that could also modulate SERCA2a activity. SERCA activity not only contributes to cardiac relaxation, but also plays a key role in providing the load of Ca^{2+} required for subsequent contraction. In heart failure, diminished peak systolic Ca^{2+} level, prolonged diastolic Ca^{2+} sequestration, and elevated diastolic Ca^{2+} levels were clearly demonstrated which indicate malfunction of Ca^{2+} uptake activities (Houser et al., 2000; Frank et al., 2002). Changes in SERCA2a and phospholamban protein contents may account for the suppressed Ca^{2+} uptake function in human failing heart. A

reduction in phospholamban phosphorylation at Ser^{16} was also found to contribute to changes in SERCA activity (Schwinger et al., 1999; Dash et al., 2001).

4. Na^+/ Ca^{2+} exchanger

The major physiological role of the Na^+/Ca^{2+} exchanger is to extrude Ca^{2+} from the cell which then contributes significantly to the relaxation of the heart. The cardiac Na^+/Ca^{2+} exchanger consists of 970 amino acids, composing of an NH_2-terminal cleaved signal sequence, 11 hydrophobic (putative transmembrane) domains, and a hydrophilic (intracellular) loop (Nicoll et al., 1990). There are at least three different genes coding for different Na^+/Ca^{2+} exchanger molecules with a number of spliced variants (Ruknudin et al.,, 1997). The Na^+/Ca^{2+} exchanger extrudes one calcium ion for three sodium ions using the electrochemical sodium gradient (Ruknudin et al., 1997; Philipson, 1990). However, the Na^+/Ca^{2+} exchanger is also voltage dependent and can reverse its mode during the action potential (Ruknudin et al., 1997; Philipson and Nicoll, 1992). In its influx or reverse mode it couples Ca^{2+} ion entry to Na^+ ion efflux which explains the observed inotropic effects of cardiac glycosides. It has also been suggested that the Ca^{2+} entry via the exchanger can directly trigger sarcoplasmic reticulum Ca^{2+} release (Eisner et al., 1998).

H. β-Adrenergic receptors of the hearts

β-adrenergic receptor belongs to the superfamily of G protein-coupled receptors (GPCRs), key molecules for modulating cardiac contractile function (Rockman et al., 2002). There are three identified β-adrenergic receptor subtypes, designated β_1, β_2, and β_3, which have distinct molecular functional and pharmacological characteristics (Caron and Lefkowitz, 1993). In the human myocardium, β_1-adrenergic receptor is the predominant subtype, representing approximately 70% to 80% of the total β-adrenergic receptor density. On the other hand, β_2-adrenergic receptors are present about 20% of all β-adrenergic receptors with a minor contribution of β_3-adrenergic receptors (Brodde, 1993). Stimulation of myocardial β_1-adrenergic receptors with catecholamine plays a physiologically significant role in enhancing cardiac contractile activity through modifications of Ca^{2+} flow during the process of excitation contraction coupling and on myofilament sensitivity (Bers, 2002) (Figure 2). β_1-

adrenergic receptors are the G-protein coupling receptors signaling through both cAMP-dependent and cAMP-independent pathways (Bishopric et al., 1992; Steinberg, 1999; Zhu et al., 2003). In the heart, agonist occupancy of β_1-adrenergic receptors leads to the primary activation of adenylyl cyclase stimulatory G proteins (G_s) resulting in an increased intracellular cyclic-AMP (cAMP). Cyclic-AMP acts as secondary messengers to activate PKA which will phosphorylate many proteins as mentioned earlier. In the cAMP-independent pathway, the signal is transmitted through nuclear receptors to stimulate or inhibit the transcriptional process (Bishopric et al., 1992; Schafer et al., 2000). Therefore, effects of β_1-adrenergic stimulation could be indicated by cellular phosphorylation levels as well as protein expression levels.

Alterations in the responsiveness to β_1-adrenergic stimulation depend on two contributions, including receptor activation and downstream signaling processes. Under normal physiological condition, there is a basal β_1-adrenergic receptor stimulation. Alterations in β_1-adrenergic receptors stimulation will disturb the cardiac cellular homeostasis (Dorn et al., 2000; Schafer et al., 2000; Engelhardt et al., 2001). The increased adrenergic drive either through upregulation of β_1-adrenergic receptors or an increased signaling process is known to be toxic to the heart (Engelhardt et al., 1999; Lefkowitz et al., 2000; Post et al., 1999). In a transgenic mice model, overexpression of human β_1-adrenergic receptors in the heart produces a short-lived improvement of cardiac function but ultimately induces a cardiomyopathic phenotype with dilation and depressed contractile functions (Bisognano et al., 2000; Engelhardt et al., 1999). On the other hand, during the development of HF, several molecular changes progressively take place within cardiomyocytes that yield significant derangements of the β-adrenergic receptor signaling system (Brodde 1993). Decreases in the expression of β_1-adrenergic receptors in cardiac membrane of the failing hearts have also been reported (Anthonio et al., 2000; Beau et al., 1993). This receptor downregulation was considered to be a compensatory response to an increase in catecholamine levels in heart failure (Dong et al., 1999).

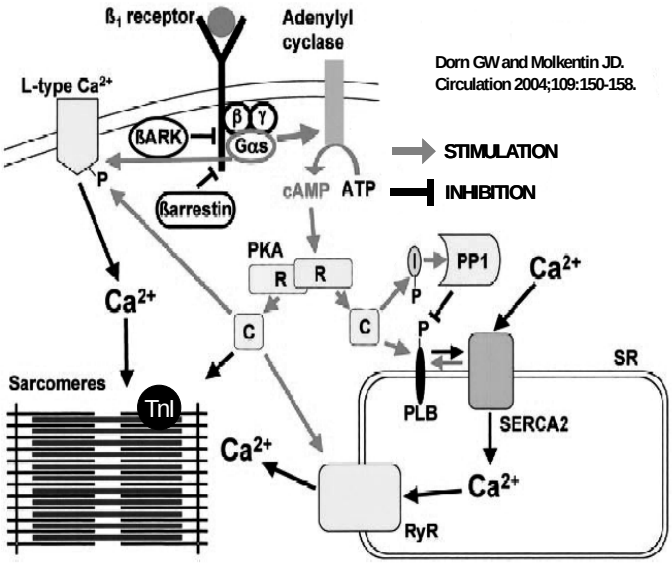


Figure 2 Diagram of β_1-adrenergic signaling pathways directly regulating myocardial contraction and cardiac output.

I. Heat shock proteins

Heat shock proteins (HSPs) are a group of protective proteins induced by many stimuli, such as hyperthermia, exercise, ischemia, hypoxia and inflammation (Hutter et al., 1996; Marber et al., 1995; Chen et al., 2002). HSPs are "molecular chaperones" that recognize and form a complex with incorrectly folded or denatured proteins, and ultimately lead to the correct folding, compartmentalization, or degradation. Many HSPs have been found in cardiac, such as HSP90, HSP72 and HSP65. Each HSP is named according to its mass in kilodaltons. The synthesis of HSPs is highly conserved between different species. The expression of the HSP genes encoding different HSPs is regulated by the heat shock transcription factors (HSFs), which are normally bound to HSPs within the cytosol. When cells are exposed to stress, HSFs are phosphorylated and form trimers that enter the nucleus and bind the heat shock elements located within the promoter of heat shock genes to activate the HSP synthesis (Nishizawa et al., 2002). Three isoforms of HSF, namely HSF1, HSF2, and HSF4, have been detected in the human heart and are encoded by distinct genes. Only HSF1 is efficiently activated with reduced oxygen species in ischemic-reperfused heart (Melling et al., 2004) and has been suggested to be involved in

protection of the heart against ischemia-reperfusion injury (Lau et al., 1997; Martin et al., 1997). In contrast, three other heat shock transcription factor isoforms (HSF2–4) are not sufficient to preserve the heat shock response in the absence of HSF1. HSP 70s including HSP72 and HSP73 are suggested to play an important role in the cardio-protection against stress-induced functional damage. Myocardial tolerance against ischemia/reperfusion injury is increased when myocardial HSP72 is accumulated after an exposure of normal animals to heat shock (Qian et al., 1998). Post-ischemic contractile recovery is improved in the perfused heart of HSP72-overexpressed mice. In contrast, reductions in the production of HSP70s are associated with the decrease in contractile function during the development of heart failure (Tanonaka et al., 2001).

CHAPTER III
MATERIALS AND METHODS

A. Animals

Female Sprague-Dawley rats (7 Weeks of age) weighing between 160-180 g were obtained from the National Laboratory Animal Center at Salaya Campus, Mahidol University. The rats were housed in hanging stainless steel cage in temperature and humidity-controlled room with 12 hour, dark-light cycle. Water and rat chow (C.P., Thailand) were fed *ad libitum*. The animal protocol was approved by the Experimental Animal Committee, Faculty of Science, Mahidol University, in accordance with National Laboratory Animal Centre, Thailand.

B. Experimental protocols

Model of study

The ovarian sex hormone deficiency was induced by ovariectomy in post-pubertal female Sprague-Dawley rats weighing between 180-200 g (approximately 8 weeks of age). Diabetic animal was experimentally induced by intraperitoneal injection of streptozotocin (STZ), in a dose of 65 mg/kg body weight and a fixed volume of 0.2 ml of citrate buffer (pH 4), two weeks after ovariectomy. The rats were sacrificed 10 weeks after ovariectomy. The uterus was weighed to verify the condition of ovarian sex hormone deprivation while urinary glucose was checked 24 hr after diabetic induction and before sacrifice using glucose strip to confirm the diabetic status. Sham animals were prepared by the same operation procedure as that performed in ovariectomized rats except that both ovaries were kept intact. The control group for diabetic condition received only citrate buffer injection instead of streptozotocin.

Protocol 1. To evaluate the influence of diabetes on changes in response to Ca^{2+} of cardiac myofilament from OVX rats.

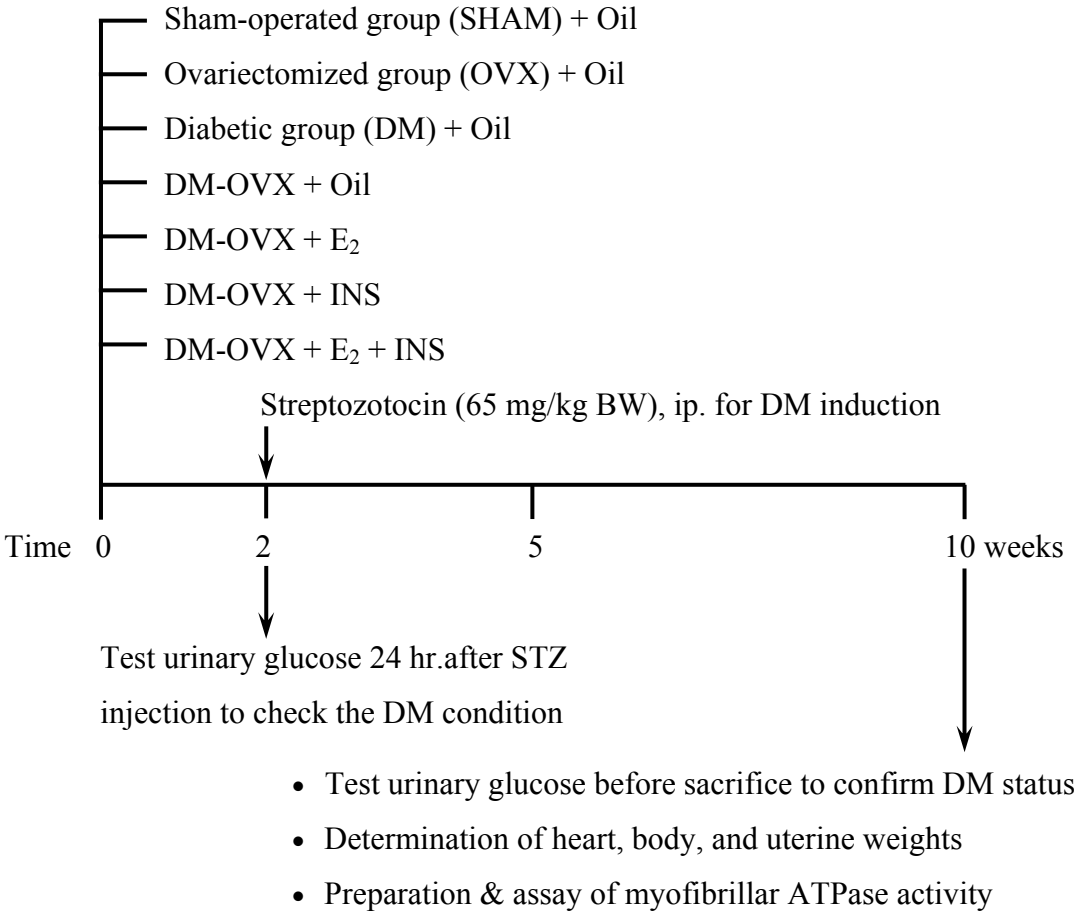


Figure 3 Diagram of experimental protocol 1

Rats were divided into seven groups as shown in Figure 3. Rats were bilaterally ovariectomized or sham-operated. Sham-operated rat was prepared by the same operating procedure as that performed in ovariectomized rats except that both ovaries were kept intact. Two days after surgery, sham rats were subcutaneously injected with 0.1 ml of corn oil whereas ovariectomized rats were randomly divided into two groups and subcutaneously injected with either 0.1 ml of corn oil with or without 5 μg of 17β-estradiol three times per week for 10 weeks. Two weeks after surgery, both sham and ovariectomized rats were further randomly divided into non-diabetic and diabetic groups. Diabetic rats were induced by intra-peritoneal injection of freshly prepared streptozotocin (STZ), in a dose of 65 mg/kg body weight and a fixed volume

of 0.2 ml, while non-diabetic groups were injected with only citrate buffer instead of STZ. Three days after diabetic induction, diabetic-ovariectomized rats were subcutaneously injected with 5 units of human insulin on a daily basis for the whole experimental period. Ten weeks after surgery, animals were sacrificed and left ventricles were used for determining the myofibrillar ATPase activity. The –log free Ca^{2+} concentration (pCa)-myofilament ATPase relationship of isolated myofibrillar was then performed.

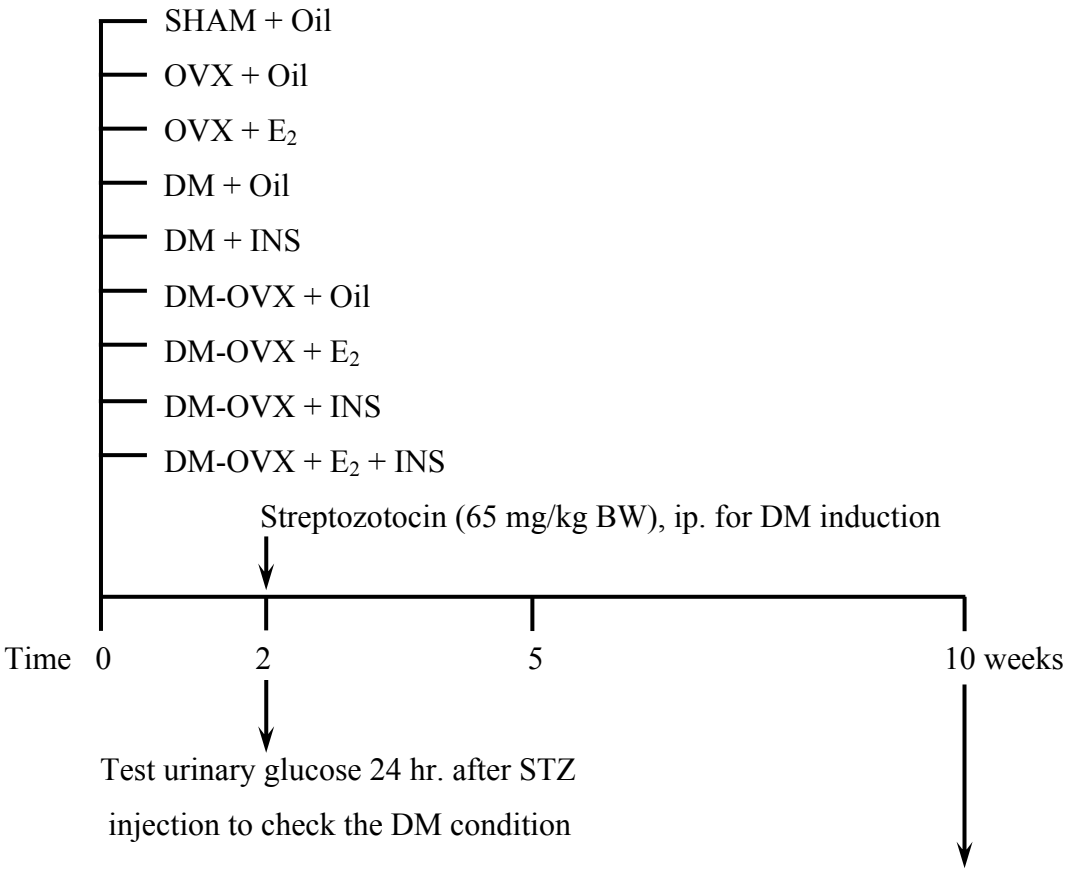
Protocol 2. To investigate the interactive effect of E_2 and INS deprivation on β-adrenergic receptor and HSP72 expression.

Rats were prepared as described in protocol 1. The density and binding affinity of β_1-adrenergic receptor was analyzed using cardiac sarcolemmal preparation and receptor binding assay. SDS-gel electrophoresis and Western blot analysis were also performed to determine quantity of the amounts of β_1-adrenergic receptor and HSP72.

Protocol 3. To investigate the regulatory effect of E_2 on the crossbridge cycling kinetics of the cardiac contractile filament.

Protocol 3.1 To evaluate the correlation between cardiac maximum myofilament ATPase activity and the MHC isoform in E_2- and/or INS-deficient rats.

Rats were divided into nine groups as shown in Figure 4 and were prepared as described in protocol 1. Upon the complete duration of study, rats were sacrificed for preparations of cardiac myofilament, measurements of myofilament ATPase activity, and determination of MHC isoforms. Maximum Ca^{2+}-dependent actomyosin Mg^{2+}-ATPase activity at pH 7.0 was measured using cardiac myofibrillar preparations. MHC isoforms were also analyzed using SDS-gel electrophoresis. Relations of the percentage of α-MHC and the maximum myofilament ATPase activity from each group were plotted and the linear regression was analyzed for each experimental group based on the combined data with sham group.



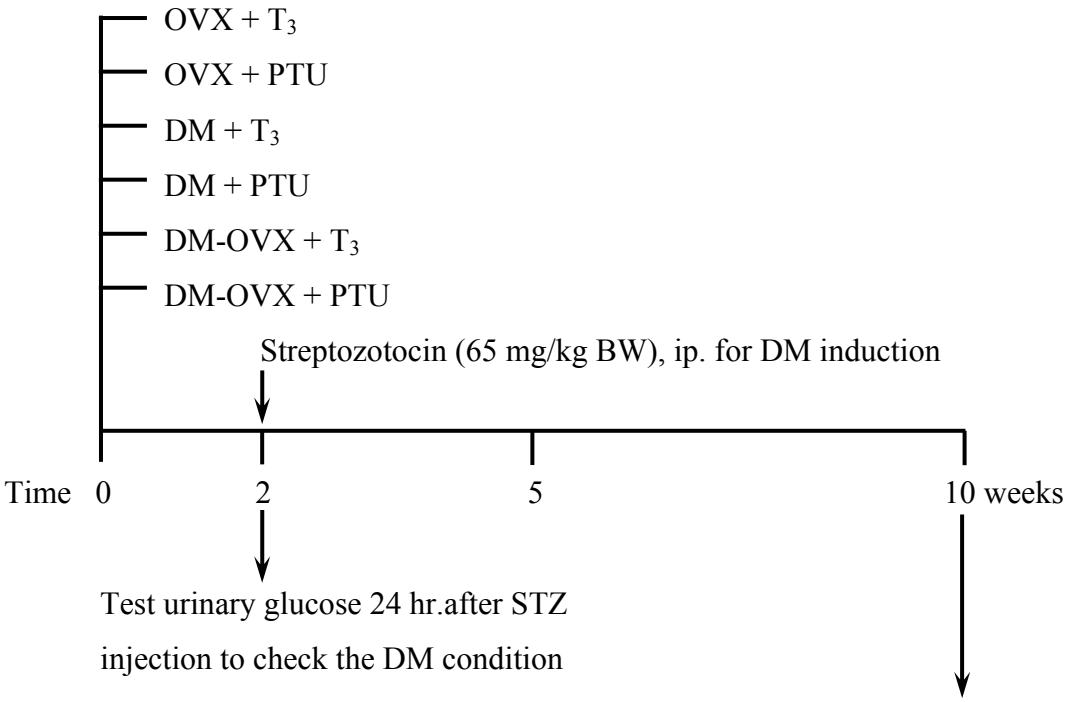
- Test urinary glucose before sacrifice to confirm DM status
- Determination of heart, body, and uterine weights
- Preparation & assay of maximum myofibrillar ATPase activity at pH 7.0
- Determination of MHC isoforms

Figure 4 Diagram of experimental protocol 3.1

Protocol 3.2 To test whether changes in the cardiac crossbridge cycling kinetics were regulated only to the MHC isoform.

In order to evaluate the crossbridge cycling kinetics of the cardiac myofilament at a wide scale of α-MHC, the thyroid status of OVX, DM and DM-OVX rats were then manipulated to induce different amount expression of α-MHC. OVX, DM and DM-OVX rats were prepared as described in protocol 1. Suppression of plasma thyroid hormone was induced by oral-administration of 6-n-propyl-2-thiouracil (PTU) on a daily basis in a dose of 0.05 - 0.1 g starting three days of the diabetic induction.

On the other hand, increments of thyroid hormone were induced by injection by subcutaneous injection of triiodothyronine (T_3) 15 μg/kg body weight on a daily basis. Thyroid status was verified by the plasma free T_3 level before sacrifice.



- Test urinary glucose before sacrifice to confirm DM status
- Determination of heart, body, and uterine weights
- Measurement of free T_3 in plasma
- Preparation & assay of maximum myofibrillar ATPase activity at pH 7.0
- Determination of MHC isoforms

Figure 5 Diagram of experimental protocol 3.2

Protocol 4 To evaluate the significant role of E_2 in the regulation of intracellular Ca^{2+} handling in cardiac myocytes by the sarcoplasmic reticulum under diabetic complication.

Rats were divided into eight groups as shown in Figure 6 and were prepared as described in protocol 1. Ten weeks after surgery, the animals were sacrificed, and the left ventricles homogenates and SR membrane vesicle were used for quantification of the SR Ca^{2+} uptake and the SR Ca^{2+} ATPase (SERCA) activity, respectively. SR Ca^{2+}

uptake activity was performed using left ventricular homogenate. The SERCA activity was also determined using coupled enzyme reaction. The protein contents of SERCA2a, total phospholamban (PLB), phosphor-Ser[16] phospholamban, phospho-Thr[17] phospholamban and calsequestrin on the SR membrane were also quantitated using Western blot analysis technique.

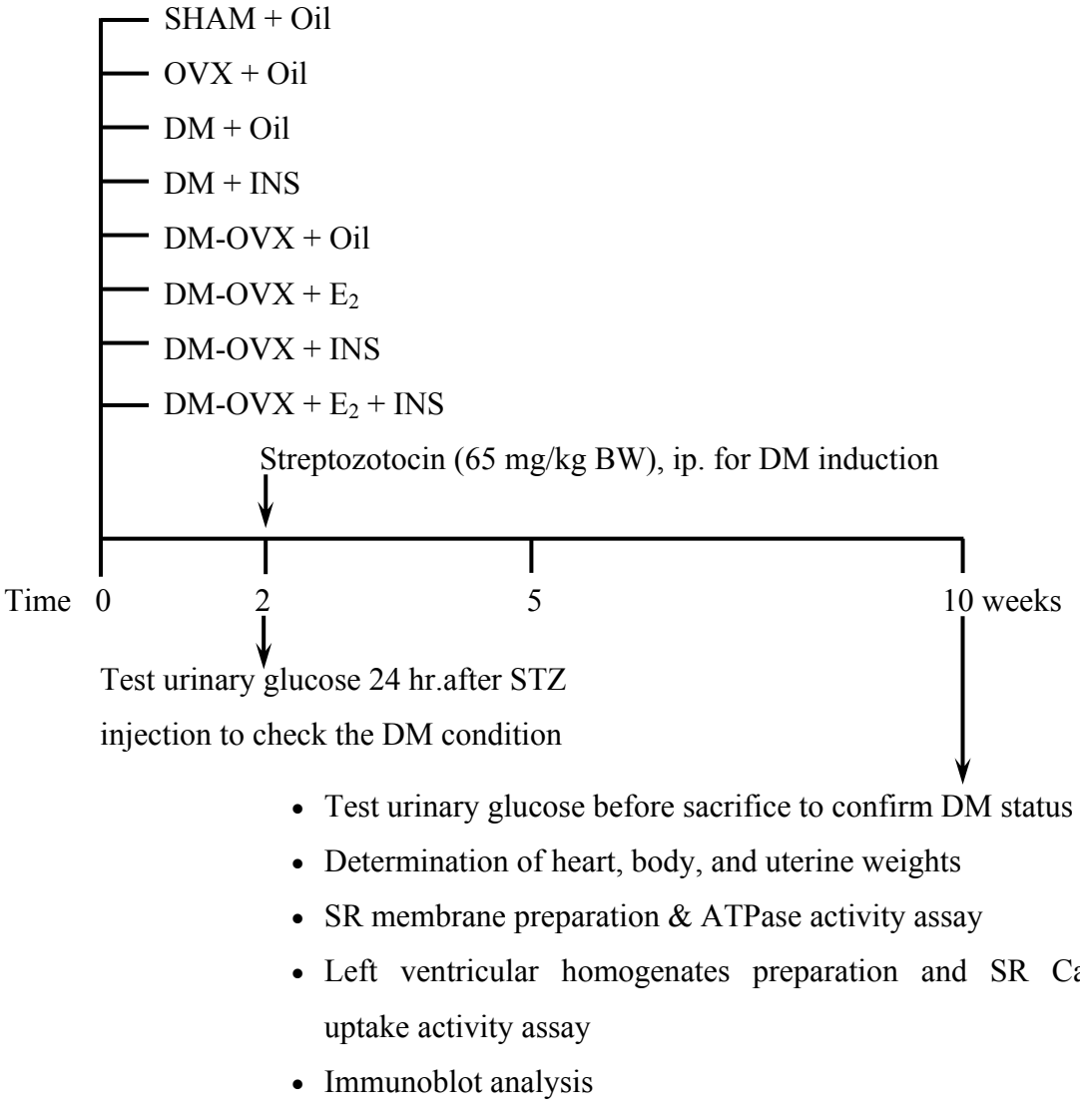


Figure 6 Diagram of experimental protocol 4

C. Materials

Most of the chemicals were purchased from Sigma, St Louis, MO, and Fisher Scientific, Pittsburgh, PA. Millipore filters (Millex-HA, 0.45 μm) were purchased

from Millipore, Molseim, France. Chemicals for SDS-PAGE and immunoblotting were obtained from Bio-Rad, Hercules, CA. [^{3}H]-dihydroalprenolol, $^{45}CaCl_2$, enhanced chemiluminescence detection kit and hyperfilm were obtained from Amersham Pharmacia Biotech, Buckinghamshire, England. Human insulin was purchased from Eli Lilly, Indianapolis, IN. Glucose strips were purchased from Roche, Indianapolis, IN.

Monoclonal and polyclonal antibodies of β_1-adrenergic receptor, calsequestrin, phospholamban, and SERCA2a were obtained from Affinity Bioreagents, Golden, CO. Polyclonal antibodies of HSP72 were purchased from Stressgen, Victoria, BC. Polyclonal antibodies of phospho-Ser^{16} and phospho-Thr^{17} phospholamban were acquired from Badrilla Leeds, UK. Horseradish peroxidase-conjugated donkey anti-mouse was purchased from Research Diagnostic Inc., Flanders NJ. Horseradish peroxidase-conjugated goat anti-rabbit was purchased from Zymax Reagents, San Francisco, CA.

D. Methods

1. Cardiac membrane preparation

Isolated cardiac membranes were prepared from the left ventricle using the method of Baker and Potter with modification (Appendix D). The heart was rapidly rinsed in ice-cold normal saline solution after excision. Left ventricle was homogenized in 10 ml of 10 mM Tris-HCl pH 8.0 twice with Omni Macro homogenizer. After homogenization, 30 ml of 1 M KCl was added in the homogenate and then left on ice for 20 minutes to dissolve the myofilament proteins and to reduce the non-specific binding debris. The homogenate was then filtered through six layers of cheesecloth to remove debris. The filtrate was centrifuged at 43,900 g at 4^{o}C for 20 minutes to precipitate the membrane. The supernatant was discarded and the pellet was resuspended in 35 ml Tris-buffer. The homogenate was resedimented with the same speed of centrifugation. The final pellet was dispersed with 10 ml of 50 mM HEPES buffer, pH 8.0 using Teflon-glass homogenizer. Protein concentration was finally determined by Bradford assay (Appendix B). Cardiac membrane suspension was immediately used for receptor binding assay.

2. β_1-Adrenergic receptor binding assay

The binding assay for β_1-adrenergic receptor was performed under an equilibrium condition based on the procedure of Cervoni et al. (Appendix E). Approximately 0.05 mg of fresh cardiac membrane protein was added into each assay tube containing 50 mM HEPES buffer (pH 8.0) with 4 mM $MgCl_2$ and various concentrations of [^{3}H]-dihydroalprenolol (DHA) ranging from 0.5-40 mM. The reaction was incubated for 20 minutes at 25^{o}C in metabolic shaking water bath set at 60 oscillations per minute. The reaction was stopped by adding of 4 ml ice-cold washing solution (25 mM HEPES with 4 mM $MgCl_2$) and immediately filtered through GF/B glass filter paper. The filter was rinsed twice with the washing solution to remove the unbound ligand. The membrane was transferred into scintillation vial, blown dry, and 7 ml of scintillation fluid (Appendix F) was then added into the vial. The vial was left overnight to let the membrane dissolved and then transferred 6 ml of the scintillation fluid to a new vial. The radioactivity was measured using β-counter and defined as the total binding of [^{3}H]-DHA on the membrane. Non-specific DHA binding on the membrane protein was determined in a parallel set of assay containing 10 μM (-)-alprenolol in the incubation mixture. The specific binding of [^{3}H]-DHA on the membrane was calculated by subtracting the non-specific binding from the total binding.

Relations between specific [^{3}H]-DHA binding (fmole/mg protein) and free [^{3}H]-DHA (nM) were plotted by non-linear least square regression analysis based on the binding isotherm equation using GraphPad Inplot, ISI software version 4.0 to determine a saturation binding. The maximum binding (B_{max}) and dissociation constant (K_d) of the receptor were obtained from a linear transformation of data to a Scatchard plot of bound/free. The x-axis interception is an estimate of B_{max} and the slope provides an estimate of $-1/K_d$.

3. Cardiac myofibrillar preparation

Hearts were rapidly removed from the rats under ether anesthesia and placed in ice-cold saline. Cardiac myofibrils were prepared from the left ventricles as described by Pagani and Solaro (1984). Two to three left ventricles were minced and suspended

in 40 ml of 30 mM imidazole with protease inhibitor (0.5 mg/ml Leupeptin, 0.5 mg/ml pepstatin A, and 0.45 mM phenylmethylsulfonyl fluoride). The suspension was homogenized twice by Omni Macro Homogenizer. The homogenate was centrifuged at 12,000g, 4°C for 13 min. The supernatant was discarded and the pellet was resuspended, homogenized, and centrifuged (10 min at 3,000g) in many segmential steps with different solutions as follows: three washing steps in standard buffer containing 60 mM KCl, 30 mM imidazole, and 2.5 mM $MgCl_2$ along with protease inhibitors; one washing step in standard buffer with 1 mM EGTA followed by two membrane extracting steps in standard buffer with 1% Triton-X100 using glass-glass homogenizer; three final washing steps in standard solution. The final pellet was suspended in approximately 3-4 ml of standard buffer per heart. Myofibrillar protein concentration was finally determined by the method of Lowry et al. (Appendix A) using bovine serum albumin (BSA) as standard protein.

4. Cardiac myofibrillar Ca^{2+}-dependent actomyosin MgATPase activity

Maximum cardiac myofibrillar Ca^{2+}-dependent actomyosin MgATPase activity

Ca^{2+}-dependent MgATPase activity of isolated myofibrils was assayed by determination of inorganic phosphate (Pi) released in 10 min linear reaction at 30˚C in 2 mM Mg^{2+}, 60 mM imidazole, 5 mM $MgATP^{2-}$, and ionic strength of 120 mM. Assays were run in various the free Ca^{2+} concentrations from pCa 7.5 to 4.875 at pH 7.0 (Appendix G). Total concentrations of $CaCl_2$, EGTA, KCl, $MgCl_2$, and ATP were calculated using a computer program generated from the dissociation constants given by Fabiato (Fabiato, 1988). The concentration of Pi was measured by the method of Carter and Karl (Appendix H). The relations between pCa and MgATPase activity were plotted using non-linear least square regression based on Hill equation.

5. Sample preparation for myosin heavy chain isoform analysis

Sample preparations were preformed for gel electrophoresis as described with some modifications (Martin et al., 2002). Before cardiac myofilament preparation, papillary muscle was isolated from the left ventricle soaked in saline at 4°C. The

isolated muscles was frozen immediately in liquid nitrogen, pulverized while frozen, and added with sample buffer containing 50 volume (v/w) of 50 mM Tris pH 6.8, 2.5% SDS, 10% glycerol, 1mM DTT, 1 μg/ml Leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF, and 5 μg/ml Aprotinin. The sample was then sonicated for 1 hr at 4°C and finally centrifuged with microcentrifuge at 10,000 rpm for 5 min. The supernatant was collected and the total protein concentration was measured by the bicinchoninic acid method (Appendix C). Then, 2-mercaptoethnol was added into the sample to a final concentration of 5% (v/v). The sample was aliquoted and stored at -80°C.

6. Myosin heavy chain separation

Cardiac MHC isoform was separated in discontinuous gel containing 5% acrylamide in the stacking gels and 6.5% acrylamide in the resolving gels as previously described with some modification (Martin et al., 2002). The acrylamide and biscrylamide ratio was 100:1. Samples were diluted to a protein concentration of 0.5 μg/μl and were loaded into wells with 5 μg. Gels were run at a constant current for 3.5 hr, and then changed to constant voltage until 15 hr running. Upon the completion of the electrophoresis, gels were stained with a modified Coomassie Blue stain for 8 hours, and destained with water for 1 to 5 hours. Gels were scanned with a GS800 densitometer (Bio-Rad) to determine the relative amount of α- and β-MHC to the total MHC in each sample.

7. Determination of the SR Ca^{2+} uptake activity

Whole left ventricle was homogenized in 25 mM imidazole using a Teflon-glass homogenizer with a motor driven pestle at 600 rpm for 20-40 passes. The homogenate was filtered through six layers of cheesecloth and the filtrate was collected for the SR Ca^{2+} uptake measurement. An amount of 50 mg of left ventricular homogenate was diluted with 25 mM imidazole to make a final concentration of 1 mg of protein per ml of reaction (Appendix I). The reaction was run in various concentrations of Ca^{2+} ranging from pCa 8.0 to 4.875, pH 7.0 at 37°C with 0.1% of $^{45}CaCl_2$. The Ca^{2+} uptake assay was initiated by addition of ATP to a final concentration of 5 mM and then incubated for 3 minutes at 37°C with 60 rpm

shaking. The reaction was stopped by rapid cooling in ice. A volume of 0.3 ml of the reaction mixture was filtrated through 0.45 μm Millipore filter (Millex HA). The radioactivities of the sample in the filtrated and non-filtrated fraction were determined using liquid scintillation spectrometer (Appendix F). Subtraction of radioactivity in the filtrate from the non-filtrated portion represents the amount of SR Ca^{2+} uptake. Protein concentration was confirmed by Bradford assay (Appendix B). The relationships between pCa and the SR Ca^{2+} uptake were plotted using non-linear least square regression based on Hill equation.

8. Isolation of cardiac SR vesicular membrane

SR vesicle was prepared from the left ventricular homogenate in 8 ml of 10 mM $NaHCO_3$, pH6.8. The homogenate was centrifuged at 8,000 g, 4°C for 10 minutes and the supernatant was then centrifuged at 10,000 g, 4°C for 20 minutes to precipitate myofibrils. KCl powder was added into the supernatant to a final concentration of 0.6 M KCl and the mixed supernatant was then left on ice for 15 minutes. SR vesicle was then precipitated by centrifugation at 45,000 g, 4°C for 45 minutes. The pellet was resuspended in 0.25 M sucrose buffer followed by ultracentrifugation at 100,000 g, 4°C for 60 minutes. The final pellet was then resuspended in 2 ml of 0.4 M sucrose, 5 mM HEPES, 5 mM Tris-base, pH 7.2. The final suspension was immediately frozen and stored at –80°C. Protein concentration of the SR vesicle was determined using Bradford assay (Appendix B).

9. Determination of the SR Ca^{2+} ATPase (SERCA) activity

SR vesicle was used for the assay of SERCA activity. The reaction was analyzed by triple enzyme assay (SERCA, pyruvate kinase, and lactate dehydrogenase) to quantify NADH contents after oxidation as previously described by Chu and his group (Appendix K). The reaction was run at various concentrations of Ca^{2+} ranging from pCa 8.0 to 5.0, pH 7.0 at 37°C. The SERCA activity was determined by incubating 5 μg of SR vesicular protein in 1 ml reaction mixture containing 21 mM MOPS, 4.9 mM NaN_3, 0.06 mM EGTA, 0.1 M KCL, 3 mM $MgCl_2$, 1 mM phospho(enol) pyruvate, 0.2 mM NADH, pyruvate kinase 8.4 unit/ml,

and lactate dehydrogenase 12 unit/ml. The reaction was initiated by addition of ATP to a final concentration of 1 mM. Kinetic reaction of NADH degradation was monitored with spectrophotometer at 340 nm for 350 second. SERCA activity was calculated from the optical density using extinction coefficient of NADH (Appendix K). The non-specific SERCA activity was also measured in the reaction mixture with an addition of 0.1 mM thapsigargin. Relations between pCa and SERCA activity were plotted using non-linear least square regression based on Hill equation.

10. General methods and statistics

Amounts of SERCA2a, total phospholamban (PLB), phosphor-Ser^{16} PLB, phospho-Thr^{17} PLB, and calsequestrin (CSQ) of cardiac contractile proteins in left ventricular homogenate were determined by general immunoblot protocol. Dilutions for monoclonal antibodies of SERCA2a and PLB were 1:1,000 and 1:5,000, respectively. Polyclonal antibodies of phosphor-Ser^{16} and phosphor-Thr^{17} PLB were used at a dilution of 1:10,000 and 1:2,000, respectively. The blotting membrane used for determining SERCA2a and phospholamban were re-probed with polyclonal antibody of CSQ at a dilution of 1:20,000. Secondary antibody conjugated with horseradish peroxidase was used and the specific protein bands were detected on hyperfilm using chemiluminescence-labeling system. The relative amounts of SERCA2a and PLB were normalized by the amount of CSQ in the same lane of the blot. The relative amount of actin was used as a loading control for phosphor-Ser^{16} and phosphor-Thr^{17} PLB detections.

Relations of pCa to Ca^{2+} uptake or SERCA activity were fit to the Hill equation using non-linear least square regression analysis (PRISM) to derive the EC_{50} (half maximal activating [Ca^{2+}]) and Hill coefficient (n) (Appendix L). Data are presented as means ± SE. One-way analysis of variance (ANOVA) was used for determining the difference among groups with the significance set at $P < 0.05$. Student Newman-Keuls multiple comparison was used for determining significant difference between the two means.

CHAPTER IV
RESULTS

1. Interactive effect of estrogen and insulin deficiency on the cardiac myofilament response to Ca^{2+}.

As shown in Table 1, the condition of ovarian sex hormone deficiency was clearly indicated by significant decreases in uterine weight of OVX and DM-OVX groups compared to sham. Supplementation of E_2 could prevent the reduction in uterine weights. The uterine weight was also significantly lower in DM rats than shams but in a smaller magnitude compared to ovarian sex hormone-deficient groups. While a significant increase in both heart and body weights was demonstrated in OVX rats, a significant decrease in both heart and body weights was detected in DM rats. A decrease in body weight was still observed in the DM-OVX rats. Similarly, hypertrophy of the heart represented by an increased heart weight/body weight ratio was demonstrated in DM and DM-OVX groups. The decreased body weight and cardiac hypertrophy in DM-OVX rats could be prevented by either E_2 or INS supplementation but the coadministration of E_2 and INS.

Relationships of pCa-myofilament ATPase among the various groups were compared to evaluate the interactive effect of E_2 and INS deprivation on the cardiac myofilament response to Ca^{2+}. As shown in Figure 7C, maximum myofibrillar ATPase activity of OVX and DM rats were significantly depressed from that of shams to the same magnitude of suppression (22.7% and 32.9%, respectively). Maximum myofibrillar ATPase activity was also depressed in DM-OVX rat hearts to a similar degree (29.43%) and was completely restored upon supplementation with both E_2 and INS (Figure 7D). On the other hand, the leftward shift in the pCa-ATPase activity relations, representing an increase in the myofilament sensitivity to Ca^{2+} (reported as pCa_{50}), was only detected in OVX (pCa_{50} = 6.06 ± 0.02) but not in DM (pCa_{50} = 5.96 ± 0.02) groups when compared to sham (pCa_{50} = 5.93 ± 0.02) (Figure 8C).

The cardiac myofilament Ca^{2+} hypersensitivity detected in sex hormone-deficient rats was also observed in DM-OVX rats (pCa_{50} = 6.01 ± 0.02, Figure 8C) in which could be reversed upon E_2 or E_2 + INS supplementation (Figure 8D). Among all groups, there was no significant difference in the slope or Hill coefficient of the pCa-myofilament ATPase relationship (Appendix L). These results indicated that E_2 and INS affect the cardiac contractile activation partly through a common final pathway in the regulation of maximum myofilament ATPase activity. Moreover, it is the E_2 deficiency that induces an adaptive response of the myofilament to become more sensitive to Ca^{2+} activation even under diabetes complication.

TABLE 1 Body weight (BW), heart weight (HW), uterine weight (UW) and % heart weight/ body weight (% HW/BW)

GROUPS	BW (g)	HW (g)	UW (g)	% HW/BW
SHAM	270 ± 3	0.90 ± 0.01	0.41 ± 0.01	0.33 ± 0.02
OVX	340 ± 6 *	1.00 ± 0.02 *	0.09 ± 0.01 *	0.29 ± 0.01 *
DM	228 ± 9 *#	0.81 ± 0.01 *#	0.31 ± 0.01 *#	0.36 ± 0.02 *#
DM-OVX	246 ± 9 *#	0.92 ± 0.03 #	0.09 ± 0.00 *	0.37 ± 0.02 *#
DM-OVX + E_2	233 ± 5 *#	0.86 ± 0.02 *#	0.37 ± 0.03 #	0.37 ± 0.02 *#
DM-OVX + INS	355 ± 8 *	1.01 ± 0.01 *	0.09 ± 0.01 *	0.29 ± 0.01 *
DM-OVX + E_2 + INS	263 ± 4 #	0.90 ± 0.01 #	0.40 ± 0.01 #	0.34 ± 0.01 #

Values are means ± SE of 7 rats each group. SHAM, sham-operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic-ovariectomized; E_2, estrogen; INS, insulin. $P < 0.05$, significant difference from SHAM (*) and OVX (#) groups.

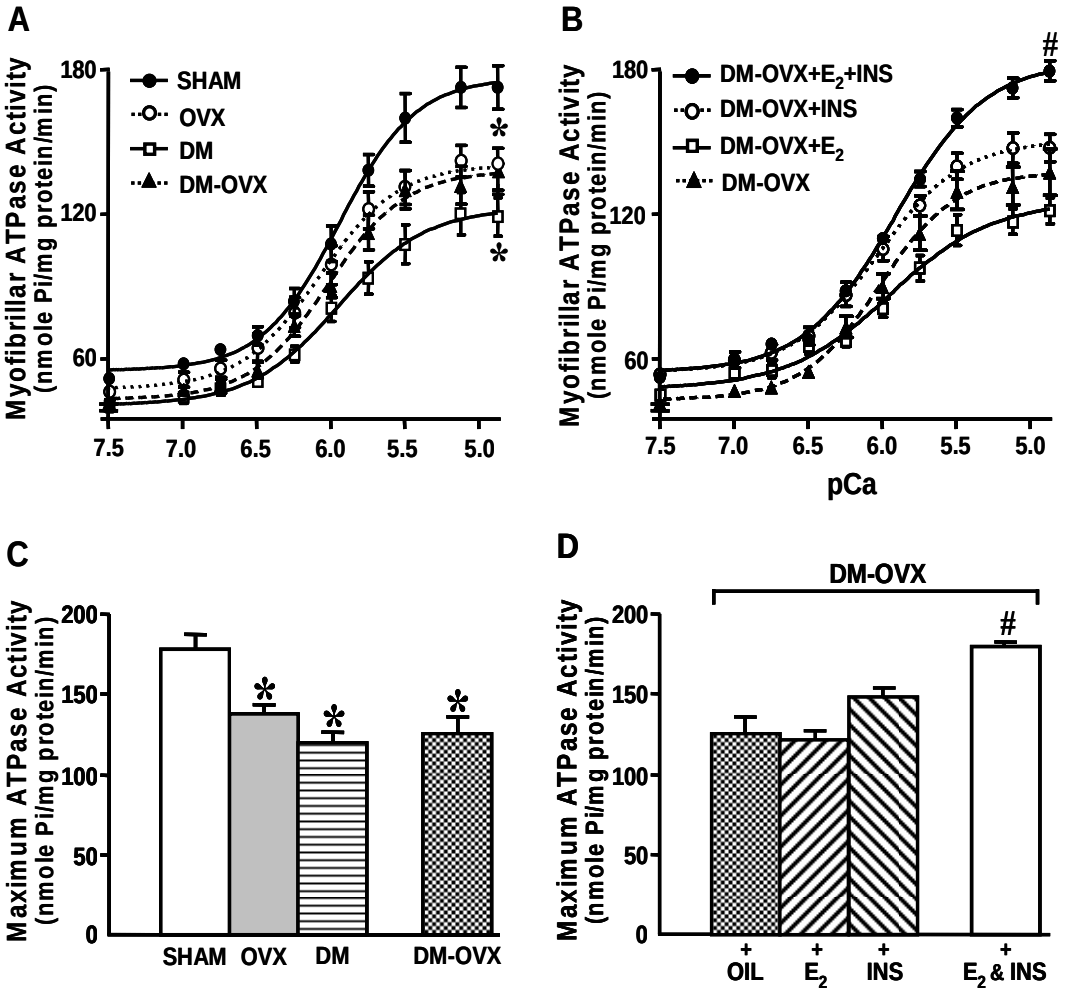


Figure 7 -log free Ca^{2+} concentration (pCa)-myofibrillar ATPase activity relation from sham (SHAM), ovariectomized (OVX), diabetic (DM) and diabetic-ovariectomized (DM-OVX) rats (A), and DM-OVX rats with estrogen (E_2) and/or insulin (INS) supplementation (B). Comparison of the maximum Ca^{2+}-dependent actomyosin Mg^{2+}-ATPase activities of cardiac myofibrillar preparations from SHAM, OVX, DM, DM-OVX rats (C) and DM-OVX rats with estrogen and/or insulin supplementation (D). Data are means ± SE from 6-10 preparations. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

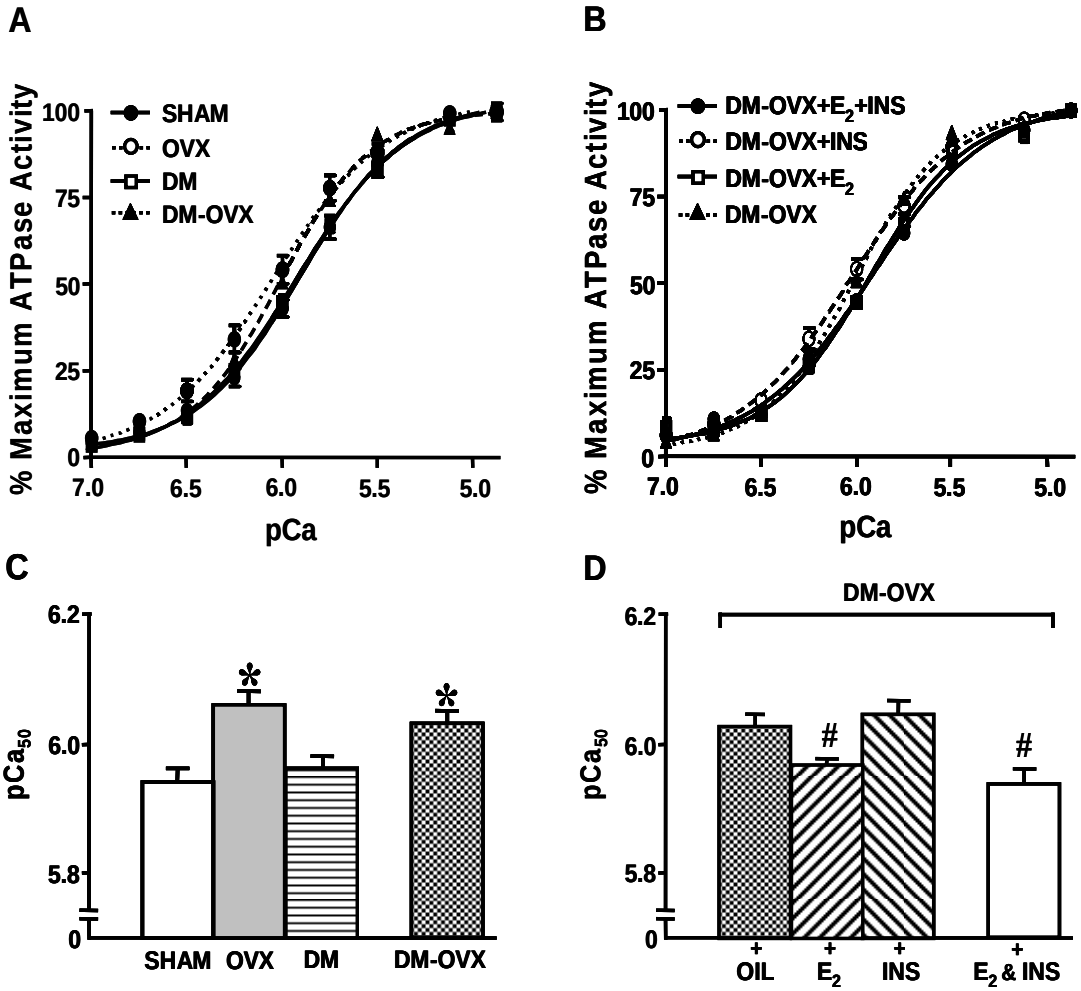


Figure 8 pCa-%maximum ATPase activity relation from SHAM, OVX, DM and DM-OVX rats (A) and DM-OVX rats with estrogen and/or insulin supplementation (B). Comparison of –log of the Ca^{2+} concentration producing half-maximal activation (pCa_{50}) from SHAM, OVX, DM, DM-OVX rats (C) and DM-OVX rats with estrogen and/or insulin supplementation (D). Data are means ± SE from 6-10 preparations. $P <$ 0.05, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

2. Interactive effect of estrogen and insulin deficiency on the cardiac β_1-adrenergic receptors and HSP 72.

Alteration in the regulatory effect of β_1-adrenergic receptors stimulation appears to play important roles in the cardiac myofilament response to Ca^{2+} (Bers, 2002). A significant upregulation of β_1-adrenergic receptors density only in OVX rat hearts (257 ± 7.6 fmole/mg protein) but not in DM rat hearts (216 ± 9.2 fmole/mg protein) was demonstrated when compared to sham (209 ± 4.3 fmole/mg protein) (Figure 9). The same magnitude of enhancement in the receptor density was also observed in DM-OVX rat hearts (245 ± 5.8 fmole/mg protein). Supplementation of E_2 but not INS could restore the upregulation of the receptor in DM-OVX groups. Results from Western blot analysis (Figure 10) also demonstrated a significant upregulation of the protein content in OVX (~48%) and DM-OVX rat hearts (~49%) but not in DM rats. The receptor upregulation in DM-OVX rats could be completely restored by E_2 supplementation. On the other hand, the binding affinity of β-adrenergic receptor was not different among the various experimental groups as summarized in Figure 11. Thus, these results indicated that upregulation of β_1-adrenergic receptor is specifically induced in ovarian sex hormone-deprived rat hearts even under DM complication. The close relation between the increased β_1-adrenergic receptor and the enhanced myofilament sensitivity to Ca^{2+} in OVX rat hearts implies that the alteration of β_1-adrenergic receptor may, in part, underlie changes in the cardiac myofilament response to Ca^{2+} after estrogen deprivation.

To further investigate whether changes in the myofilament response to Ca^{2+} were associated with loss in the cardioprotective effect through expression of HSP72, the amount of HSP72 was determined in various left ventricular homogenates and summarized in Figure 12. A significant decrease in HSP72 content was detected in OVX (~27%) and DM rat hearts (~25%) as compared to sham. Similar to OVX and DM rats, the expression of HSP72 in DM-OVX group was also suppressed to a comparable degree (~22%) compared to sham. Supplementation with E_2 or INS in DM-OVX group could not restore the decrease in HSP72 but the combined administration did. The loss in cardioprotective effect through the suppressed HSP72 expression in E_2- or INS-deficient rats paralleled the suppression of maximum

myofibrillar ATPase activity which then indicates a potential common target, HSP72, in the controlling process of the two hormones in regulating myofilament contractile activity.

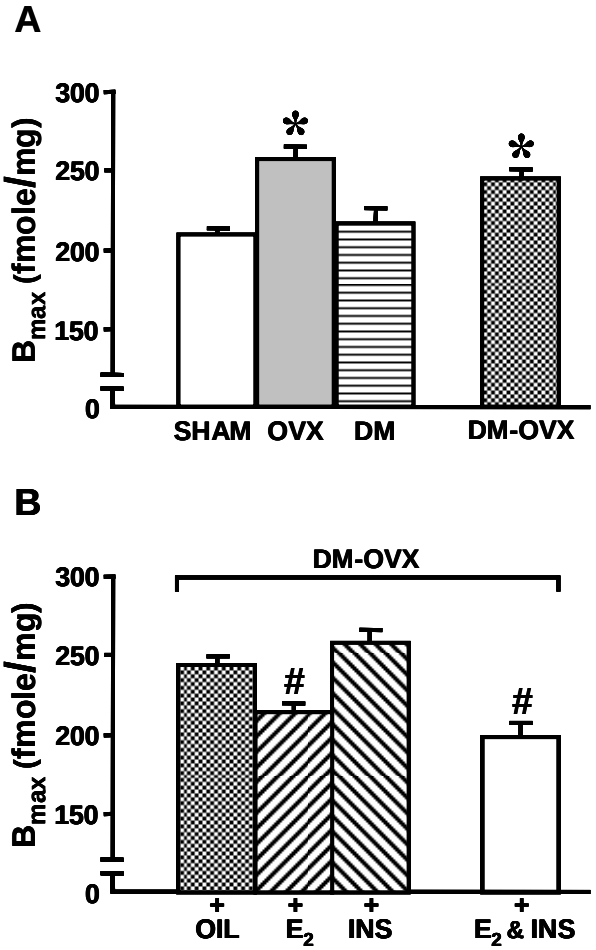


Figure 9 A: comparison of the density (B_{max}) of the cardiac β_1-adrenergic receptors (β_1-AR) in left ventricular membrane preparations from SHAM, OVX, DM and DM-OVX rats. B: comparison of the B_{max} of the cardiac β_1-AR in left ventricular membrane preparations from DM-OVX rats with estrogen and/or insulin supplementation. Data are means ± SE from 8 preparations. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

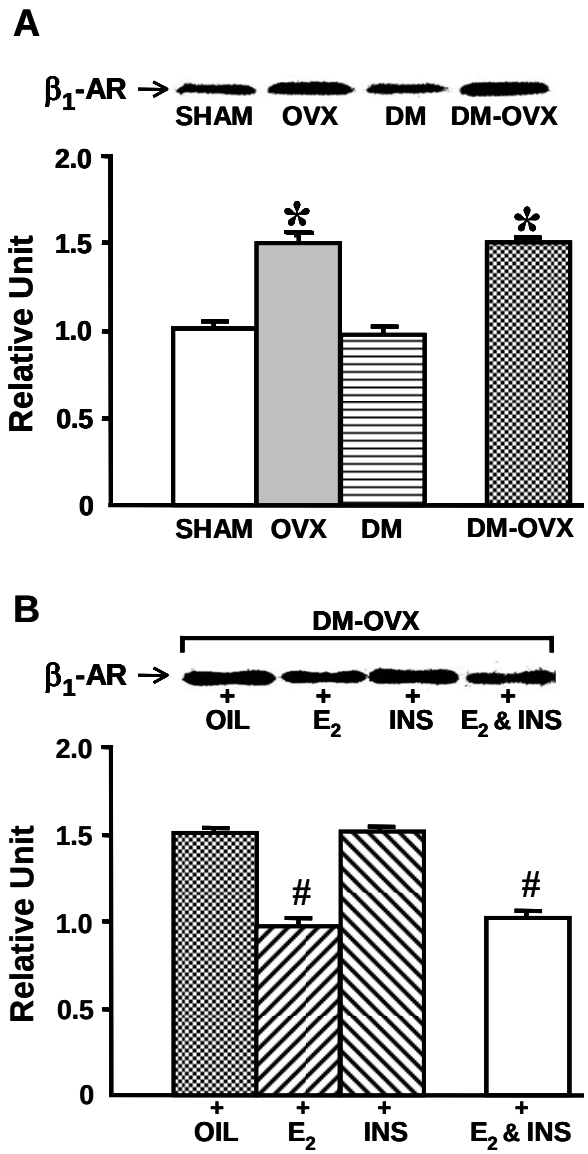


Figure 10 A: immunoblot analysis of β_1-AR proteins from left ventricular homogenates and comparison of the relative band intensity from SHAM, OVX, DM, and DM-OVX rats. B: immunoblot analysis of β_1-AR proteins and comparison of the relative band intensity from DM-OVX rats with estrogen and/or insulin supplementation. Data are means ± SE of 8 hearts. *$P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

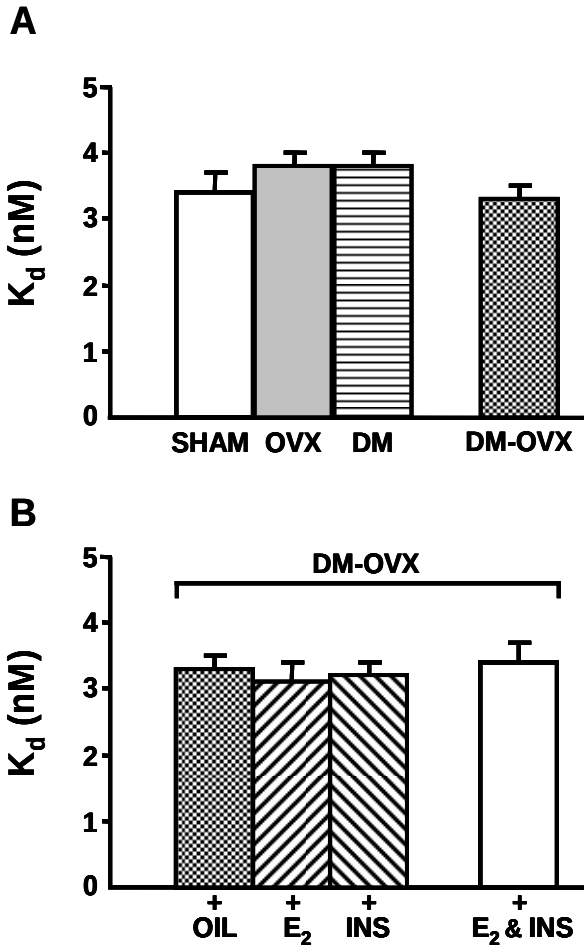


Figure 11 A: comparison of the dissociation constant (K_d) of cardiac β_1-AR in left ventricular membrane preparations from SHAM, OVX, DM, and DM-OVX rats. B: comparison of the K_d of β_1-AR in left ventricular membrane preparations from DM-OVX rats with estrogen and/or insulin supplementation. Data are means ± SE from 8 preparations.

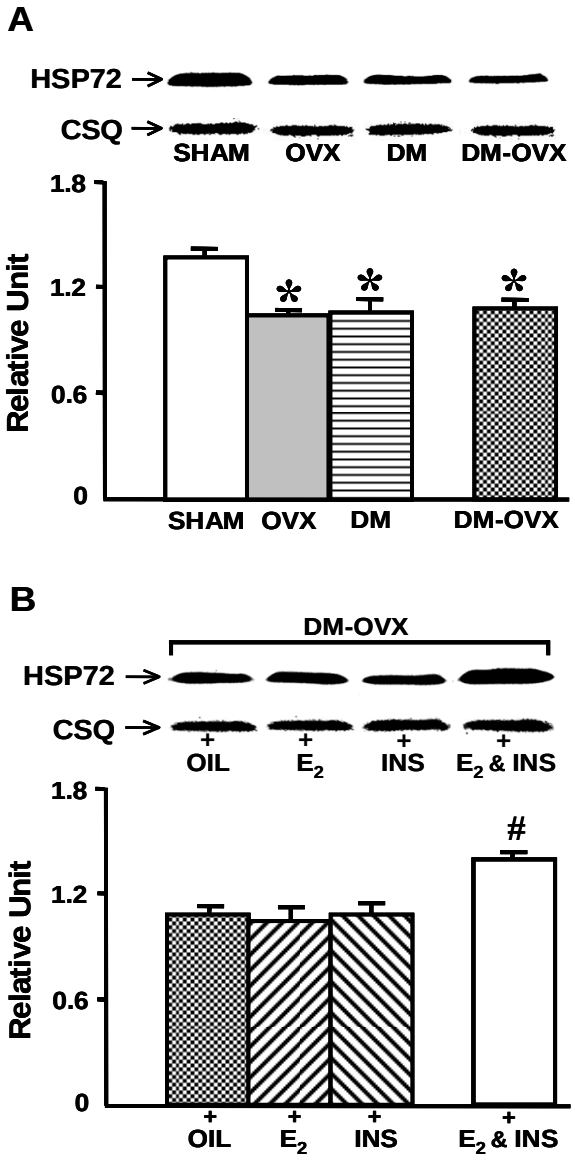


Figure 12 A: immunoblot analysis of heat shock protein (HSP) 72 and calsequestrin (CSQ) and comparison of the band intensity expressed as a ratio of HSP72:CSQ of left ventricular homogenates from SHAM, OVX, DM, and DM-OVX rats. B: immunoblot analysis of HSP72 and CSQ and comparison of the band intensity expressed as a ratio of HSP72:CSQ of left ventricular homogenates from DM-OVX rats with estrogen and/or insulin supplementation. Data are means ± SE from 8 preparations. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

3. Regulatory effect of estrogen on crossbridge cycling kinetics of the cardiac contractile filament.

3.1 Correlations of estrogen and insulin on the maximum myofilament ATPase activity and MHC isoform.

A significant decrease in uterine weights was clearly observed in OVX and DM-OVX rats in which could be completely restored by E_2 supplementation (Appendix M in Table IV). A significant reduction in uterine weights of DM rats was also observed in a smaller magnitude compared to OVX rats which could be completely restored by INS supplementation. Hypertrophy of the heart was still demonstrated in DM and DM-OVX rats which could be prevented by INS supplementation and coadministration of E_2 and INS, respectively. While the plasma T_3 level was not changed in OVX rats, hypothyroid was induced in DM and DM-OVX rats, which could be completely restored by INS supplementation.

Maximum myofilament ATPase activity and % α-MHC isoform were measured and summarized in Figure 13 and 14, respectively. As shown in Figure 13, the same degree of suppression in maximum ATPase activity demonstrated in the hearts of OVX (29%) and DM (30%) rats was also observed in DM-OVX rats (29%) compared to sham. The suppression in maximum ATPase activity was prevented by E_2 and INS supplementation in OVX and DM rats, respectively, but only by coadministration of E_2 and INS in DM-OVX rats. On the other hand, different degrees of suppression in the relative α-MHC isoform expression in the heart of OVX (~19%) and DM (~36%) rats was detected as compared to sham and completely reversed upon supplementation with E_2 and INS, respectively (Figure 14A). The pronounced suppression of the relative α-MHC isoform expression detected in DM rat hearts was also detected in the hearts of DM-OVX (~34%) rats (Figure 14B). Similar to myofibrillar ATPase activity, the isoform shift in MHC could be prevented by E_2 and INS in OVX and DM rats, respectively, whereas coadministration of E_2 and INS was needed for complete prevention in DM-OVX rats (Figure 14). These results indicated differential effects of E_2 and INS in regulating maximum myofibrillar ATPase activity.

To further prove the different effects of the E_2 and INS on CB cycling kinetics, relationships of maximum ATPase activity and % α-MHC in experimental groups was

plotted. The linear regression analysis of data from sham to that of individual experimental group was fitted to determine the direct effect of α-MHC isoform on CB cycling kinetics. As shown in Figure 15A, the different linear regression lines in OVX (dash line, slope = 2.60 ± 0.16) or DM (solid line, slope = 1.49 ± 0.09) to sham were observed. Regression analysis fitting data of OVX+E_2 to that of OVX group gave rise to the same slope as that of OVX and SHAM (Figure 15B). Similarly, regression analysis of DM+INS data from DM group was the same as that of DM and SHAM groups. Interestingly, a similar slope of linear regression line from DM-OVX and SHAM (semi-dash line, slope = 1.55 ± 0.08) to that of DM and SHAM group was detected (Figure 15C). As expected, regression analysis from data of DM-OVX and DM-OVX+E_2+INS groups was similar to that of DM and DM+INS groups. These results indicated that E_2 and INS regulated the CB cycling kinetics partly through expression of α-MHC isoform, whereas the different linear relations from DM and OVX rat hearts indicated the different regulatory effects of E_2 and INS on CB cycling kinetics.

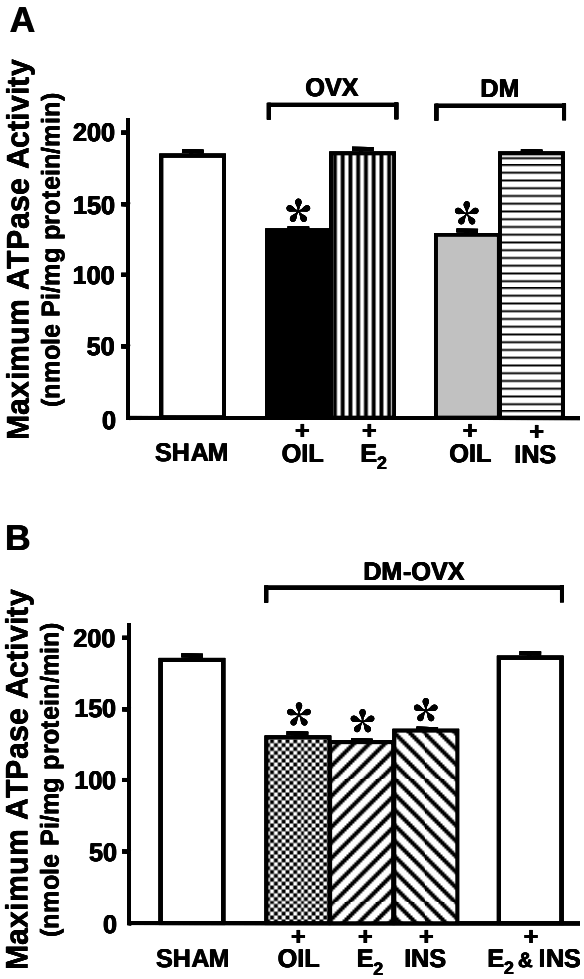


Figure 13 Comparison of maximum myofilament ATPase activity from SHAM, OVX rats with and without estrogen supplementation, DM rats with and without insulin supplementation (A), and DM-OVX rats with estrogen and/or insulin supplementation (B). Data are means ± SE from 10-12 preparations. *$P < 0.05$ = significantly different from SHAM, using Student Newman-Keuls test after ANOVA.

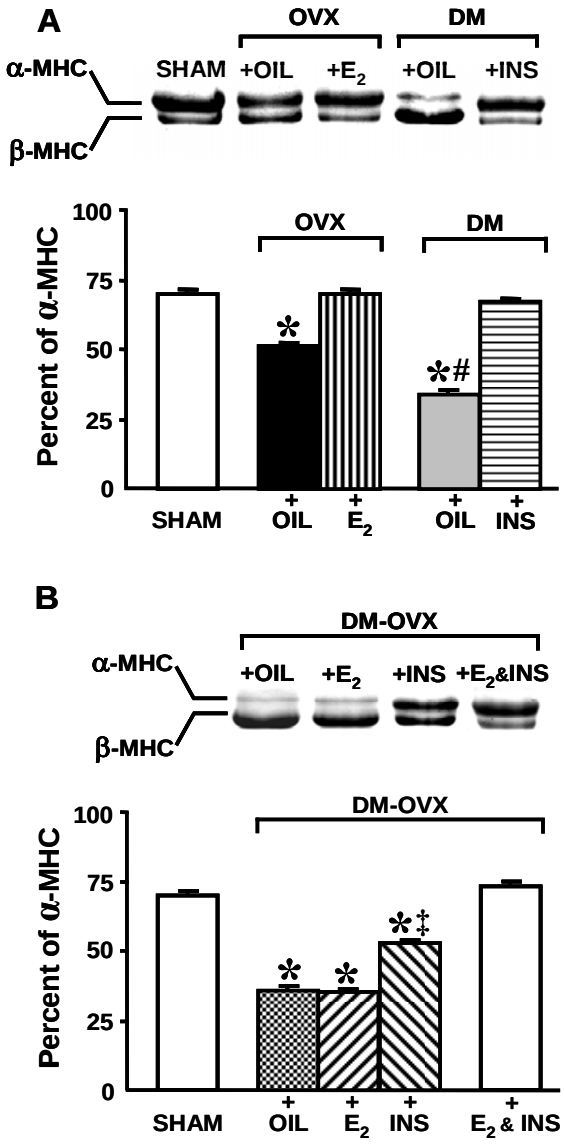


Figure 14 Myosin heavy chain (MHC) region on SDS gels and the relative amount of α-MHC (as a percentage of total MHC) of left ventricular papillary muscle from SHAM, OVX rats with and without estrogen supplementation, DM rats with and without insulin supplementation (A), and DM-OVX rats with estrogen and/or insulin supplementation (B). Data are means ± SE from 10-12 preparations. $P < 0.05$, significant difference from SHAM (*), OVX (#) and DM-OVX (‡) groups, respectively, using Student Newman-Keuls test after ANOVA.

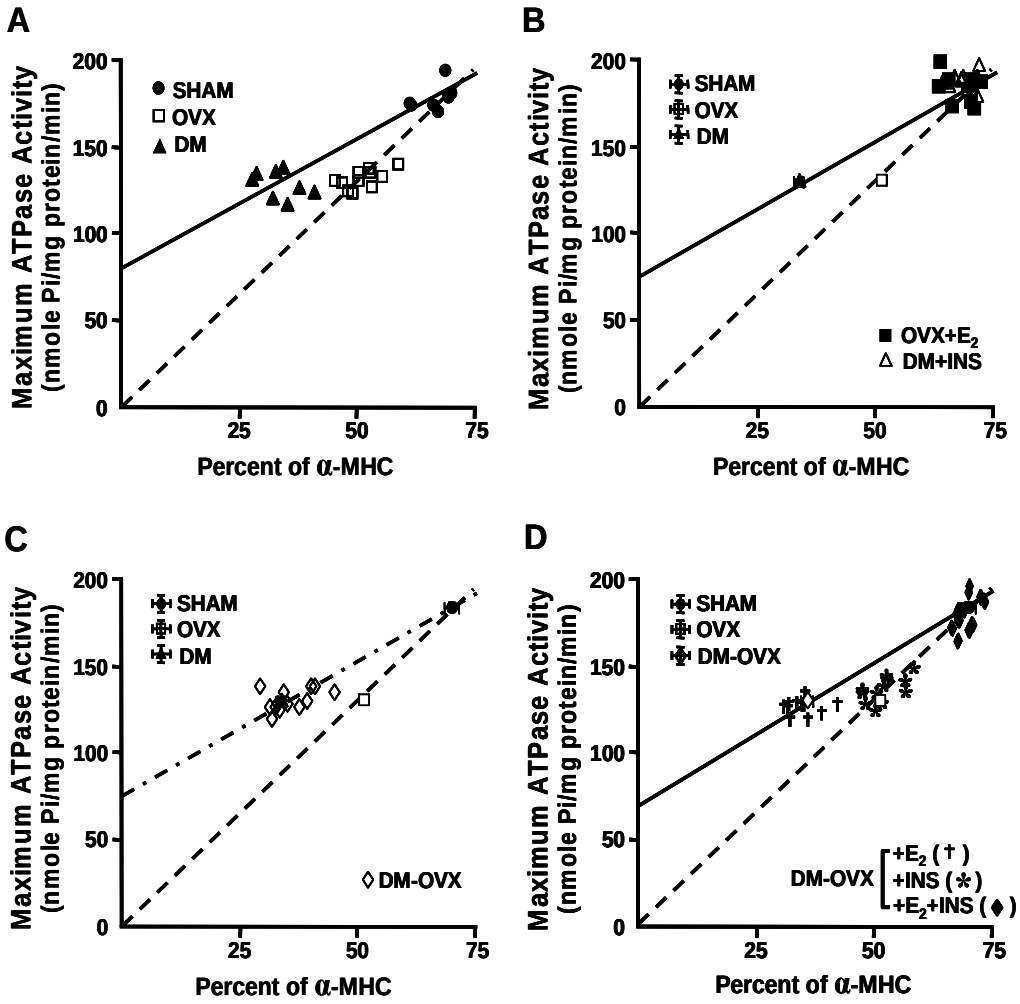


Figure 15 Relationships between maximum Ca^{2+}-dependent actomyosin MgATPase activity and % α-MHC isoform from (A) SHAM, OVX, and DM rats with linear plot of combined data of SHAM with OVX (dash line) or DM (solid line) group, (B) from OVX+E_2 and DM+INS, (C) from DM-OVX group with linear plot of combined data with SHAM (semi-dash line), (D) from DM-OVX rats supplemented with E_2, INS and E_2+INS with linear plot of combined data of DM-OVX+E_2 (solid line), and DM-OVX+INS (dash line) with SHAM. Data are means ± SE from 10-12 preparations.

3.2 Shifts in MHC isoforms determined the sole changes in CB cycling kinetics in the heart of INS- but not E_2-deficient rats.

To further specify the underlying mechanism of E_2 and INS in regulating cardiac CB cycling kinetics, different levels of α-MHC expression were then varied by inducing different plasma thyroid concentrations. General characteristics of the experimental rats were summarized in Appendix M, Table VII. As expected, significant reductions in plasma T_3 level were observed in OVX, DM and DM-OVX rats after PTU treatment. On the other hand, T_3 treatment induced euthyroid in DM and DM-OVX rats, but hyperthyroid in OVX rats. There was no effect of T_3 or PTU on the uterine weight of any groups. Hypertrophy of the heart in DM and DM-OVX rats was enhanced after the treatment of T_3. Moreover, the cardiac hypertrophy in OVX group was induced after T_3 treatment but not in PTU treatment.

As expected, maximum myofibrillar ATPase activity and % α-MHC were further suppressed in the hypothyroid rat hearts of every group (Figure 16). On the other hand, euthyroid induction in DM and DM-OVX rats by T_3 injection completely reversed the suppressed maximum ATPase activity and the reduced % α-MHC expression. A linear relationship in both DM (slope = 1.46 ± 0.05) and DM-OVX (slope = 1.46 ± 0.06) groups with different thyroid levels (Figure 17A and 17B, respectively) was demonstrated to have the same slope value as those previous regression of DM vs SHAM (slope = 1.49 ± 0.09) and DM-OVX vs SHAM (slope = 1.55 ± 0.08) (Figure 15). Similarly, significant increases in maximum myofibrillar ATPase activity and % α-MHC of OVX rat were observed after T_3 treatment. However, the correlation of maximum ATPase activity to % α-MHC expression at different thyroid status of OVX rats demonstrated a concave shape relationship fitted by the second degree polynomial equation (Figure 17C). These results indicated that E_2, in contrast to INS, plays an additional regulatory role on the CB cycling kinetics through changes in the myofilament protein besides its effect on the shift expression of MHC isoform. The results also indicated that insulin indirectly regulates the CB cycling kinetics through the direct effect of thyroid hormones on α-MHC expression.

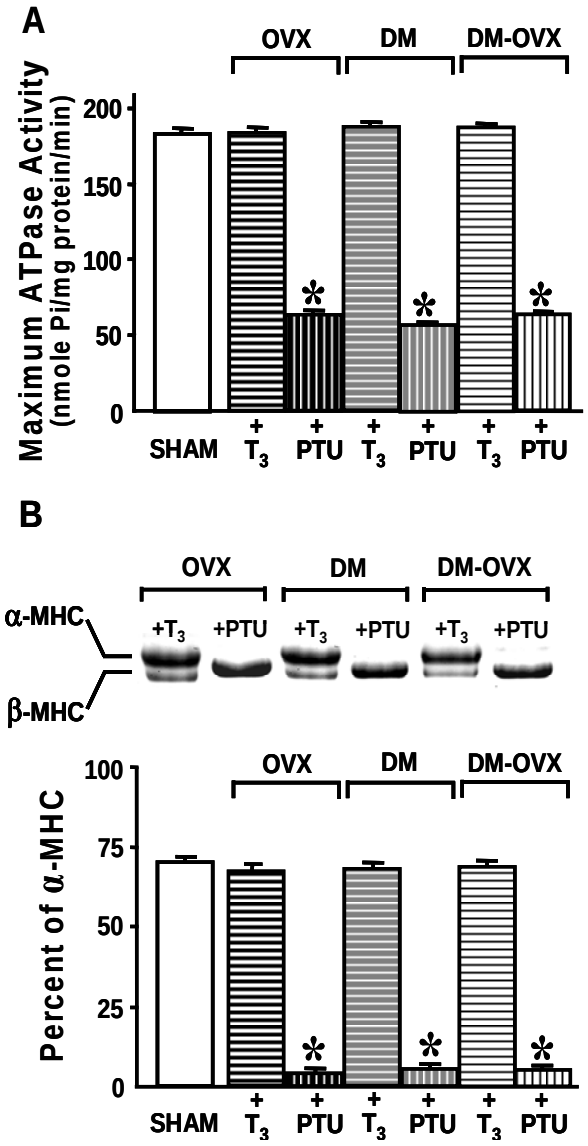


Figure 16 Comparison of maximum myofilament ATPase activity (A) and MHC region on SDS gels and the relative amount of α-MHC (as a percentage of total MHC) of left ventricular papillary (B) from SHAM, OVX, DM and DM-OVX rats with triiodothyronine (T_3) or propyl-2 thiouracil (PTU) treatment. Data are means ± SE from 12 preparations. *$P < 0.05$ = significantly different from SHAM, using Student Newman-Keuls test after ANOVA.

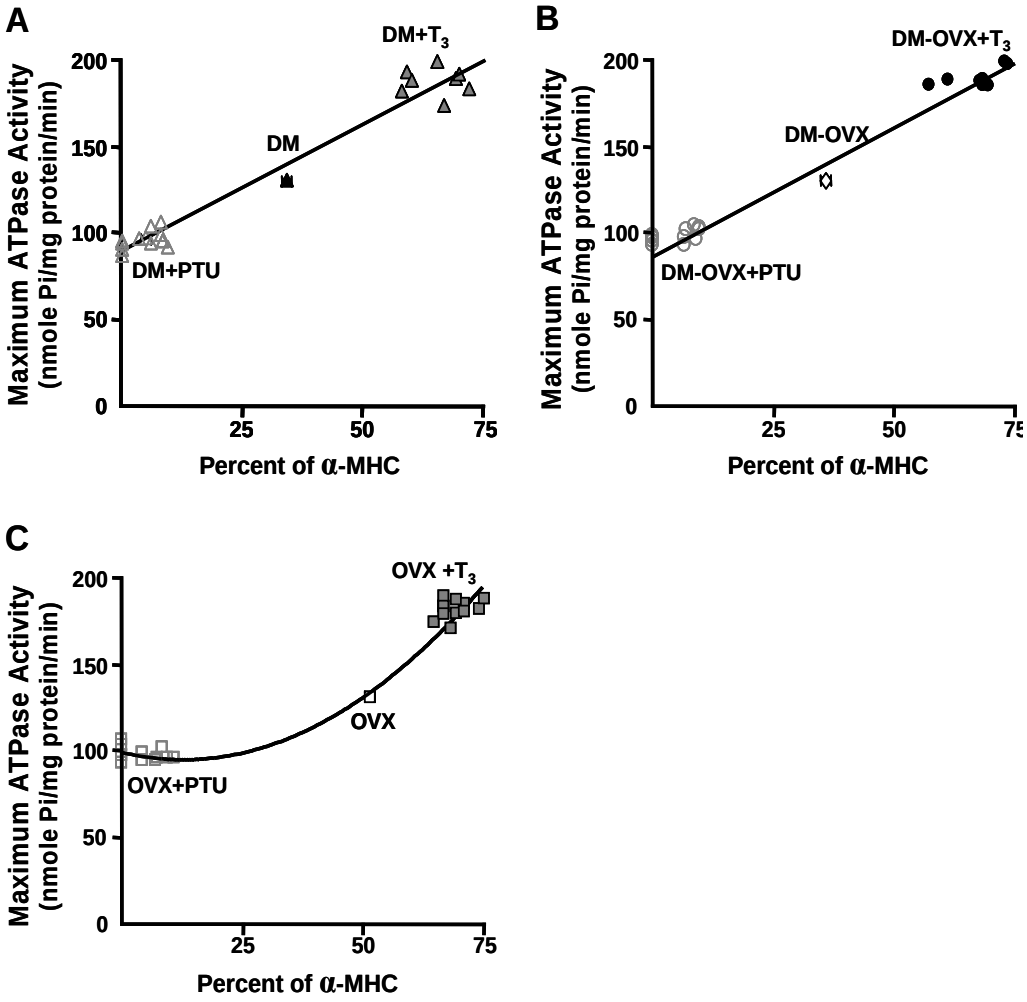


Figure 17 Relationship between maximum Ca^{2+}-dependent actomyosin MgATPase activity and % α-MHC isoform in DM (A), DM-OVX (B) and OVX (C) groups with triiodothyronine or propyl-2 thiouracil treatment. Data are means ± SE from 12-17 preparations.

4. Interactive effect of estrogen and insulin deficiency in the intracellular calcium handling in cardiac myocytes by SR.

As expected, significant decrease in uterine weights was observed in OVX and DM-OVX rats in which could be completely restored by E_2 supplementation (Appendix M in Table X). A significant reduction in uterine weight of DM rats was also observed in a smaller magnitude compared to OVX rats which could be completely restored by INS supplementation. Hypertrophy of the heart was still demonstrated in DM and DM-OVX rats which could be prevented by INS and coadministration of both E_2 and INS supplementation, respectively.

The SR Ca^{2+}-uptake activity was measured from left ventricular homogenates at various free Ca^{2+} concentrations. As shown in Figure 18B, maximum SR Ca^{2+}-uptake activity of OVX (56.3 nmole/mg protein/min) and DM (55.4 nmole/mg protein/min) rats were significantly lower than that of sham (80.9 nmole/mg protein/min) with the same magnitude of suppression (30.1% and 32.1%, respectively). Maximum SR Ca^{2+}- uptake activity was also reduced in DM-OVX rats (54.7 nmole/mg protein/min, 32.1%) without additive suppression when compared to OVX and DM rats. The suppression of SR Ca^{2+}-uptake activity in DM-OVX and DM rats was completely restored by supplementation of E_2 and INS, and INS, respectively (Figure 18D). In contrast, the SR Ca^{2+}-uptake sensitivity was increased in OVX (ECa_{50} = 0.48 ± 0.01) and DM (ECa_{50} = 0.50 ± 0.02) rats compared to sham (ECa_{50} = 0.66 ± 0.02) (Figure 19B). The increased SR Ca^{2+}-uptake sensitivity was also observed in DM-OVX rats (ECa_{50} = 0.48 ± 0.01). The increased SR Ca^{2+}-uptake sensitivity could be prevented by supplementation of INS and coadministration of E_2 and INS in DM and DM-OVX groups, respectively (Figure 19D). Despite changes in the maximum SR Ca^{2+}-uptake rate and SR Ca^{2+}-uptake sensitivity in OVX rat hearts, the Hill slope (Appendix L) was not different among the experimental groups. These results indicate no dominant effect of E_2 deficiency on the SR Ca^{2+}-uptake function. Moreover, E_2 and INS may interactively affect the cardiac SR Ca^{2+}-uptake function.

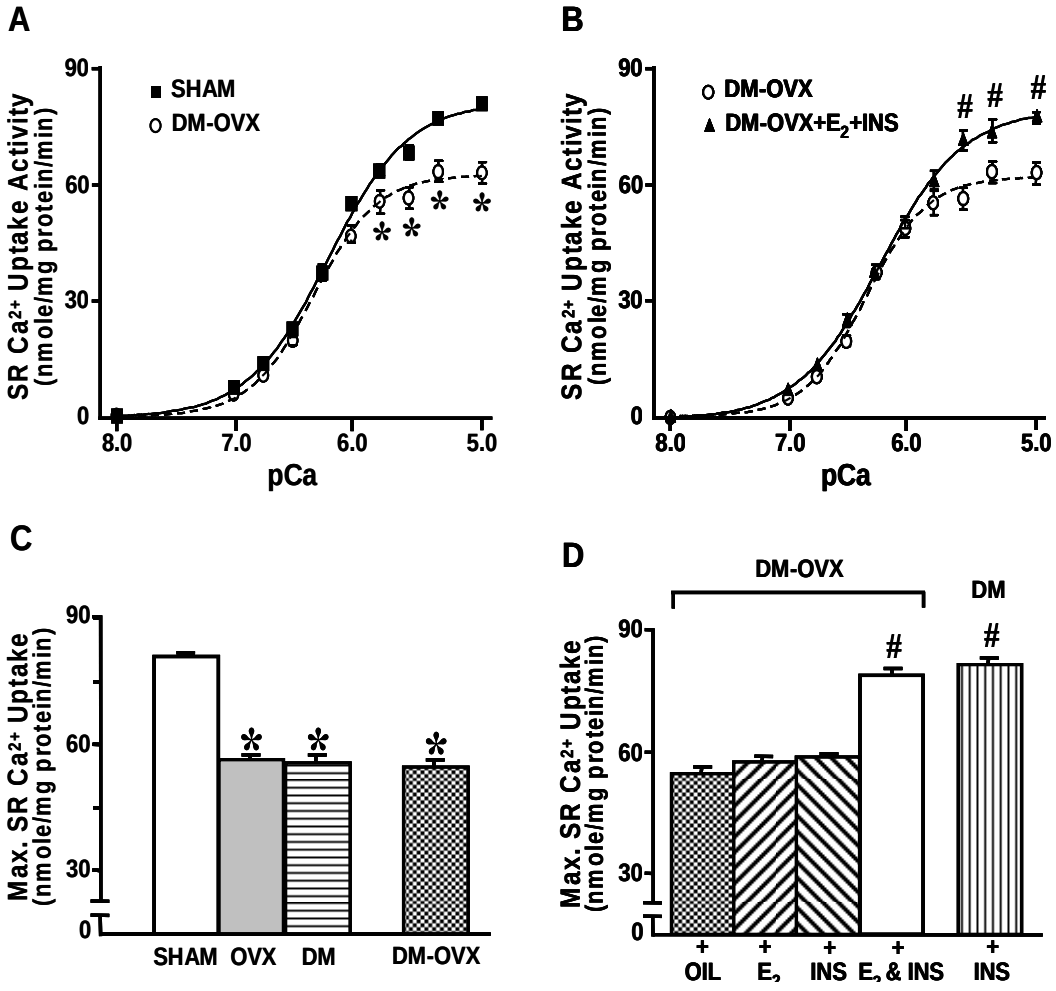


Figure 18 pCa-SR Ca^{2+}-uptake activity relation of left ventricular homogenates from SHAM and DM-OVX rats (A) and DM-OVX rats with and without estrogen and/or insulin supplementation (B). Comparison of maximum SR Ca^{2+}-uptake activity from SHAM, OVX, DM, and DM-OVX rats (C) and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (D). Data are means ± SE from 12 preparations. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

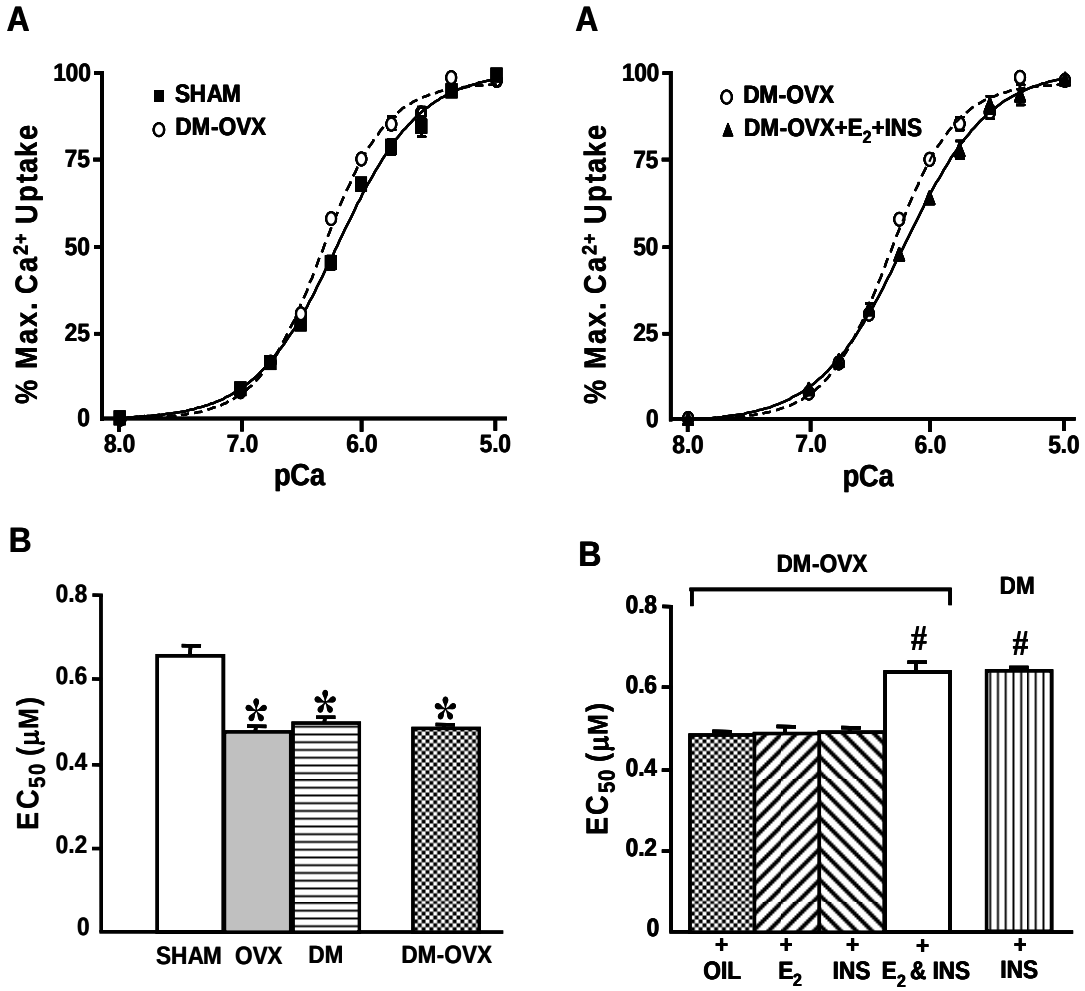


Figure 19 pCa-%maximum SR Ca^{2+}-uptake activity relation of left ventricular homogenates from SHAM and DM-OVX rats (A) and DM-OVX rats with and without estrogen and/or insulin supplementation (B). Comparison of Ca^{2+} concentration producing half-maximal activation (EC_{50}) from SHAM, OVX, DM, and DM-OVX rats (C) and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (D). Data are means ± SE from 12 preparations. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

To further specify the underlying mechanism of changes in the SR Ca^{2+}-uptake activity, SR Ca^{2+} ATPase or SERCA activity was then determined using cardiac SR membrane vesicles. Similar to those changes in the SR Ca^{2+}-uptake activity, maximum SERCA activity of OVX (0.92 ± 0.04 μmole Pi/mg protein/min) and DM (0.93 ± 0.03 μmole Pi/mg protein/min) rats were significantly depressed from sham (1.11 ± 0.04 μmole Pi/mg protein/min) to the same magnitude of suppression (31.3% and 30.6%, respectively, Figure 20B). Maximum SERCA activity was also depressed in DM-OVX rats (0.90 ± 0.02 μmole Pi/mg protein/min, 32.8%) with no additive suppression when compared to OVX and DM rats and was completely restored upon supplementation with both E_2 and INS (Figure 20D). Supplementation of INS in DM rats could prevent the suppression of maximum SERCA activity. The sensitivity of SERCA to Ca^{2+} activation was also increased in OVX (ECa_{50} = 0.74 ± 0.08) and in DM (ECa_{50} = 0.76 ± 0.10) rat hearts when compared to sham (ECa_{50} = 1.44 ± 0.12) (Figure 21B). The increased SERCA sensitivity was also observed in DM-OVX group (ECa_{50} = 0.73 ± 0.07) with the same degree as that detected in OVX and DM rats. The increase in SERCA sensitivity in DM and DM-OVX groups could be reversed by supplementation with INS and coadministration of E_2 and INS, respectively (Figure 21D). There was no difference in the Hill coefficients of pCa-SERCA activity relationship among the experimental groups. The similar change in the SR Ca^{2+}-uptake and SERCA activities thus suggested that changes in the SERCA activity may account for changes in the SR Ca^{2+}-uptake function of E_2- and INS-deficient rat hearts.

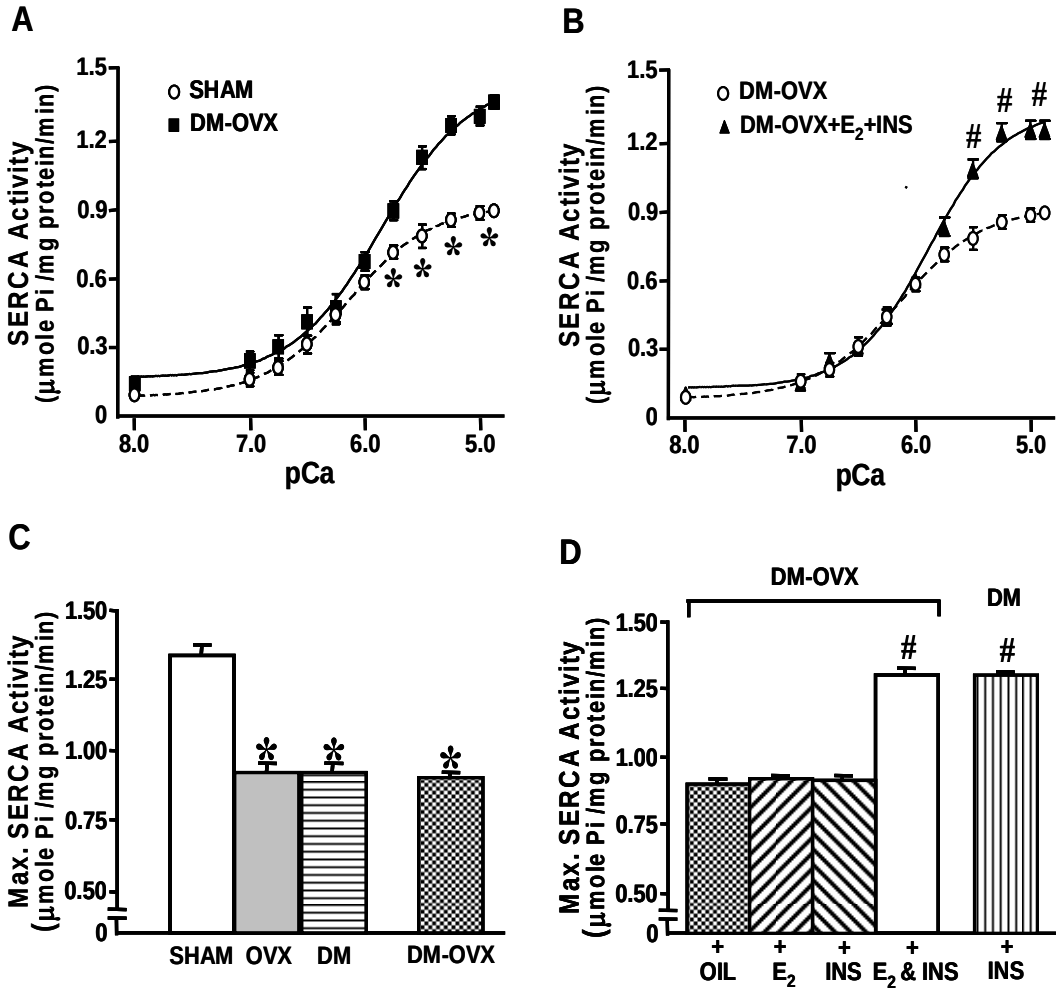


Figure 20 pCa- SR Ca^{2+}-ATPase (SERCA) activity relation of SR membrane vesicles from SHAM and DM-OVX rats (A) and DM-OVX rats with and without estrogen and/or insulin supplementation (B). Comparison of maximum SERCA activity from SHAM, OVX, DM, and DM-OVX rats (C) and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (D). Data are means ± SE from 12 preparations. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

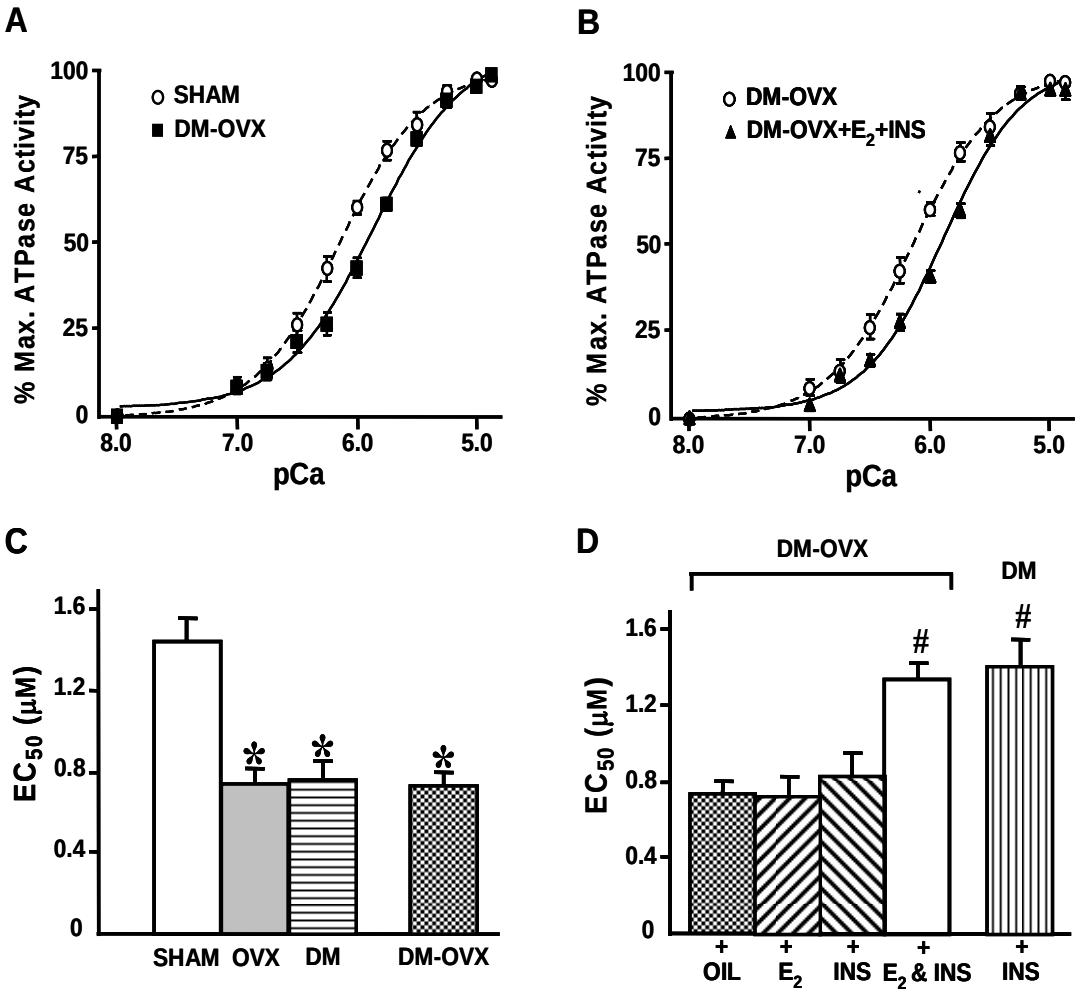


Figure 21 pCa-%maximum SERCA activity relation of SR membrane vesicles from SHAM and DM-OVX rats (A) and DM-OVX rats with and without estrogen and/or insulin supplementation (B). Comparison of Ca^{2+} concentration producing half-maximal activation (EC_{50}) from SHAM, OVX, DM, and DM-OVX rats (C) and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (D). Data are means ± SE from 12 preparations. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

Role of E_2 and INS in regulating the cardiac SR Ca^{2+}-uptake activity was further analyzed on both qualitative and quantitative mechanistic changes in SERCA proteins. Effects of E_2 and INS deficiencies on the expression of both the SERCA and the SR Ca^{2+}-uptake associated proteins were then evaluated. Figure 22A shows results of immunoblot analysis of SERCA, phospholamban, and calsequestrin (CSQ) in sham, OVX, DM, and DM-OVX rats. The protein bands of CSQ were used as loading control. Relative amounts of SERCA and phospholamban to CSQ in the experimental rats were summarized in figure 22C and 22E, respectively. As shown in figure 22C, the amount of SERCA proteins in OVX and DM rats was significantly lower (24.34% and 24.32%, respectively) than that of sham. Downregulation of SERCA proteins was also observed in DM-OVX group (24.34%) without additive suppression when compared to OVX and DM groups. Based on the inhibitory effect of phospholamban on the SERCA activity, changes in the level and/or the phosphorylated/ dephosphorylated state of phospholamban could affect the SERCA activity. In contrast to SERCA expression, upregulation of phospholamban protein was detected in DM (150%) and DM-OVX (153.8%) rat hearts but not in OVX rat (Figure 22E). As a result, the SERCA to phospholamban ratio, which indicates a greater inhibitory effect of phospholamban on the SERCA activity, decreased in every group from sham with a higher magnitude of reduction in DM (48.6%) and DM-OVX (50.5%) groups than that in OVX (22.4%) rat (Figure 22G). All these changes detected in DM and DM-OVX rat hearts could be reversed after INS supplementation and E_2 and INS coadministration, respectively (Figure 22D, 22F and 22H). These results indicated that the suppression of maximum SR Ca^{2+}-uptake activity in E_2- and INS-deprived rat hearts was due to the decreased expression of SERCA in accompany with the greater inhibitory effect of phospholamban.

To further elucidate the modulating effect of phospholamban on the responsiveness of SERCA to Ca^{2+} in E_2- and INS-deficient rat hearts, the level of the phosphorylated form of phospholamban at either Ser^{16} or Thr^{17} site was analyzed. Actin bands on SDS-PAGE were used as loading control and the relative amount of the phospho-Ser^{16} phospholamban and the phospho-Thr^{17} phospholamban were summarized in Figure 23 and 24, respectively. Downregulation of the phospho-Ser^{16} phospholamban was detected in DM (28.8 %) and DM-OVX (29.7%) groups (Figure

23A) in which could be reversed by INS supplementation (Figure 23B). Despite the unchanged phospho-Ser^{16} phospholamban, a significant downregulation of the phospho-Thr^{17} phospholamban was detected in OVX rats (32.2%) (Figure 24A). Downregulation of the phospho-Thr^{17} phospholamban was also observed in DM (32.2%) and DM-OVX (33.0%) groups in the same degree as that detected in OVX group. Downregulation of the phospho-Thr^{17} phospholamban in DM and DM-OVX rats could be abolished by supplementation of INS and coadministration of E_2 and INS, respectively (Figure 24B). These result indicated that changes in the SERCA response to Ca^{2+} after E_2 and INS deficiency were not modulated by alterations in phospholamban phosphorylation. Therefore, the increase in Ca^{2+} sensitivity of SERCA in E_2 and INS deficient groups might be due to other mechanisms.

Besides changes in the phosphorylation level of phospholamban, changes in the structural stoichiometry of phospholamban between the monomer, an active inhibitory form, and the pentamer, an inactive inhibitory form, of phospholamban could also differentially affect the SERCA sensitivity. Immunoblot analysis of the monomeric and pentameric forms of PLB and the proportion of the monomeric form to the total amount of phospholamban were shown in Figure 25. An increase in the proportion of the monomeric phospholamban was observed in OVX rats (19.8 ± 1.3%) from sham. The increased monomeric phospholamban was also detected in DM (26.7 ± 1.4%) and DM-OVX (27.5 ± 1.1%) groups in a higher magnitude than that of OVX rat and could be normalized by INS supplementation and E_2 and INS coadministration, respectively. The increased proportion of the monomeric form of phospholamban in DM, DM-OVX and OVX groups was paralleled to the reduction in the phosphorylation level of Thr^{17}-phospholamban and/or Ser^{16}-phospholamban, respectively. These similar changes indicated an involvement of the phosphorylation status of phospholamban to shift the equilibrium from monomer to pentamer. These data confirm that the Ca^{2+} hypersensitivity of SERCA activity induced by E_2 or INS deficiency was not related to change in the quantity or the activity of phospholamban.

Taken together, these results clearly indicated an interactive effect of estrogen and insulin in regulating the SR Ca^{2+} uptake function through both the expression of SERCA proteins and the interaction of SERCA to its regulatory protein.

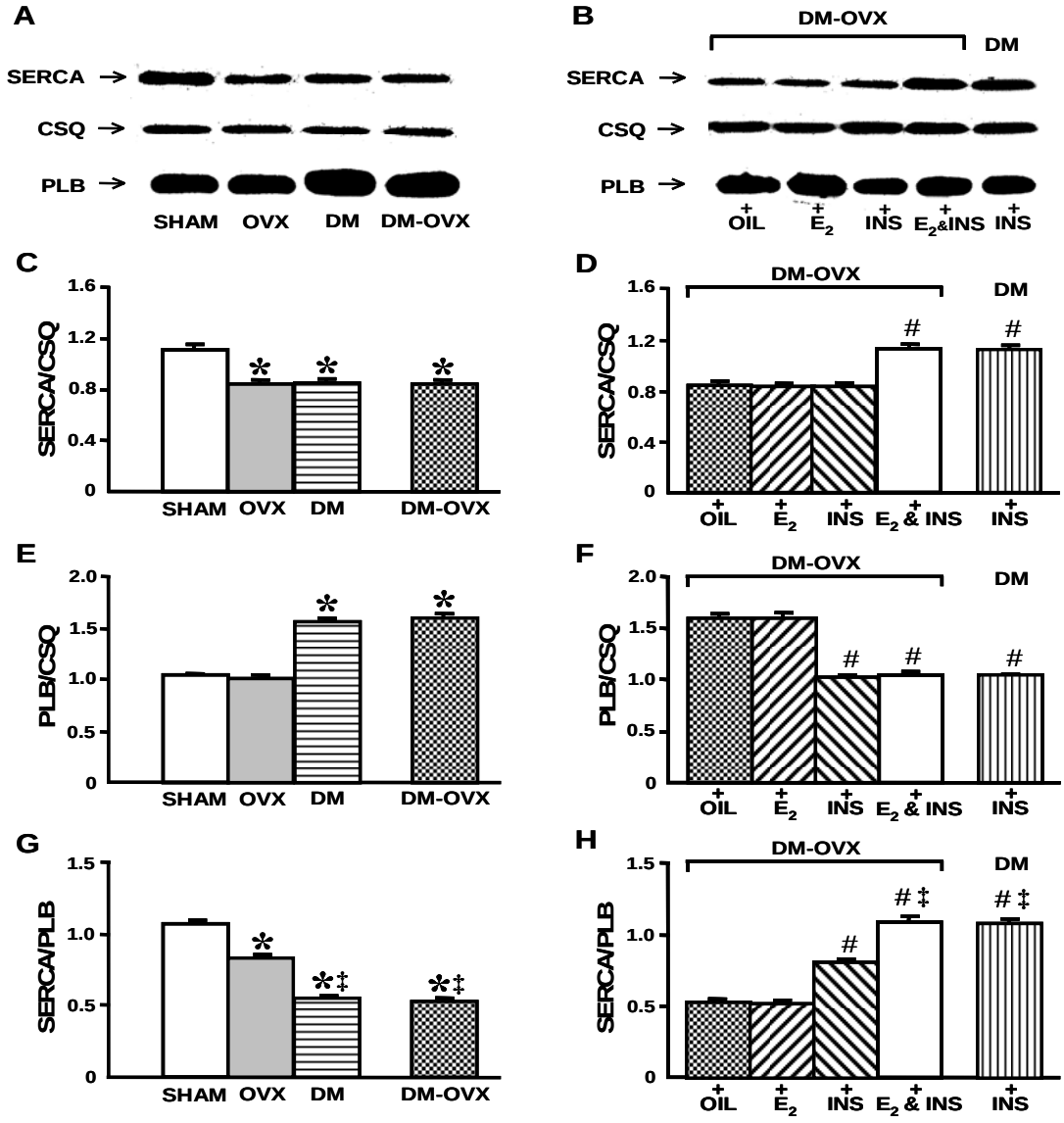


Figure 22 Immunoblot analyses of SERCA, calsequestrin (CSQ), and phospholamban (PLB) (A and B) and comparison of the band intensity expressed as a ratio of SERCA to CSQ (C and D), PLB to CSQ (E and F), and SERCA to PLB (G and H) of left ventricular homogenates from SHAM, OVX, DM, and DM-OVX rats and from DM-OVX rats with estrogen and/or insulin supplementation, and DM rat with insulin supplementation, respectively. Data are means ± SE from 6 hearts. $P < 0.05$, significant difference from SHAM (*), OVX (‡), and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

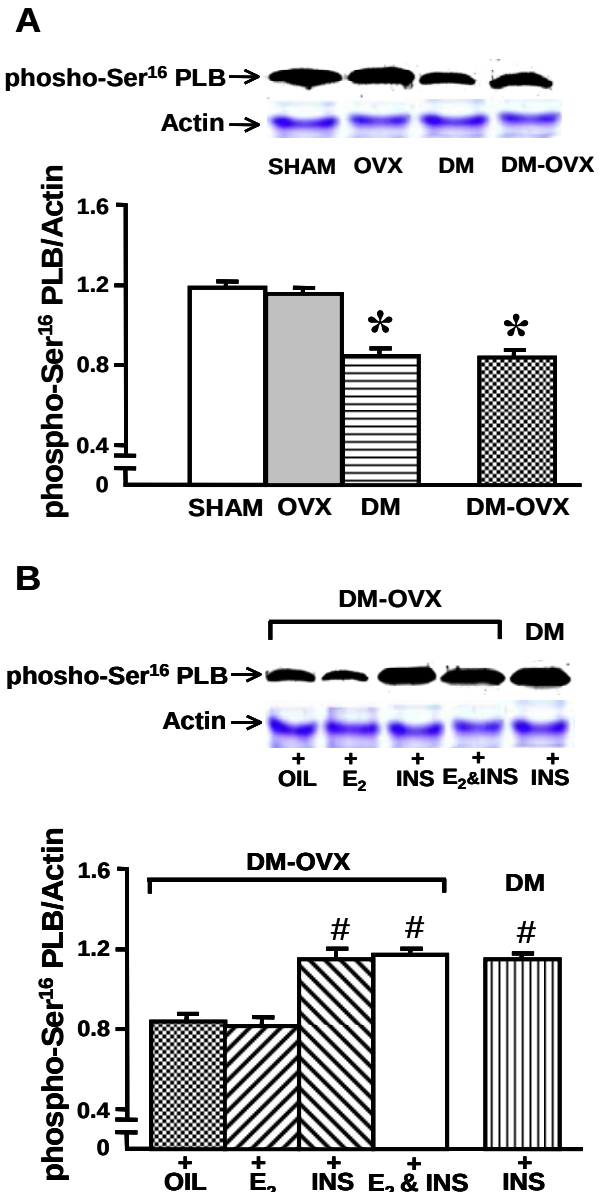


Figure 23 Immunoblot analysis of phosphorylated Serine-16 (phosphor-Ser^{16}) and actin and comparison of the band intensity expressed as a ratio of phosphor-Ser^{16}:actin of left ventricular homogenates from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (B). Data are means ± SE from 6 hearts. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

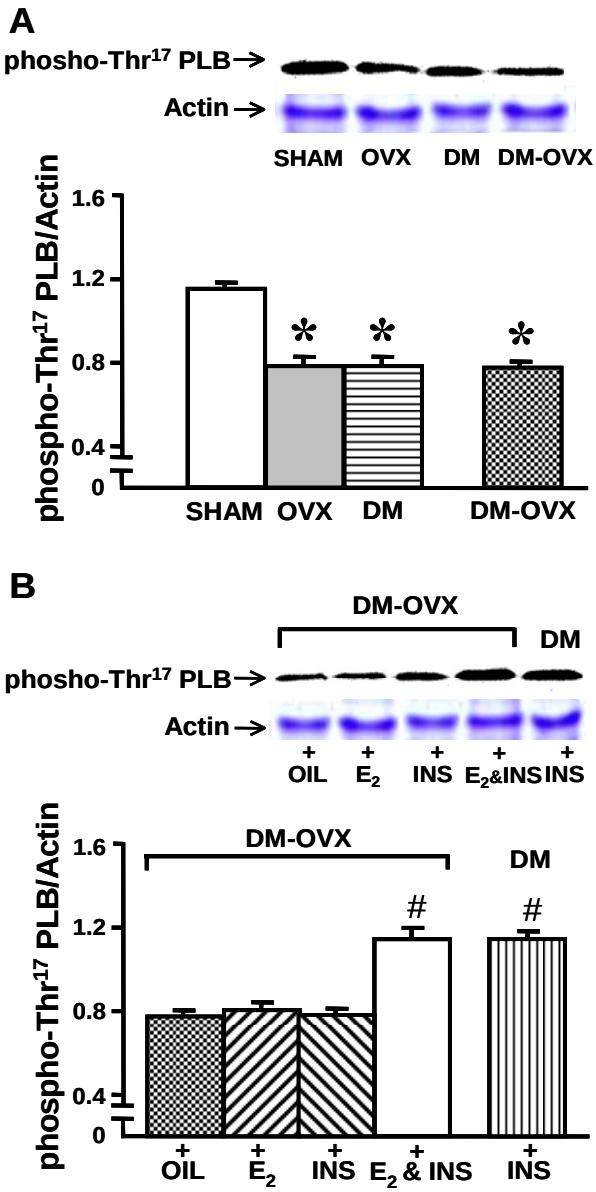


Figure 24 Immunoblot analysis of phosphorylated Threonin-17 (phosphor-Thr^{17}) and actin and comparison of the band intensity expressed as a ratio of phosphor-Thr^{17}:actin of left ventricular homogenates from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (B). Data are means ± SE from 6 hearts. $P < 0.05$, significant difference from SHAM (*) and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

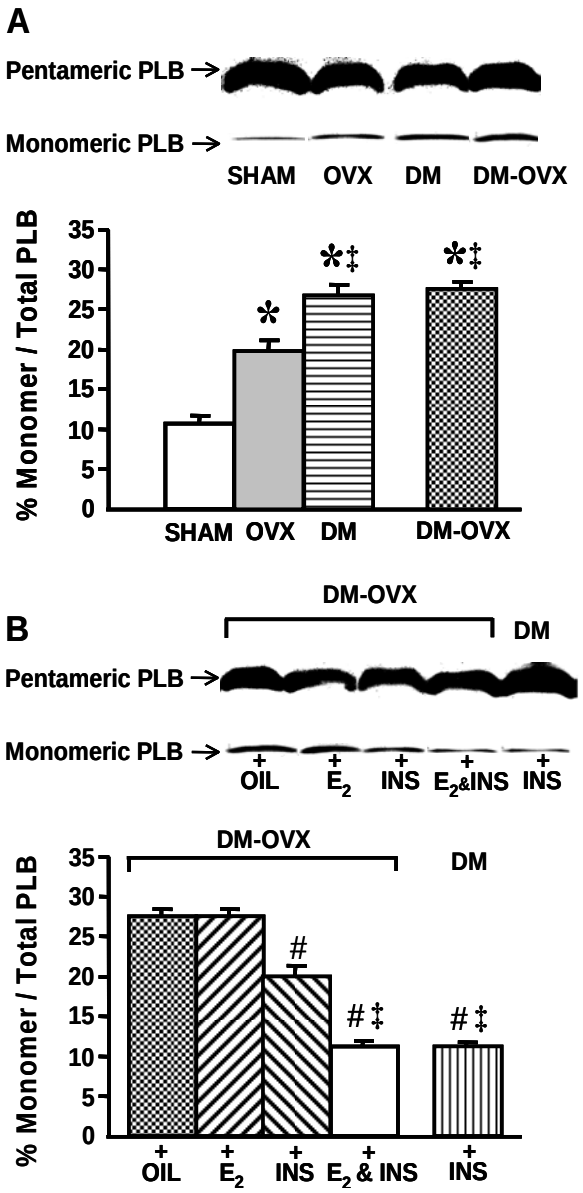


Figure 25 Amount of monomeric phospholamban expressed as percentage of total phospholamban of samples of left ventricular homogenates from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (B). Data are means ± SE from 6 hearts. $P < 0.05$, significant difference from SHAM (*), OVX (‡), and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

CHAPTER V
DISCUSSION

The present study extended our understanding on the cellular, subcellular, and molecular actions of E_2 on the cardiac myofilament activation. The cardiac myofilament Ca^{2+} hypersensitivity is found to be specifically induced in ovariectomized rat hearts even under diabetes complication. Alterations in the expression of β_1-adrenergic receptors may, in part, play a mechanistic role underlying the cardioprotective effects of E_2 that act together with Ca^{2+} hypersensitivity of the myofilament, thus, accounting for the gender difference in cardiac activation. Moreover, E_2 and INS deficiencies interactively downregulated HSP72 expression and therefore suppressed maximum myofilament ATPase activity. While INS indirectly affects the cardiac CB cycling through the action of thyroid hormone on the MHC expression, E_2 plays regulatory role in both the expression of MHC as well as the potential modifications of the contractile elements. Lastly, E_2 and INS also interactively regulate the cardiac SR Ca^{2+}-uptake activity through changes in both the quality and quantity of SERCA2a protein. Under E_2- or INS-deficient condition, an adaptive response of the SERCA2a function would be induced in almost the same condition that escaped the regulatory influence of phospholamban. Therefore, the regulatory role of E_2 on the SERCA2a represents a considerably minor contribution to the mechanisms underlying the post-hormone deficiency inducing myofilament Ca^{2+} hypersensitivity, whereas the major contribution is from the contractile protein modifications.

1. Interactive effect of estrogen and insulin on the cardiac myofilament response to Ca^{2+}.

The experiments provide the first clear evidence on the dominant effect of E_2 deficiency in inducing Ca^{2+} hypersensitivity of the cardiac myofilament under diabetes

complication. The data emphasize the significance of myofilament Ca^{2+} hypersensitivity in the pathophysiology of the heart after sex hormone deficiency. The present study also showed that E_2 and INS physiologically regulate and protect the cardiac contractile function through their interactive actions on the maximum myofibrillar ATPase activity, but not the myofilament Ca^{2+} sensitivity.

Presently, it is not clear how the increase in the mofilament response to Ca^{2+} leads to cardiac contractile dysfunction after ovariectomy. An enhanced Ca^{2+} sensitivity of the myofilament is a common feature detected in most cardiomyopathy patients (Gomes and Potter, 2004) and heart failure models (Heyder et al, 1995; Wolff et al., 1996; Wolff et al., 1995). An increased myofilament response to Ca^{2+} is a cellular mechanism proposed for explaining the underlying alterations in the Starling force of the heart (Rice and de Tombe, 2004) and could provide a therapeutic approach in the search for Ca^{2+}-sensitizing agents (Kass and Solaro, 2006). An elevated Ca^{2+} regulation of cardiac muscle activation has been shown to be the primary mechanism contributing to pathogenesis of troponin T-linked familial hypertrophic cardiomyopathy (Harada and Potter, 2004). Increased affinity of Ca^{2+} bound to myofilament occurring with mutant cardiac troponin I could also cause a threat for arrhythmic activity associated with cardiomyopathy (Kobayashi and Solaro, 2006). Moreover, a chronic increase in the cardiac myofilament response to Ca^{2+} could cause hypertrophic induction in association with mutations in sarcomeric proteins (Gomes and Potter, 2004). Besides the reported shift in cardiac MHC isoforms in ovariectomy (Bupha-Intr and Wattanapermpool, 2004; Wattanapermpool and Reiser, 1999) that is more likely to underlie the suppressed maximum myofibrillar ATPase activity, evidence for changes in other sarcomeric proteins that subsequently alter the myofilament response to Ca^{2+} awaits future studies.

Differential effects of E_2 and INS interaction on cardiac contractile function despite the presence of both receptors in the myocardium (Grohe et al., 1997; Velloso et al., 1998) suggest that different mechanisms exist for the hormones on the cardiac contractile response to Ca^{2+}. In contrast to other organs, the reversal of maximum cardiac myofibrillar ATPase activity in ovariectomy combined with diabetes only results when both E_2 and insulin treatment are given, reflecting an interaction of the hormones in activating myofilament function. On the other hand, the absence of the

interaction of the hormones on the ovariectomy-associated increase in cardiac myofilament Ca^{2+} sensitivity confirms that Ca^{2+} hypersensitivity of myofilament is a specific maladaptive response of the heart induced by sex hormone deficiency. Moreover, although interactive effects of E_2 and INS on the ovariectomy associated increase in the myofilament response to Ca^{2+} are absent, E_2 demonstrates a cardioprotective effect over INS in preventing Ca^{2+} hypersensitivity of myofilaments. This absence of hormone interaction confirms that Ca^{2+} hypersensitivity of myofilaments is a specific maladaptive response of the heart induced by E_2 deficiency. How the hormones act on cardiac contractile function is not known at present.

2. Interactive effect of estrogen and insulin on β_1-adrenergic receptor and HSP72 expression.

Data presented in this study also provide information on the mechanistic adaptation underlying the cardioprotective effects of estrogen that involves elevated expression of β_1-adrenergic receptor and loss of protective factor, HSP72 in E_2-deprived rat hearts. In ovariectomy, the close relation between enhanced myofilament sensitivity to Ca^{2+} and increased β_1-adrenoceptor expression in the heart, with or without complication of diabetes, confirms the adaptation of the contractile response of the heart in a pathological direction. Previous demonstrations that in ovariectomy there are decreases in the intracellular cardiac Ca^{2+} concentration and sarcoplasmic reticulum Ca^{2+} uptake activity (Ren et al., 2003; Bupha-Intr et al., 2007; Bupha-Intr and Wattanapermpool, 2006) suggest that both myofilament Ca^{2+} hypersensitivity and upregulation of β_1-adrenoceptors are likely to be the maladaptive responses induced by sex hormone depletion. The increased adrenergic drive either through the upregulation of β_1-adrenoceptors or increase in signaling process is known to be toxic to the heart (Bristow et al., 1992; Chakraborti et al., 2000; Kaye et al., 1995; Lefkowitz et al., 2000; Post et al., 1999). In a transgenic mouse model, overexpression of human β_1-adrenergic receptors in the heart produces a short-lived improvement of cardiac function but ultimately leads a cardiomyopathic phenotype characterized by dilation and depressed contractile functions (Bisognano et al., 2000; Engelhardt et al., 1999). This harmful compensatory mechanism of the heart induced

by chronic adrenergic stimulation has provided the fundamental basis for the use of anti-adrenergic agents in the treatment of chronic heart failure (Bristow, 2000; Hunt et al., 2005; Lechat et al., 1998; Packer et al., 2001). Although the sequential induction between changes in the myofilament response to Ca^{2+} and in β_1-adrenoceptors in ovariectomy remains unclear, parallel changes in these factors even with diabetes complication provide evidence for a high potential of cardiomyopathy induction in sex hormone-deficient hearts. Physiological suppression of β_1-adrenoceptor expression and stimulation may, in part, account for the cardioprotective effect of E_2 on the contractile response to Ca^{2+}.

The cardioprotective action of E_2 on the contractile activation could also be accounted for by the stability and the quality control of protein folding after translation in cardiomyocytes using a biological chaperone, HSP72 (Benjamin and McMillan, 1998). The effect of HSP72 on the myofilament proteins after exposing to the ischemia/reperfusion condition has recently been demonstrated by protecting the degradation of cTnI and cTnT by their proteolytic cleavage via interacting with the proteins (Lu et al., 2008). Thus, paralleled changes in HSP72 level and maximum myofibrillar ATPase activity (Figure. 7 and 12) provide a potential that HSP72 may be a common target for E_2 and INS actions in regulating the contractile activity. It has been reported that both E_2 and INS control HSP72 expression via phosphorylation of the same transcription heat shock factor (HSF)-1 (Atalay et al., 2004; Knowlton and Sun, 2001; Yu et al., 2006). Although homeostatic balance of β_1-adrenergic receptor and protective factor HSP72 is physiologically regulated by E_2, it is likely that only protective factors are regulated by INS. Our data confirm the physiologically cardioprotective function of E_2 on the contractile response to Ca^{2+} even under diabetes complication. These results support the beneficial use of E_2 and β_1-blocker in preventing maladaptation of the heart to E_2 deficiency, thereby lowering the incidence of heart failure in postmenopausal women.

3. The regulatory effect of estrogen on the crossbridge cycling kinetics in myofilament activation.

Based on the determination of CB cycling kinetics by maximum myofilament ATPase activity, CB cycling kinetics was then analyzed to help answering how E_2

regulated the maximum myofilament ATPase activity. According to a report by Alpert and his group (Perterson et al., 2001; Alpert et al., 2002), the relationship of CB cycling kinetics and % α-MHC isoform could indicate the mechanistic regulation of MHC isoform and the cardiac contractile element on the CB cyclings. The linear regression of the relationship indicates a sole direct effect of MHC on the CB cycling kinetics, whereas the curve relationship points to influences of both MHC isoform and the components of the contractile proteins in regulating CB cycling kinetics. In diabetic rat, the linear regression of maximum myofilament ATPase activity and % α-MHC detected in this study was similar to that reported earlier (Rundell et al., 2004). The results indicated that INS-deficient rats affect the CB cycling kinetics by determining the α-MHC expression. The shift of α-MHC in INS-deficient rats could be underlied by three possibilities, including a direct action of INS on the α-MHC gene, an indirect action through reduction in thyroid hormone, and an indirect action through induction of hyperglycemia. Inasmuch as an absence of insulin responsive element but a presence of thyroid responsive element on the α-MHC gene (Danzi et al., 2003) together with the present finding of decreases in T_3 level and α-MHC expression in INS deficient rat thus suggested an indirect impact of INS on the expression of α-MHC through changes in T_3 concentration. Moreover, the reversal of α-MHC level in DM rats with the still presence of hyperglycemic status after T_3 injection discards the influence of hyperglycemia on the shift in α-MHC in INS-deficient rats. In contrast, the concave relations found in the E_2-deficient rats suggested that E_2 influences on both the expression of MHC as well as the potential modification in the contractile elements in affecting the kinetics of CB cycling. Although there is no information concerning the presence of E_2 responsive element (ERE) on the cardiac MHC gene, a direct regulatory effect of E_2 on the transcription of skeletal MHC gene has been reported (Piccone et al., 2005). Thus, E_2 may affect the cardiac MHC gene by hormone-mediated transactivation without direct binding of the hormone receptors. However, there is so far no report on the effect of E_2 on changes in the contractile proteins.

It is possible that the modifications of the contractile proteins by E_2 in the regulation of the CB cycling kinetics could involve many processes including isoform shifts, mutation, and phosphorylation of the contractile proteins either on the thick or

thin filament. While MHC isoforms mainly determine the CB kinetics, isoforms of MLC have been reported to act as a fine tuner of the CB cyclings (Andruchov et al., 2006). Isoform switching from ventricular form to atrial form of MLC observed in ventricles of failing heart was found to underlie the compensatory increase in the Ca^{2+} sensitivity of force and CB cycling kinetics for improving the cardiac contractile function (Abdelaziz et al., 2005; Morano et al., 1997). Besides the isoform shift, mutations of cardiac Tn complex, TnT, TnC and TnI, have also been reported to worsen the cardiac CB cycling kinetics. Tschirgi and his coworker (Tschirgi et al., 2006) have demonstrated that mutation of rat cardiac TnT could decrease the CB cycling kinetics of myofilament in skinned-fiber measurement study. A significant depression in CB kinetics of cardiac myofilament has also been reported in the single point mutation of cardiac TnC from Gly to Asp at amino acid 159 (TnC-G159D), which induced development of cardiomyopathy (Biesiadecki et al., 2007). Recently, a mutation of cardiac TnI in the cardiac stunning condition has been demonstrated to alter the CB cycling kinetics with increased myofilament Ca^{2+} sensitivity (Tachampa et al., 2008). Another new mutation of human cardiac TnI detected in hypertrophic cardiomyopathy was also shown to result in deterioration of the CB cycling kinetics (Wen et al., 2008).

Modifications of cardiac contractile proteins by phosphorylation have also been reported to be an important determinant of depressed myofilament function in various models of heart failure. For example, depression of cardiac contractile function in the failing human heart was reported to be associated with an alteration of thin filament function that was due to upregulation of phosphorylation by PKC (Noguchi et al., 2004). This finding was also supported by the work of Belin and his group (Belin et al., 2007). Upregulations in both the expression and activity of PKC-α induced hyperphosphorylation of the myofilament, which contributed to myofilament dysfunction in experimental congestive heart failure, and was restored upon protein phosphatase1 stimulation. In addition, studies by Hamdani and coworker (Hamdani et al., 2008) has recently indicated that the decrease in myofilament function in human heart failure is associated with alterations in Tn phosphorylation and other contractile proteins, such as MLC and MyBP-C. Although it is not known how E_2 modulated cardiac contractile proteins, the specific effect of E_2 deficiency in inducing

myofilament Ca^{2+} hypersensitivity implies the significant cardioprotective effect of E_2 on the cardiac contractile activation.

4. Interactive effect of estrogen and insulin deficiency in the intracellular calcium handling in cardiac myocytes by SR.

The present experiments provide clear evidence on the interactive effect of E_2- and INS-deficiency in regulating the intracellular Ca^{2+} handling by changes in the SR Ca^{2+} uptake of cardiomyocytes through reduction in both activity and content of SERCA2a protein. An alteration in the cardiac SR Ca^{2+}-uptake activity by E_2- and INS-deficiencies could underlie the overall changes in the intracellular Ca^{2+} handling, resulting in a lower magnitude but prolonged decay of the intracellular Ca^{2+}-transients in OVX and DM rat hearts (Ren et al., 2003; Bupha-Intr et al., 2007; Choi et al., 2002). However, the same suppressive effect on the SR Ca^{2+}-uptake function but different changes in the myofilament responsiveness to Ca^{2+} hypersensitivity observed in E_2-deficient condition suggests an involvement of other mechanistic adaptations of cardiac myocytes after ovariectomy. Based on results of the third objective, E_2 also plays a potential regulatory role in the modifications of the cardiac contractile components. It is therefore possible that the Ca^{2+} hypersensitivity of the myofilament after ovarian sex hormone deficiency may also be due to changes in the contractile components.

A suppressed SR Ca^{2+}-uptake activity is a common feature detected in most human and animal models of cardiomyopathy and heart failure (Prestle et al., 2003; Temsah et al., 2000). It is, however, unclear how E_2 and INS regulate the SR Ca^{2+}-uptake function. Physiological significance of E_2 and INS action on the SR Ca^{2+}-uptake function was certainly indicated in present results which showed the same magnitude of suppression in the maximum activity in both OVX and DM rats as that in heart failure (Frank et al., 2002; Houser et al., 2000). The SR Ca^{2+}-uptake activity in failing heart was apparently found to be associated with the reduction in both activity and expression of SERCA2a (Zheng et al., 2004; Armoundas et al., 2007). The parallel effects of E_2 and INS deficiencies ie., suppression of the cardiac SR Ca^{2+}-uptake function and reduction in the activity and expression of SERCA indicated a

final common pathway of these two hormone action on the SR Ca^{2+}-uptake function through SERCA2a protein.

Generally, change in the amount and property of SERCA2a could affect the protein activity to pump Ca^{2+} into the SR. Quantitatively, the amount of SERCA2a proteins could be altered by means of degradation and synthesis. Downregulation of SERCA2a expression in E_2- and INS-deficient rats could point to the possible regulatory effect of E_2 and INS on the SERCA2a synthesis and/or degradation. A significant decrease in both mRNA and protein levels of SERCA2a in E_2- and INS-deficient rats (Bupha-Intr and Wattanapermpool, 2006; Kim et al., 2001) indicated a possible interactive regulatory action of E_2 and INS on the synthesis of SERCA2a protein. However, it is currently not known how E_2 and INS regulate the expression of SERCA2a in cardiac tissue. Moreover, based on the positive regulatory effect of thyroid hormone on the SERCA2a gene (Dillmann et al., 1990), the parallel reduction in SERCA2a and the reduction in plasma T_3 level in INS-deficient rats implied an indirect regulatory effect of INS on the SERCA2a expression through the action of thyroid hormone. Although there is no information on E_2 responsive element on the SERCA2a gene, the absence of additive suppression on the SERCA2a protein in E_2- and INS-deficient rats suggested a possible regulatory effect through the hormone-mediated transactivation without direct binding of the hormone receptors on the gene, such as the activation of transcription factor called stimulating factor 1 and 3, Sp1 and Sp3, (Doyle et al., 2004; Wang et al., 1998; Rishi et al., 1995; Schultz et al., 2005; Brady et al., 2003). Likewise, the reduction of SERCA2a protein in E_2- and INS-deficient rats could involve a decrease in the protein stability. According to a report of stabilization of HSP70 on SERCA2a protein after thermal stimulation in skeletal muscle (Tupling et al., 2004), the interactive effect of E_2 and INS deficiencies in downregulating the HSP72 expression supported the potential regulatory effect of E_2 and INS on the degradation of SERCA2a protein.

Besides the quantitative effect of SERCA2a protein, the qualitative effect of SERCA2a could also regulate the SERCA2a activity through modifications of protein phosphorylation and stoichiometry. In cardiac muscle cells, the activity of SERCA2a was normally regulated by phosphorylation of SERCA2a via CaMKII or of phospholamban via CaMKII and PKA. Direct phosphorylation of SERCA at Ser^{38} by

CaMKII results in an increase in the maximal activity (V_{max}) without affecting SERCA sensitivity to Ca^{2+} (Frank et al., 2003), leading to an enhancement of the rate of cardiac muscle relaxation (Toyafuku et al., 1994; Xu et al., 1999). In contrast, phosphorylation of phospholamban by both CaMKII and PKA has been shown to relieve the inhibitory action of phospholamban on SERCA2a, resulting in an increased affinity of SERCA2a for Ca^{2+} and increase in SERCA2a activity (Hagemann and Xiao, 2002). Because of a reduction in the systolic Ca^{2+} concentration in E_2- and INS-deficient rat hearts (Ren et al., 2003; Bupha-Intr et al., 2007; Choi et al., 2002), reductions in phosphor-Thr^{17} and/or phosphor-Ser^{16} phospholamban (Figure 23 and 24) thus indicated a possible suppression in CaMKII activity in E_2 and INS deficiencies. Surprisingly, the reduction in phosphor-Thr^{17} and/or phospho-Ser^{16} phospholamban in E_2- and INS-deficient rats (Figure 23 and 24) could not explain the increased Ca^{2+} sensitivity of SERCA in both groups. Additionally, an increase in the monomeric form of phospholamban, which is an active inhibitory form, was detected in both E_2- and INS-deficient rats, which should induce a decrease rather than an increase in the SERCA Ca^{2+} sensitivity. Although these studies could imply the regulatory effect of E_2 and INS in the SR Ca^{2+}-uptake activity through SERCA2a expression and modulation, the absence of correlation of changes in the SERCA2a Ca^{2+} sensitivity and the regulatory effect of phospholamban in both E_2- and INS-deficient rats suggested that other functional adaptations of SERCA2a might also be involved. Sarcolipin has been reported to regulate the SERCA activity by decreasing the sensitivity of SERCA to Ca^{2+} (MacLennan et al., 2003; Asahi et al., 2003). Overexpression of sarcolipin in mouse heart suggests the direct inhibitory effect of sarcolipin on SERCA2a protein without changes in the phosphorylation level or stoichiometry of phospholamban (Babu et al., 2006). Whether sarcolipin contributes to the effect of E_2 and INS on the cardiac SR awaits further investigation.

CHAPTER VI
CONCLUSIONS

The objectives of this study focus on the regulatory mechanism of E_2 on the cardiac myofilament response to Ca^{2+}. The first objective was to study the possible interactive effect of E_2 and INS deficiency on the cardiac myofilament response to Ca^{2+}. Ca^{2+}-dependent actomyosin MgATPase activities of cardiac myofibrillar preparations from various experimental groups were determined at various concentrations of Ca^{2+} to obtain the maximum activity and Ca^{2+} sensitivity in each group. While there was no additive effect of E_2 and INS deficiencies in the suppression of maximum myofilament ATPase activity, E_2 deficiency showed the dominant effect in inducing the cardiac myofilament Ca^{2+} hypersensitivity even in condition of diabetic complication. All changes could be reversed by the hormones supplementation. These results indicated that E_2 and INS interactively affect the cardiac contractile activation on regulating maximum myofilament ATPase activity. Moreover, it is the E_2 deficiency that induced an adaptive response of the myofilament to become more sensitive to Ca^{2+}. To better understand how E_2 regulated the cardiac myofilament response to Ca^{2+}, in the second objective the density and binding affinity of β_1-adrenergic receptors in various cardiac membrane preparations were quantified using saturation binding isotherm assay at various concentrations of [^{3}H]-dihydroalprenolol. Expressions of β_1-adrenergic receptors and HSP72 proteins were also determined by immunoblot analysis. Similar to myofilament Ca^{2+} sensitivity, the dominant effect of E_2 deficiency was observed in inducing an upregulation of β_1-adrenergic receptors even under diabetic complication. E_2 supplementation could restore this change. Additionally, there was no additive effect of E_2 and INS deficiencies in downregulating HSP72 which was restored upon coadministration of E_2 and INS. The close relation between the increased cardiac β_1-adrenergic receptors and the enhanced myofilament sensitivity to Ca^{2+} after ovarian sex hormone deficiency implied that the alteration of β_1-adrenergic receptors may, in

part, underlie changes in the cardiac myofilament response to Ca^{2+} after estrogen deprivation. In contrast, the loss in cardioprotective effect through the suppressed HSP72 expression in E_2- or INS-deficient rats paralleled the suppression of maximum myofilament ATPase activity which then indicated a potential common target, HSP72, in the controlling process of the two hormones in regulating myofilament contractile activity. In the third objective, experiments were designed to investigate how E_2 regulated the cardiac CB cycling kinetics. Maximum myofilament ATPase activities of cardiac myofibrillar preparations and expression of cardiac MHC isoforms of papillary muscle were analyzed. The relationships of maximum ATPase activity and % α-MHC isoform were plotted. The linear regression analysis of data from individual experimental group to that of shams was then fitted to determine the direct effect of MHC isoform on the CB cycling kinetics. Different linear regressions were observed between the groups of OVX vs SHAM and DM vs SHAM. Interestingly, the linear regression line demonstrated in DM vs SHAM groups was also detected in DM-OVX vs SHAM groups. These results indicated that both E_2 and INS regulated the CB cycling kinetics partly through changes in the expression of MHC isoform. However, the different slope of the linear relationship between DM and OVX group to SHAM indicated the different degrees of the impact of E_2 and INS on the CB. To further specify the regulatory mechanism of E_2 and INS on the CB cycling, effects of various levels of α-MHC on the kinetics were then evaluated. Maximum myofilbrillar ATPase activity and % α-MHC in OVX, DM and DM-OVX groups with various thyroid hormone states were evaluated and plotted. A linear regression line was demonstrated in both DM and DM-OVX groups with different thyroid levels with the same slope value to the previous regression of DM and DM-OVX with sham. In contrast, correlation of maximum myofilament ATPase activity to % α-MHC expression at different thyroid status of OVX groups demonstrated a concave shape relationship. These results confirmed that E_2, in contrast to INS, plays a major regulatory role on CB cycling kinetics through modifications on the myofilament proteins besides its effect on the expression of MHC isoform. The results also indicated that INS indirectly regulates the CB cycling through the direct effect of thyroid hormones on α-MHC expression. The forth objective was to further evaluate the significant role of E_2 in the regulation of intracellular Ca^{2+} handling of

cardiac myocytes by the SR under diabetic complication. The SR Ca^{2+}-uptake activity of the left ventricular homogenate and the SERCA2a activity from SR membrane vesicles were determined at various concentrations of Ca^{2+} to obtain the maximum activity and the Ca^{2+} sensitivity in each group. The SR Ca^{2+}-uptake associated protein contents from left ventricular preparations were also analyzed using immunoblot technique. Results demonstrated no additive suppression in the SR Ca^{2+}-uptake function in association with decreases in both the amount and activity of SERCA proteins with a high inhibitory effect of phospholamban in DM-OVX rat hearts. These changes were restored upon E_2 and INS supplementation. These results indicated an interactive effect of E_2 and INS in regulating the SR Ca^{2+}-uptake function through both the expression of SERCA proteins and the interaction of SERCA to its regulatory protein. The detail results from these studies could be concluded as follows:

1. No additive suppression in maximum myofibrillar ATPase activity induced by ovarian sex hormone deficiency and diabetic complication and the suppression was completely reversed by coadministration of E_2 and INS.
2. E_2 deficiency induced cardiac myofilament Ca^{2+} hypersensitivity even under diabetic complication and the hypersensitivity was restored by E_2 supplementation.
3. An upregulation of β_1-adrenergic receptors was specifically induced in ovarian sex hormone-deprived rat hearts even under diabetic complication and the receptor upregulation was normalized by E_2 supplementation.
4. No additive suppression in HSP72 protein expression was detected in E_2- and INS-deficient rat hearts and the suppression was restored after coadministration of E_2 and INS.
5. Different linear regression lines of maximum myofilament ATPase activity and % α-MHC were obtained from OVX vs SHAM and DM vs SHAM.
6. A linear regression of maximum myofilament ATPase activity and % α-MHC detected in DM vs SHAM was also detected in DM-OVX vs SHAM.
7. A linear relationship was still observed in both DM and DM-OVX groups at different thyroid levels with the same slope value to the previous regression of DM and DM-OVX data with sham.

8. The correlation of maximum myofilament ATPase activity to % α-MHC expression at different thyroid status of OVX groups demonstrated a concave shape relationship.
9. No additive suppression in maximum activity with Ca^{2+} hypersensitivity of the SR Ca^{2+}-uptake function was detected in left ventricular homogenates from E_2- and INS-deficient rats and the changes were completely reversed by coadministration of E_2 and INS.
10. No additive effect of E_2 and INS deficiencies on decreases in the SERCA protein and activity was detected and these changes were completely restored by coadministration of E_2 and INS.
11. Upregulation of total phospholamban protein detected in DM but not OVX group was still observed in DM-OVX group and the upregulation was restored by INS supplementation.
12. Decreases in both the phosphor-Ser^{16} and phosphor-Thr^{17} phospholamban expression were found in DM and DM-OVX groups but only the phosphor-Thr^{17} phospholamban was decreased in OVX group. All changes were reversed by supplementation with INS and E_2, respectively.
13. Upregulation of the monomeric form of phospholamban was observed in OVX and DM groups with a more pronounced upregulation in the DM group.
14. The same pronounced degree of upregulation of the monomeric form of phospholamban in the DM group was also observed in the DM-OVX group which was restored by INS supplementation.

In conclusion, results from this study indicate the significant role of E_2 in the regulation of cardiac myofilament activation. E_2 regulated the cardiac myofialement response to Ca^{2+} through changes in the expressions of β_1-adrenergic receptor, HSP72 and MHC, modifications of the myofilament proteins, and regulation of SR Ca^{2+}-uptake function by affecting both the activity and the expression of SERCA2a protein with the greater inhibitory effect of phospholamban.

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APPENDIX

APPENDIX A

PROTEIN DETERMINATION WITH FOLIN PHENOL (Lowry et al.,1951)

Reagents

Reagent A	2% Na_2CO_3 in 0.1 NaOH
Reagent B	4% Na-K tartrate
Reagent C	2% $CuSO_4 + 5H_2O$
Reagent D	Alkaline Copper solution Mixed 20 ml of reagent A with 100 µl of reagent B and 100 µl of reagent C. This mixed solution was discarded after 1 day
Reagent E	Folin phenol in H_2O Folin & Cicocalteu's phenol reagent (Sigma Chemical, MO) was diluted with H_2O in a 1:1 ratio before use.

Standard

Protein standard solution was prepared by dissolving 0.05 g of bovine serum albumin (BSA) from Sigma Chemical, MO in 100 ml of distilled water.

Procedures

1. The sample and standard proteins were diluted to appropriate concentration with reagent A to obtain a total volume of 200 µl.
2. Then added 1 ml of reagent D.
3. Added 100 µl of reagent E and mixed by vortex immediately. This rapid mixing is important since the reagent decomposes rapidly.
4. Incubated for at least 30 minutes at room temperature.
5. Read optical density at 770 nm by spectrophotometer.

6. Protein concentration of sample was calculated using intercept and slope factors of standard curve.

Reference:

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. Protein measurement with Folin Phenol reagent. J Biol Chem. 1951; 193(1): 265-275.

APPENDIX B

PROTEIN DETERMINATION WITH BRADFORD'S ASSAY (Bradford, 1976)

Reagents

Reagent A — Bradford stock solution

Mixed 100 ml of 95% ethanol, 200 ml of 88% phosphoric acid and 350 g of Serva Blue G together and kept at room temperature.

Reagent B — Bradford working solution

Mixed 30 ml of reagent A with 15 ml of 95% ethanol, 30 ml of 88% of phosphoric acid and obtained to 500 ml. Filter the solution through Whatman No.1 paper and stored at room temperature for several weeks.

Standard

Protein standard solution was prepared by dissolving 0.05 g of bovine serum albumin (BSA) form Sigma Chemical, MO in 100 ml of distilled water.

Procedures

1. The sample and standard proteins were diluted to appropriate concentration with distill water to obtain a total volume of 100 µl
2. Then added 0.9 ml of reagent B and mixed by vortex immediately.
3. Incubated for at least 5 minutes at room temperature.
4. Read optical density at 595 nm by spectrophotometer.
5. Protein concentration of sample was calculated using intercept and slope factors of standard curve.

Reference:

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.

APPENDIX C

PROTEIN DETERMINATION WITH BCA REAGENT

(Hill and Straka, 1988)

Reagents

Reagent A	BCA Stocking solution
	Composed of 1.0% Bicinchoninic acid, 2.0% Na_2CO_3, 0.16% NaK-tartrate, 0.4% NaOH, and 0.95% $NaHCO_3$, pH 11.25
Reagent B	2% $CuSO_4 + 5H_2O$
Reagent C	BCA working solution
	Mixed reagent A and reagent B at ratio 50:1, freshly prepare.

Standard

Protein standard solution was prepared by dissolving 0.01 g of bovine serum albumin (BSA) from Sigma Chemical, MO in 100 ml of distilled water.

Procedures

1. The sample and standard proteins were diluted to appropriate concentration with distilled water to obtain a total volume of 50 µl
2. Added 1 ml of mixed reagent C and then mixed by vortex immediately.
3. Incubated for at least 30 minutes at 37° C.
4. Read optical density at 562 nm by spectrophotometer.
5. Protein concentration of sample was calculated using intercept and slope factors of standard curve.

Reference:

Hill HD and Straka JG. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. Anal Biochem 1998; 170(1): 203-208.

APPENDIX D

CARDIAC MEMBRANE PREPARATION

(Backer and Potter, 1980: with modification)

Reagent

Reagent A	10 mM Tris-HCl, pH 8.0
Reagent B	1 M KCl
Reagent C	50 mM HEPES buffer pH 8.0 (containing 4 mM $MgCl_2$)

Procedure

1. Left ventricle was cut into small pieces and homogenized with Omni-Mixer Macrohomogenizer speed 5.5 (one minute twice) in 10 ml ice-cold Tris-HCl buffer.
2. 30 ml of 1 M KCl was added and incubated at 4^{o}C for 20 min to dissolve the myofilament proteins.
3. Then, homogenate was filtered through six layers of cheesecloth followed by centrifugation at 43,900 g, 4^{o}C for 20 minutes.
4. The pellet was resuspended in 35 ml of Tris-HCl buffer and resedimented with the same speed of centrifugation.
5. The final pellets were resuspended in 10 ml of ice-cold 50 mM HEPES containing 4 mM $MgCl_2$.
6. Protein concentration of cardiac membrane preparation was determined by Bradford's protein assay (Appendix B).

Reference:

Baker SP and Potter LT. Purification and partial characterization of cardiac plasma membranes rich in β-adrenoceptors. Membr Biochem. 1980; 3: 185-205.

APPENDIX E

β-ADRENERGIC RECEPTOR ASSAY

(Cervoni et al., 1981: with modification)

Incubation solutions	**Final concentration**
HEPES, semi sodium salt, pH 8.0 (N-[2-Hydroxyethyl] piperazine-N-[2-ethane sulfonic acid)	50 mM
$MgCl_2$	4.0 mM
(-)-Alprenolol	10 μM

Washing Solution

Iced-cold 25 mM HEPES, pH 8.0 and 4 mM $MgCl_2$

Procedure

The assay was run at various concentrations of [^{3}H]-DHA from 0.5-40 nM with or without (-)-alprenolol pursuing the following steps.

1. 100 μl of incubation buffer was preconditioned at 25^{o}C for 1 minute.
2. Binding reaction was started by adding 100 μl of cardiac membrane preparation (~50 μg protein) incubating at 25^{o}C for 20 min with shacking at 60 oscillations/min.
3. After 20 min, 4 ml ice-cold washing solution was added to the reaction.
4. The incubation mixture was immediately filtered through GF/B glass fiber filter (Whatman) using high performance vacuum pump.
5. The filter was rinsed twice with 4 ml washing solution to remove the unbound [^{3}H]-DHA and then blown dry
6. Membrane protein and [^{3}H]-DHA on dried filter was re-dissolved in scintillation fluid without Triton X-100 overnight.
7. Equal amount of Scintillation was counted radioactivity (Appendix F)

Reference:

Cervoni P, Herzlinger H, Lai FM, and Tanikella T. A comparison of cardiac reactivity and β-adrenergic receptor number and affinity between aorta-coarcted hypertensive and normotensive rats. Br J Pharmacol 1981;74(3): 517-523.

APPENDIX F

DETERMINATION OF RADIOISOTOPE

Principle

^{3}H and ^{45}Ca used in the study are the radioactive isotopes that decay radioactivity by emitting beta particles. To determine their radioactivity, a liquid scintillation, which converts the energy absorbed from the beta particle into light photon, is used to dissolve standards and sample. The liquid scintillation contains two solutes, 2,5-diphenyl oxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP) in toluene solvent. The emitted electron from nucleus of radioisotopes is taken gained up by toluene and make them unstable state that must eventually lose the energy gained by emission of photons. The photon energy is transmitted to solutes (sometimes called fluors). A primary flours, PPO, converted excitation energy to short wavelength light photon. Short wavelength photon is then absorbed by secondary fluors, POPOP. Excited state of secondary solute emits the long wavelength energy, which is converted to photoelectrons at the photocathode of the photomultiplier tube. The radioactivity is presented as counts per minute (cpm) as compared to standard.

Instrument

Liquid scintillation counter LKB Wallac 1219 Rackbeta.

Reagents

Scintillation fluid 1 litter contains;

5 g 2,5-diphenyl oxazole (PPO)

0.3 g 1,4-bis[2-(5-phenyloxazole)]benzene (POPOP)

Toluene (with 500 ml of triton x-100 when the sample containing water)

Procedures

For dry filter:

1. Put a dry filter, containing radioactive receptor complex, into the vial and added with 7 ml of scintillation fluid without triton x-100 and shacked.
2. Left at room temperature overnight. Then, transferred 6 ml of scintillation fluid in the new vial.
3. Counted the radioactivity in Liquid Scintillation counter.

For aqueous solution:

1. Placed sample in the 2 ml microcentrifugal tube with 1.5 ml of scintillation fluid. Mixed the suspension until it clear.
2. Put the microcentrifugal tube into the counting vial.
3. Counted the radioactivity in Liquid scintillation counter.

The standard radioisotope was performed in the same way with known amounts. Counting duration per vial was set at 10 and 3 min for ^{3}H and ^{45}Ca, respectively. Amounts of ion were calculated using the intercept and slope factors of standard curve.

Reference:

Osterman LA. Isotopes, Scintillation and Scintillation Counters. In: Methods of protein and nucleic research (V2.). Edited by Osterman LA. Germany: Springer-Verlag Berlin Heidenberg, 1984: 67-78.

APPENDIX G

THE Ca^{2+}-DEPENDENT MYOFIBRILLAR ACTOMYOSIN Mg^{2+}-ATPase ACTIVITY ASSAY

(Pagani and Solaro, 1987)

Incubation solutions	**Final concentration**
Potassium chloride	60 mM
Imidazole	30 mM
Magnesium chloride	7.5 mM
ATP	5.0 mM

Procedure

The assay was run at various Ca^{2+} concentrations ranging from pCa (-log of molar calcium free) 7.5 to 4.875 in pH 7.0 pursuing the following steps.

1. 50 µl of myofibrillar protein (concentration ranging between 3.8-4.2 mg/ml) was added to the incubation solutions with various concentrations of $CaCl_2$ at 5 mM EGTA to make a volume of 0.474 ml.
2. Mixed and preincubated at 30°C for 3 minutes in a temperature-controlled dry-bath.
3. The reaction would start by adding ATP to a final volume of 0.5 ml. Mixed and left at 30°C for another 10 min.
4. After 10 minutes, 0.5 ml of ice-cold 10% trichloroacetic acid (TCA) was added to stop reaction. Mixed and rapidly placed the tube on ice.
5. Each tube of sample was then centrifuged at 3,000 rpm, 4°C for 10 minutes. After that 50 µl of supernatant was taken for Pi assay (Appendix H).

6. For a blank tube, the incubation mixture contained no $CaCl_2$. 10% TCA was added before adding ATP and left the tube on ice without incubation.

Reference:

Pagani ED and Solaro RJ. Method for measuring function properties of sarcoplasmic reticulum and myofibrils in small samples of myocardium. In: Schawartz A, editor. Methods in pharmacology, Vol. 5 New York : Plenum Publishing Crop, 1984: 44-61.

APPENDIX H

DETERMINATION OF INORGANIC PHOSPHATE

(Carter and Karl, 1982)

Reagents

Solution A	Mixing of 2N HCl and 0.1 M Na_2MoO_4 in the volume ratio of 4:3
Solution B	0.042% (w/v) malachite green dye and 1% (w/v) polyvinyl alcohol.
Solution C	7.8% (v/v) H_2SO_4 solution.

Standard solution

0.5 mM of KH_2PO_4 stored at 4°C

Procedure

1. 50 µl of sample and standard Pi was added into 0.35 ml of solution A and 0.15 ml of solution B. Immediately mixed and allowed to stand for exactly 2 minutes for color development.
2. Added 1 ml of solution C and mixed thoroughly.
3. Allowed to stand for 60 minutes at room temperature to complete color development.
4. The optical density was measured at 625 nm by spectrophotometer.
5. Pi concentration of sample was calculated using intercept and slope factors of standard curve.

Reference:

Carter SG and Karl DW. Inorganic phosphate assay with malachite green: an improvement and evaluation. J Biochem Biophys Methods. 1982; 7(1): 7-13.

APPENDIX I

DETERMINATION OF SARCOPLASMIC RETICULUM Ca^{2+} UPTAKE

(Pagani and Solaro, 1987: with modification)

Incubation solution	**Final concentration**
Potassium chloride	100 mM
Imidazole	20 mM
Magnesium chloride	5 mM
Sodium azide	5 mM
Potassium oxalate	5 mM
EGTA	5 mM
Ruthenium red	5 μM

Procedure

The assay was run at various Ca^{2+} concentrations ranging from pCa (-log of molar calcium free) 8.0 to 4.875, pH 7.0. The ratio of $CaCl_2$ and $^{45}CaCl_2$ in the reaction was equal to 1000: 1. Three minutes of reaction was run at 37°C pursuing the following steps;

1. 50 μl of left ventricular homogenate (~0.5 mg) was added to the incubation solutions with various concentrations of $CaCl_2$ to make a volume of 0.475 ml.
2. Mixed and preconditioned at 37°C for 3 minutes in a temperature controlled shaking water bath with 60-oscillations/min shaking.
3. The reaction would start by adding 25 μl of 100 mM ATP to a final volume of 0.5 ml. Mixed and left at 37°C for another 3 minutes.

4. Uptake reaction was stopped by putting the reaction tube on ice-cold for 1 minute followed by filtration through 0.45 μm Millipore filter (Millex HA).
5. 100 μl of both filtrated and non-filtrated solution were dissolved in 1.5 ml scintillation fluid with triton X-100 and determined for the radioactivity (Appendix F).

Reference:

Pagani ED and Solaro RJ. Method for measuring function properties of sarcoplasmic reticulum and myofibrils in small samples of myocardium. In: Schwartz A, editor. Methods in pharmacology, Vol. 5 New York: Plenum Publishing Corp, 1984; 44-61.

APPENDIX J

CARDIAC SARCOPLASMIC RETICULAR MEMBRANE PREPRATION

(Jones et. al., 1979: with modification)

Reagent

Reagent A	10 mM NaHCO3, pH6.8
Reagent B	KCl (powder)
Reagent C	0.25 M sucrose buffer (containing 0.3 M KCl, 0.05 M sodium pyrophosphate, and 0.1 M Tris), pH 7.2.
Reagent D	0.8 M sucrose buffer (containing 0.3 M KCl, 0.05 M sodium pyrophosphate, and 0.1 M Tris), pH 7.2
Reagent E	sample solution containing 400 mM sucrose, 5 mM HEPES, and 5 mM Tris, pH 7.2.

Procedure

1. Left ventricle was chopped into small pieces and homogenized with Ystral Macrohomogenizer speed 3 (one minute twice) in 8 ml of ice-cold reagent A.
2. The homogenate was centrifuged at 8,000 g, 4°C for 10 minutes to precipitate large debris and the supernatant was further centrifuged at 10,000 g, 4°C for 20 minutes.
3. KCl was added into the supernatant to reach a concentration of 0.6 M KCl and then put on ice for 15 minutes.
4. Membrane vesicle was then precipitated from the high salt supernatant by centrifugation at 45,000 g, 4 °C for 45 minutes.

5. The pellet was re-suspended in 7 ml of reagent C using Taflon-glass homogenizer.
6. The suspension was poured on top of 15 ml reagent D in the ultracentrifugal tube and then centrifuged at 100,000 g, 4 °C for 1 hr.
7. The pellet was re-suspended in 2 ml of reagent E with glass homogenizer, immediately dipped in liquid nitrogen, and kept at –80 °C until use.
8. The amount of proteins on the SR-enriched membrane vesicles was determined using Bradford's protein assay (appendix B).

Reference:

Jones LR, Besch HR, Jr., Fleming JW, McConnaughey MM, Watanabe AM. Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. Comparative biochemical analysis of component activities. J Biol Chem 1979; 254(2): 530-539.

APPENDIX K

SARCOPLASMIC RETICULUM Ca^{2+}-ATPase ACTIVITY ASSAY

(Chu et al, 1988: with modification)

Incubation solution	**Final concentration**
MOPS	21 mM
NaN_3	4.9 mM
EGTA	0.06 mM
KCl	100 mM
$MgCl_2$	3.0 mM
NADH	0.2 mM
Phospho(enol)pyruvate	1.0 mM
Pyruvate kinase	8.4 u/ml
Lactate dehydrogenase	12 u/ml

Procedure

The reaction was run in various Ca^{2+} concentrations ranging from pCa (-log of molar calcium free) 8.0 to 5.0, pH 7.0, and observed at 37°C pursuing the following steps;

1. 5 μg of SR-enriched membrane vesicles was added to the incubation solution in various concentrations of $CaCl_2$ to make a volume of 0.980 ml.
2. Mixed and preconditioned the reaction mixture at 37°C for 2 minutes.
3. The reaction was started by adding 20 μl of 50 mM ATP. Mixed and continuously measured with spectrophotometer at 37°C for 6 minutes.
4. The SERCA activity was calculated from the kinetic change at 349 nm during 250 to 350 sec after starting.

5. Another set of reaction was performed with addition of thapsigargin, SERCA inhibitor, 1.0 μM in 0.1 μl of DMSO to determine the non-specific activity.

Calculation:

$$\text{SERCA activity (μmole/mg protein/min)} = \frac{[\Delta OD/min] \times \text{dilution factor}}{[6.22] \times \text{mg protein}}$$

Reference:

Chu A, Dixon MC, Saito A, Seiler S, Fleischer S. Isolation of sarcoplasmic reticulum fraction referable to longitudinal tubules and junctional terminal cisternae from rabbit skeletal muscle. Methods Enzymol. 1988; 157: 36-46.

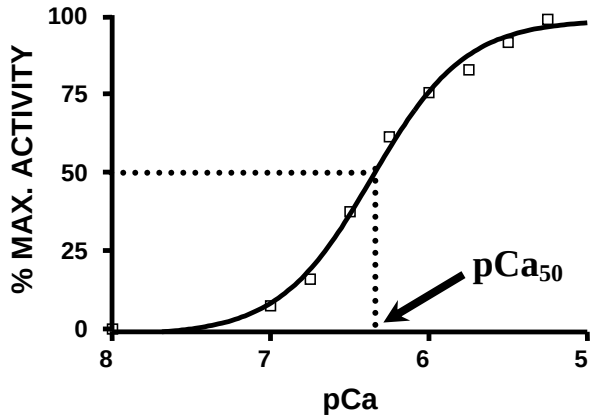
APPENDIX L

HILL EQUATION

Hill equation describes a cooperative behavior for the non-linear regression enzyme activity or force development.

$$v = V_{max} \cdot S^n / (S_{0.5}^n + S^n)$$

where; v is the initial velocity, V_{max} is the maximum velocity, S is the concentration of varied substrate, $S_{0.5}$ is the substrate concentration where $v = 0.5\ V_{max}$, and n is the Hill coefficient. As shown in the figure below, the substrate is Ca^{2+}.



$$\text{Relative activity} = \frac{[Ca^{2+}]^n}{K + [Ca^{2+}]^n}$$

K = $(EC_{50})^n$ = pCa_{50} (at n = 1) = The Ca^{2+} concentration at half maximum ATPase activity representing the sensitivity of the active protein (s) to Ca^{2+} activation.

n = Hill coefficient = the tangent of the upstroke slope which represents the cooperative function of the functional units.

Reference:

Segel IH. Multisite and allosteric enzyme. In: Segel IH editor. Biochemical calculations (2nd ed.) John Wiley & Sons 1976; 303-316.

APPENDIX M

Table I-Table XVII

Table I Maximum and pCa_{50} of myofilament ATPase activity from sham (SHAM), ovariectomized (OVX), diabetic (DM), diabetic-ovariectomized (DM-OVX) rats, and DM-OVX rats with estrogen (E_2) and/or insulin (INS).

No.	SHAM + Oil	OVX + Oil	DM + Oil	DM-OVX + Oil	DM-OVX + E_2	DM-OVX + INS	DM-OVX + E_2 + INS
Maximum ATPase Activity (nmole Pi/ mg protein/ min)							
1	149.0	133.8	102.8	133.2	142.1	172.6	169.2
2	193.6	147.9	113.1	152.6	114.9	141.3	186.3
3	176.7	145.7	135.9	135.3	124.8	163.6	169.8
4	197.7	108.7	125.5	102.0	143.6	129.6	184.2
5	181.8	132.7	123.5	87.7	101.8	128.3	184.4
6	161.6	136.5	122.3	127.2	127.9	149.2	183.2
7	-	155.1	-	134.5	107.5	159.7	188.2
8	-	-	-	-	119.9	138.8	189.2
9	-	-	-	-	-	-	188.4
10	-	-	-	-	-	-	203.1
MEAN	176.8	137.2*	120.5*	124.7*	122.8*	147.9*	184.6
SE	7.6	5.7	4.6	8.4	5.3	5.7	3.1
n	6	7	6	7	8	8	10
pCa_{50}							
1	5.97	6.12	5.86	6.02	5.99	6.01	5.87
2	5.95	6.04	5.92	6.09	5.94	6.11	5.96
3	5.98	5.98	5.94	5.89	5.99	6.07	5.97
4	5.89	5.99	5.96	6.03	5.94	6.05	5.99
5	5.89	6.09	5.92	5.97	5.92	5.93	5.95
6	5.96	6.12	5.99	6.07	6.01	6.03	5.96
7	-	6.11	-	5.98	6.02	6.07	5.85
8	-	-	-	-	5.96	6.16	5.97
9	-	-	-	-	-	-	5.86
10	-	-	-	-	-	-	5.97
MEAN	5.94	6.06*	5.93	6.01*	5.97	6.05*	5.94
SE	0.02	0.02	0.02	0.02	0.01	0.02	0.02
n	6	7	6	7	8	8	10

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference form SHAM using Student Newman-Keuls test after ANOVA

Table II Maximum density (B_{max}) and dissociation constant (K_d) of the cardiac β_1-adrenergic receptor (β_1AR) in left ventricular membrane preparations from SHAM, OVX, DM, DM-OVX rats and DM-OVX rats with estrogen and/or insulin supplementation.

No.	SHAM + Oil	OVX + Oil	DM + Oil	DM-OVX + Oil	DM-OVX + E_2	DM-OVX + INS	DM-OVX + E_2 + INS
B_{max} (fmole/ mg protein)							
1	199.3	256.3	243.6	227.6	201.6	220.3	217.4
2	192.2	251.6	238.0	238.1	226.0	229.1	184.9
3	216.5	247.6	177.2	232.1	229.4	296.0	191.7
4	227.9	245.9	241.5	250.3	229.7	257.7	201.3
5	210.7	292.9	229.4	252.5	223.1	264.7	244.7
6	220.8	244.2	186.3	256.3	192.8	263.8	176.4
7	200.7	233.1	211.5	228.0	190.3	255.0	216.8
8	205.1	287.6	200.1	274.5	224.3	279.2	153.9
MEAN	209.1	257.1*	215.9	244.9*	214.6	258.2*	198.4
SE	4.3	7.6	9.2	5.8	5.9	8.7	9.9
n	8	8	8	8	8	8	8
K_d (nM)							
1	3.6	3.9	3.2	2.3	3.2	3.3	3.1
2	4.2	4.9	3.1	3.9	3.9	3.0	2.5
3	2.4	3.0	3.3	2.5	3.9	3.2	2.5
4	3.6	3.5	3.5	3.3	2.9	4.1	4.1
5	3.5	3.6	3.2	4.0	4.0	3.4	4.7
6	4.6	4.5	2.2	3.5	2.0	2.4	3.1
7	2.3	3.8	3.0	3.5	2.6	4.0	4.3
8	2.9	3.0	3.3	3.1	2.4	2.6	2.9
MEAN	3.4	3.8	3.8	3.3	3.1	3.2	3.4
SE	0.3	0.2	0.8	0.2	0.3	0.2	0.3
n	8	8	8	8	8	8	8

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference form SHAM using Student Newman-Keuls test after ANOVA

Table III Relative intensity of β_1-AR and HSP72 proteins to calsequestrin (CSQ) of left ventricular homogenates from SHAM, OVX, DM, DM-OVX rats and DM-OVX rats with estrogen and/or insulin supplementation.

No.	Protein Expression (Relative Intensity)						
	SHAM	OVX	DM	DM-OVX			
	+ Oil	+ Oil	+ Oil	+ Oil	+ E_2	+ INS	+ E_2 + INS
β_1-adrenergic receptor							
1	199.3	256.3	243.6	227.6	201.6	220.3	217.4
2	192.2	251.6	238.0	238.1	226.0	229.1	184.9
3	216.5	247.6	177.2	232.1	229.4	296.0	191.7
4	227.9	245.9	241.5	250.3	229.7	257.7	201.3
5	210.7	292.2	229.4	252.5	223.1	264.7	244.7
6	220.8	244.2	186.3	256.3	192.8	263.8	176.4
7	200.7	231.3	211.5	228.0	190.3	255.0	216.8
8	205.1	287.6	200.1	274.5	224.3	279.2	153.9
MEAN	209.1	257.1*	215.9	244.9*	214.6	258.2*	198.4
SE	4.3	7.6	9.2	5.8	5.9	8.7	9.9
n	8	8	8	8	8	8	8
HSP72/CSQ							
1	1.33	0.92	1.18	1.08	1.15	1.12	1.41
2	1.36	.98	1.03	1.07	0.99	0.89	1.34
3	1.46	1.05	1.09	1.12	0.95	1.10	1.38
4	1.38	0.99	0.95	0.99	0.95	0.84	1.31
5	1.30	1.06	0.78	0.93	0.78	0.89	1.28
6	1.25	0.95	1.06	0.87	0.86	1.03	1.38
7	1.24	0.92	1.14	1.14	1.12	1.10	1.21
8	1.40	0.94	1.09	1.09	1.13	1.15	1.44
MEAN	1.34	0.98*	1.01*	1.04*	0.99*	1.02*	1.34
SE	0.03	0.02	0.05	0.03	0.05	0.04	0.03
n	8	8	8	8	8	8	8

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference form SHAM using Student Newman-Keuls test after ANOVA

Table IV Body weight (BW), heart weight (HW), uterine weight (UW), % heart weight/ body weight (% HW/BW), and plasma T_3 level

GROUPS	BW (g)	HW (g)	UW (g)	% HW/BW	Plasma T_3 (ng/ml)
SHAM	270 ± 4	0.87 ± 0.01	0.42 ± 0.02	0.32 ± 0.01	65.7 ± 2.6
OVX	345 ± 6*	0.98 ± 0.01 *	0.09 ± 0.01*	0.28 ± 0.01*	62.4 ± 2.8
OVX + E_2	266 ± 5$^{\#}$	0.87 ± 0.01 $^{\#}$	0.43 ± 0.04$^{\#}$	0.33 ± 0.01$^{\#}$	64.4 ± 1.6 $^{\#}$
DM	226 ± 5*$^{\#}$	0.81 ± 0.01 *$^{\#}$	0.31 ± 0.03*$^{\#}$	0.36 ± 0.01*$^{\#}$	45.2 ± 2.8*$^{\#}$
DM + INS	274 ± 6$^{\#}$	0.91 ± 0.02 $^{\#}$	0.41 ± 0.01$^{\#}$	0.32 ± 0.01$^{\#}$	65.1 ± 1.5$^{\#}$
DM-OVX	235 ± 4*$^{\#}$	0.88 ± 0.01 $^{\#}$	0.09 ± 0.01*	0.38 ± 0.01*$^{\#}$	48.1 ± 1.3*$^{\#}$
DM-OVX + E_2	230 ± 5*$^{\#}$	0.81 ± 0.02 *$^{\#}$	0.39 ± 0.02$^{\#}$	0.36 ± 0.01*$^{\#}$	46.1 ± 1.2*$^{\#}$
+ INS	352 ± 4*	1.01 ± 0.01 *	0.09± 0.03*	0.29 ± 0.01*	62.9 ± 1.3
+ E_2 + INS	269 ± 3$^{\#}$	0.89 ± 0.01 $^{\#}$	0.40 ± 0.01$^{\#}$	0.33 ± 0.01$^{\#}$	65.6 ± 1.7$^{\#}$

Values are means ± SE of 8 rats each group. SHAM, sham-operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic-ovariectomized; E_2, estrogen; INS, insulin; T_3, triiodothyronine. $P < 0.05$, significant difference from SHAM (*) and OVX ($^{\#}$) groups

Table V Maximum myofilament ATPase activity from SHAM, OVX rats with and without estrogen supplementation, DM rats with and without insulin supplementation.

No	Maximum ATPase Activity (nmole Pi/ mg protein/ min)								
	SHAM	OVX		DM		DM-OVX			
	+ Oil	+ Oil	+ E_2	+ Oil	+ INS	+ Oil	+ E_2	+INS	+ E_2 + INS
1	173.7	137.0	187.9	108.3	185.2	128.7	127.1	133.5	180.6
2	175.5	123.9	172.3	128.2	175.6	127.2	120.0	133.4	172.3
3	170.0	123.2	193.1	136.2	196.1	137.7	121.0	126.8	185.9
4	204.9	128.5	185.2	113.9	182.5	126.6	126.0	142.4	195.1
5	193.1	139.5	195.1	138.6	188.3	138.5	134.6	134.3	198.4
6	194.9	129.6	198.4	134.8	187.9	134.8	128.5	135.8	196.1
7	180.1	130.3	173.7	127.1	187.1	119.6	126.4	140.7	187.4
8	181.4	132.4	187.3	138.0	186.0	127.7	123.8	127.1	187.3
9	193.1	126.2	188.3	137.6	185.0	129.9	128.6	146.8	181.1
10	173.7	134.7	175.6	120.2	188.0	138.9	127.0	139.7	182.3
11	178.7	134.6	-	131.2	-	126.5	-	123.9	184.0
12	194.2	123.4	-	128.5	-	125.5	-	134.5	-
13	-	129.1	-	129.1	-	130.5	-	-	-
14	-	131.0	-	128.1	-	129.3	-	-	-
15	-	131.4	-	125.3	-	131.7	-	-	-
16	-	126.3	-	128.5	-	128.3	-	-	-
17	-	125.7	-	-	-	129.6	-	-	-
MEAN	184.4	130.9*	185.7	128.6*	186.2	130.5*	126.3*	134.6*	186.4
SE	3.2	1.6	2.9	3.1	1.6	1.6	1.3	1.7	2.3
N	12	17	10	16	10	17	10	14	11

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference form SHAM using Student Newman-Keuls test after ANOVA

Table VI Relative amount of α-MHC to total MHC expression of left ventricular papillary muscle from SHAM, OVX rats with and without estrogen supplementation, DM rats with and without insulin supplementation.

No	% α-MHC								
	SHAM	OVX		DM		DM-OVX			
	+ Oil	+ Oil	+ E_2	+ Oil	+ INS	+ Oil	+ E_2	+INS	+ E_2 + INS
1	61.5	52.6	65.5	35.6	63.3	33.2	31.7	50.5	73.4
2	61.2	48.4	71.1	33.1	70.3	37.6	32.2	47.8	71.1
3	67.2	49.3	76.1	41.0	70.8	41.1	36.2	51.8	81.4
4	77.1	47.0	63.3	28.7	64.6	31.5	35.6	52.7	78.7
5	76.2	59.0	78.7	37.9	70.5	29.5	35.4	56.9	64.0
6	75.5	45.5	64.0	34.6	65.6	45.2	31.9	52.0	78.0
7	69.4	50.5	66.3	32.4	67.0	31.7	35.5	56.7	71.6
8	70.1	55.4	72.5	28.0	67.1	35.1	38.8	48.3	72.5
9	76.1	53.2	70.5	33.1	64.3	39.5	42.2	58.6	70.4
10	66.3	50.4	70.3	36.0	67.5	40.4	31.0	52.9	74.9
11	69.3	53.1	-	33.4	-	32.6	-	50.1	71.5
12	68.8	51.5	-	34.6	-	33.5	-	47.8	-
13	-	51.1	-	35.7	-	34.2	-	56.4	-
14	-	48.6	-	34.5	-	47.9	-	56.4	-
15	-	46.9	-	34.1	-	48.3	-	-	-
16	-	51.0	-	33.9	-	48.4	-	-	-
17	-	51.4	-	-	-	45.3	-	-	-
MEAN	69.9	51.3*	69.8	35.8*#	67.1	48.9*#	35.1*#	52.8*	73.4
SE	1.6	1.2	1.6	1.3	0.9	0.8	1.1	1.0	1.4
N	12	17	10	16	10	17	10	14	11

Results are means ± SE from each group. n = number of rats. $P < 0.05$, significant difference from SHAM (*), and OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

Table VII Body weight (BW), heart weight (HW), uterine weight (UW), % heart weight/ body weight (% HW/BW), and plasma T_3 level

GROUPS	BW (g)	HW (g)	UW (g)	% HW/BW	Plasma T_3 (ng/ml)
SHAM	270 ± 4	0.87 ± 0.01	0.42 ± 0.02	0.32 ± 0.01	65.7 ± 2.6
OVX	345 ± 6*	0.98 ± 0.01	0.09 ± 0.01*	0.28 ± 0.01	62.4 ± 2.8
OVX + T_3	326 ± 6*$^{\#\dagger}$	1.31 ± 0.07*$^{\#\dagger}$	0.10 ± 0.03*	0.40 ± 0.02*$^{\#}$	393.1 ± 3.5*,$^{\#}$
OVX + PTU	292 ± 5*$^{\#\dagger}$	0.88 ± 0.02	0.09 ± 0.02*	0.30 ± 0.02	20.3 ± 1.8*$^{\#\dagger}$
DM	226 ± 5*$^{\#\dagger}$	0.81 ± 0.01$^{\#}$	0.31 ± 0.03*$^{\#}$	0.36 ± 0.01*$^{\#}$	45.2 ± 2.8*$^{\#}$
DM + T_3	200 ± 9*$^{\#\dagger}$	0.92 ± 0.03	0.31 ± 0.03*$^{\#}$	0.46 ± 0.02*$^{\#\dagger}$	60.3 ± 1.6†
DM + PTU	228 ± 7*$^{\#}$	0.82 ± 0.01*$^{\#\dagger}$	0.31 ± 0.02*$^{\#}$	0.37 ± 0.02*$^{\#}$	17.1 ± 0.9*$^{\#\dagger}$
DMOVX	235 ± 4*$^{\#}$	0.88 ± 0.01	0.09 ± 0.01*	0.38 ± 0.01*$^{\#}$	48.1 ± 1.3*$^{\#}$
DM-OVX + T_3	249 ± 7*$^{\#\dagger}$	1.01 ± 0.03$^{\#\dagger}$	0.09 ± 0.01*	0.41 ± 0.02*$^{\#}$	62.5 ± 0.8†
DM-OVX + PTU	275 ± 8*$^{\#\dagger}$	0.98 ± 0.02†	0.09 ± 0.01*	0.36 ± 0.01*$^{\#}$	16.3 ± 0.7*$^{\#\dagger}$

Values are means ± SE of 8 rats each group. SHAM, sham-operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic-ovariectomized; E_2, estrogen; INS, insulin; T_3, triiodothyronine; PTU, propyl-2 thiouracil. $P < 0.05$, significant difference from SHAM (*), OVX ($^{\#}$), and DM (†) groups, respectively.

Table VIII Maximum myofilament ATPase activity from SHAM and, OVX, DM, and DM-OVX rats with triiodothyronine (T_3) or propylthiouracil (PTU) treatment.

No	Maximum ATPase Activity (nmole Pi/ mg protein/ min)						
	SHAM	OVX		DM		DM-OVX	
	+ Oil	+ T_3	+ PTU	+ T_3	+ PTU	+ T_3	+ PTU
1	173.7	180.5	97.7	189.2	91.0	195.1	102.3
2	175.5	188.0	103.2	190.9	92.6	187.6	96.5
3	170.0	172.7	96.7	198.2	96.0	188.7	96.9
4	204.9	182.5	98.0	184.3	94.0	185.9	96.9
5	193.1	180.5	95.6	199.6	96.0	189.6	92.3
6	194.9	171.3	102.1	193.0	87.0	192.5	96.7
7	180.1	196.7	93.5	199.7	95.0	198.6	103.9
8	181.4	190.1	106.3	193.7	96.7	199.5	98.5
9	193.1	192.7	94.3	183.5	96.0	203.5	101.5
10	173.7	193.2	95.7	189.2	104.3	186.3	101.2
11	178.7	187.0	95.8	174.3	106.2	186.3	92.3
12	194.2	187.1	92.5	201.5	99.5	189.2	95.1
MEAN	184.4	180.1	97.6*	191.4	96.2*	191.9	97.8*
SE	3.2	2.1	1.2	2.3	1.5	1.7	1.1
n	12	12	12	12	12	12	12

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significance difference form SHAM using Student Newman-Keuls test after ANOVA

Table IX Relative amount of α-MHC to total MHC expression of left ventricular papillary muscle from SHAM, and OVX, DM, and DM-OVX rats with triiodothyronine or propylthiouracil treatment

No	% α-MHC						
	SHAM	OVX		DM		DM-OVX	
	+ Oil	+ T_3	+ PTU	+ T_3	+ PTU	+ T_3	+ PTU
1	61.5	61.1	0.0	76.1	0.0	78.7	9.8
2	61.2	75.5	00	69.0	9.5	68.3	8.9
3	67.2	68.3	0.0	76.4	8.3	67.8	0.0
4	77.1	71.1	4.2	71.7	6.0	69.6	0.0
5	76.2	72.7	7.2	79.7	0.0	60.9	0.0
6	75.5	64.6	8.3	69.6	0.0	78.6	6.5
7	69.4	66.3	7.0	65.1	0.0	73.6	8.6
8	70.1	70.5	0.0	59.0	3.5	73.1	0.0
9	76.1	79.6	4.2	57.7	8.5	72.3	9.8
10	66.3	68.4	9.6	60.0	5.9	57.2	6.9
11	69.3	76.9	10.7	66.7	7.9	67.8	6.5
12	68.8	71.9	0.0	72.9	8.0	68.5	0.0
MEAN	69.9	68.3	4.3*	68.7	4.8*	69.7	4.8*
SE	1.6	2.9	1.2	2.1	1.1	1.8	1.3
n	12	12	12	12	12	12	12

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference from SHAM using Student Newman-Keuls test after ANOVA.

Table X Body weight (BW), heart weight (HW), uterine weight (UW) and % heart weight/ body weight (% HW/BW)

GROUPS	BW (g)	HW (g)	UW (g)	% HW/BW
SHAM	270 ± 3	0.90 ± 0.01	0.41 ± 0.01	0.33 ± 0.02
OVX	340 ± 6 *	1.00 ± 0.02 *	0.09 ± 0.01 *	0.29 ± 0.01 *
DM	228 ± 9 *#	0.81 ± 0.01 *#	0.31 ± 0.01 *#	0.36 ± 0.02 *#
DM-OVX	246 ± 9 *#	0.92 ± 0.03 #	0.09 ± 0.00 *	0.37 ± 0.02 *#
DM-OVX + E_2	233 ± 5 *#	0.86 ± 0.02 *#	0.37 ± 0.03 #	0.37 ± 0.02 *#
DM-OVX + INS	355 ± 8 *	1.01 ± 0.01 *	0.09 ± 0.01 *	0.29 ± 0.01 *
DM-OVX + E_2 + INS	263 ± 4 #	0.90 ± 0.01 #	0.40 ± 0.01 #	0.34 ± 0.01 #
DM + INS	268 ± 5 #	0.91 ± 0.01 #	0.41 ± 0.01 #	0.34 ± 0.01 #

Values are means ± SE of 8 rats each group. SHAM, sham-operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic-ovariectomized; E_2, estrogen; INS, insulin. $P < 0.05$, significant difference from SHAM (*) and OVX (#) groups.

Table XI Maximum of SR Ca^{2+}-uptake activity of left ventricular homogenate from SHAM, OVX, DM rats with and without insulin supplementation, DM-OVX rats with estrogen and/or insulin supplementation.

No	Maximum SR Ca^{2+}-Uptake Activity (nmole/ mg protein/ min)							
	SHAM	OVX	DM		DM-OVX			
	+ Oil	+ Oil	+ Oil	+ INS	+ Oil	+ E_2	+ INS	+ E_2 + INS
1	130.4	102.4	84.7	143.7	100.9	99.0	95.8	121.0
2	140.1	87.4	96.9	131.8	107.5	88.2	106.8	132.6
3	133.7	95.0	100.2	129.3	108.7	99.0	98.1	137.6
4	141.6	90.0	99.8	121.5	98.2	102.6	98.5	133.0
5	136.9	89.9	102.6	130.7	74.5	102.7	98.6	136.9
6	136.1	91.8	91.6	130.9	88.8	103.0	101.5	136.6
7	133.0	98.8	71.5	127.0	82.0	97.4	103.1	131.9
8	140.6	103.2	77.4	135.3	80.8	90.1	100.7	127.2
9	134.1	97.9	92.4	142.3	92.2	101.0	100.6	123.5
10	142.4	95.2	99.1	138.0	87.7	85.5	103.5	121.4
11	136.1	98.0	93.4	139.7	92.5	93.5	96.7	133.5
12	134.4	86.0	95.0	138.5	92.5	92.5	94.5	135.0
MEAN	136.6	94.6*	92.0*	134.0	92.2*	96.2*	99.8*	130.8
SE	1.09	1.63	2.77	1.92	3.01	1.75	1.02	1.75
n	12	12	12	12	12	12	12	12

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference from SHAM using Student Newman-Keuls test after ANOVA.

Table XII EC_{50} of SR Ca^{2+}-uptake activity of let ventricular homogenate from SHAM, OVX, DM rats with and without insulin supplementation, DM-OVX rats with estrogen and/or insulin supplementation.

No	EC_{50} (μM)							
	SHAM	OVX	DM		DM-OVX			
	+ Oil	+ Oil	+ Oil	+ INS	+ Oil	+ E_2	+ INS	+ E_2 +INS
1	6.17	6.38	6.28	6.18	6.30	6.35	6.29	6.24
2	6.20	6.30	6.28	6.24	6.32	6.31	6.35	6.19
3	6.17	6.33	6.34	6.21	6.26	6.31	6.34	6.18
4	6.11	6.32	6.36	6.23	6.30	6.27	6.40	6.25
5	6.24	6.30	6.32	6.21	6.30	6.28	6.37	6.20
6	6.22	6.36	6.24	6.18	6.31	6.32	6.32	6.19
7	6.22	6.34	6.33	6.20	6.28	6.38	6.32	6.26
8	6.20	6.31	6.29	6.21	6.32	6.35	6.37	6.17
9	6.21	6.31	6.35	6.19	6.31	6.34	6.43	6.19
10	6.24	6.30	6.40	6.24	6.30	6.31	6.29	6.23
11	6.22	6.30	6.25	6.22	6.30	6.31	6.26	6.24
12	6.24	6.33	6.31	6.24	6.29	6.30	6.30	6.19
MEAN	6.20	6.32*	6.31*	6.21	6.30*	6.32*	6.33*	6.21
SE	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01
n	12	12	12	12	12	12	12	12

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference from SHAM using Student Newman-Keuls test after ANOVA.

Table XIII Maximum SERCA activity in SR membrane vesicles form SHAM, OVX, DM rat with and without insulin supplementation, DM-OVX rats with estrogen and/or insulin supplementation.

No	Maximum SERCA Activity (nmole Pi/ mg protein/ min)							
	SHAM	OVX	DM		DM-OVX			
	+ Oil	+ Oil	+ Oil	+ INS	+ Oil	+ E_2	+ INS	+ E_2 + INS
1	1.31	0.51	0.72	1.15	0.84	0.78	0.90	1.09
2	1.03	0.57	0.72	1.12	0.79	0.78	0.82	1.10
3	1.11	0.80	1.00	1.05	0.89	0.80	0.77	1.10
4	1.08	0.91	0.89	1.06	0.74	0.89	0.72	1.14
5	1.10	0.80	0.74	1.08	0.77	0.86	0.72	1.09
6	1.09	0.82	0.91	1.09	0.81	0.92	0.80	1.13
7	1.17	0.83	0.80	1.11	0.86	0.78	0.84	1.10
8	1.09	0.89	0.88	1.18	0.86	0.80	0.79	1.08
9	1.14	0.94	0.63	1.10	0.89	0.80	0.97	1.14
10	1.23	0.92	0.87	1.13	0.79	0.89	0.88	1.14
11	1.17	0.98	0.63	1.15	0.78	0.87	0.82	1.07
12	1.09	0.89	0.89	1.16	0.77	0.85	0.84	1.29
MEAN	1.13	0.82*	0.81*	1.11	0.82*	0.84*	0.82*	1.12
SE	0.02	0.04	0.03	0.01	0.01	0.01	0.02	0.02
n	12	12	12	12	12	12	12	12

Results are means ± SE from each group. n = number of rats. *$P < 0.05$, significant difference from SHAM using Student Newman-Keuls test after ANOVA.

Table XIV EC_{50} of SERCA activity in SR membrane vesicles from SHAM, OVX, DM rats with and without insulin supplementation, DM-OVX rats with estrogen and/or insulin supplementation.

No	EC_{50} (μM)							
	SHAM	OVX	DM		DM-OVX			
	+ Oil	+ Oil	+ Oil	+ INS	+ Oil	+ E_2	+ INS	+ E_2 + INS
1	5.73	6.29	6.34	5.89	6.32	6.22	6.26	5.92
2	5.70	6.01	6.35	5.98	6.10	6.38	6.21	5.85
3	5.88	6.32	6.11	5.87	6.12	5.92	6.10	5.79
4	5.85	6.04	6.15	5.97	6.23	6.21	6.18	5.84
5	5.88	6.12	6.18	5.95	6.22	6.09	5.99	5.82
6	5.97	6.13	6.34	5.96	6.18	6.25	6.24	5.96
7	5.94	6.04	6.31	5.54	6.44	6.41	6.07	5.70
8	5.70	6.30	6.01	5.80	6.07	5.97	6.17	5.91
9	5.89	6.30	6.38	5.85	6.02	6.04	6.38	5.74
10	6.02	6.31	6.19	5.89	6.10	6.26	6.28	6.07
11	5.91	6.13	6.23	5.67	6.12	6.28	6.21	5.77
12	5.82	6.24	6.06	5.87	6.21	6.29	6.24	6.00
MEAN	5.86	6.19*	6.22*	5.85	6.18*	6.18*	6.19*	5.86
SE	0.03	0.03	0.04	0.04	0.03	0.04	0.03	0.03
N	12	12	12	12	12	12	12	12

Results are means ± SE from each group. n = number of rats. *$P < 0.05$ = significant difference form SHAM using Student Newman-Keuls test after ANOVA.

Table XV Relative intensity of SERCA2a and phospholamban proteins to CSQ of left ventricular homogenates from SHAM, OVX, DM rats with and without insulin supplementation, DM-OVX rats with estrogen and/or insulin supplementation.

No	Protein Expression (Relative Intensity)							
	SHAM	OVX	DM		DM-OVX			
	+ Oil	+ Oil	+ Oil	+ INS	+ Oil	+ E_2	+ INS	+ E_2 + INS
SERCA/CSQ								
1	1.15	0.96	0.92	1.15	0.86	0.86	0.92	1.24
2	1.03	0.85	0.92	1.00	0.79	0.81	0.82	1.09
3	0.97	0.88	0.93	1.11	0.79	0.89	0.82	1.03
4	1.28	0.79	0.86	1.32	0.94	0.74	0.91	1.19
5	1.02	0.95	0.79	1.08	1.00	0.63	0.91	1.11
6	1.23	0.86	0.75	1.19	0.78	0.87	0.87	1.15
MEAN	1.11	0.84*	0.85*	1.12	0.84*	0.83*	0.83*	1.13
SE	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03
n	6	6	6	6	6	6	6	6
Phospholamban/CSQ								
1	1.13	1.08	1.33	1.06	1.73	1.59	1.14	0.97
2	1.08	1.17	1.66	1.00	1.64	1.80	0.92	0.89
3	0.99	1.00	1.67	1.11	1.63	1.48	0.99	0.82
4	1.09	0.94	1.59	1.07	1.43	1.33	1.07	1.19
5	1.02	0.99	1.73	0.95	1.68	1.57	1.00	1.11
6	0.98	1.05	1.46	1.10	1.45	1.65	1.14	1.15
MEAN	1.04	1.01	1.56*	1.04	1.60*	1.60*	1.02	1.04
SE	0.02	0.03	0.04	0.02	0.04	0.05	0.03	0.04
n	6	6	6	6	6	6	6	6

Results are means ± SE from each group. n = number of rats. *$P < 0.05$ = significant difference form SHAM using Student Newman-Keuls test after ANOVA.

Table XVI Relative intensities of the phosphor-Ser[16] and the phosphor-The[17] phospholamban to actin of left ventricular homogenates from SHAM, OVX, DM rats with and without insulin supplementation, DM-OVX rats with estrogen and/or insulin supplementation.

No	Protein Expression (Relative Intensity)							
	SHAM	OVX	DM		DM-OVX			
	+ Oil	+ Oil	+ Oil	+ INS	+ Oil	+ E_2	+ INS	+ E_2 + INS
Phospho-Ser[16] phospholamban/Actin								
1	1.17	1.13	0.85	1.10	1.08	0.84	1.26	1.28
2	1.21	1.33	0.74	1.08	0.74	0.94	1.08	1.12
3	1.14	1.03	0.99	1.07	0.83	0.85	1.02	1.20
4	1.18	1.18	0.92	1.29	0.86	0.66	1.30	1.06
5	1.29	1.13	0.85	1.11	0.74	0.97	1.23	1.25
6	1.20	1.08	0.96	1.08	0.86	0.84	1.06	1.18
MEAN	1.18	1.15	0.84*	1.14	0.83*	0.81*	1.14	1.16
SE	0.03	0.03	0.04	0.03	0.04	0.04	0.05	0.03
n	6	6	6	6	6	6	6	6
Phospho-Thr[17] phospholamban/Actin								
1	1.11	0.77	0.66	1.04	0.70	0.75	0.77	1.17
2	1.06	0.80	0.66	0.95	0.75	0.62	0.61	1.05
3	1.21	0.96	0.90	1.13	0.91	1.03	0.95	0.99
4	1.03	0.82	0.86	1.06	0.93	0.75	0.70	0.97
5	1.14	0.56	0.76	1.16	0.67	0.82	0.81	1.08
6	1.21	0.78	0.85	1.10	0.72	0.75	0.74	1.28
MEAN	1.15	0.78*	0.78*	1.14	0.77*	0.80*	0.78*	1.14
SE	0.03	0.04	0.04	0.04	0.03	0.04	0.03	0.05
n	6	6	6	6	6	6	6	6

Results are means ± SE from each group. n = number of rats. *$P < 0.05$ = significant difference form SHAM using Student Newman-Keuls test after ANOVA.

Table XVII The proportion of the monomer phospholamban of left ventricular homogenates prepared from SHAM, OVX, DM rats with and without insulin supplementation, DM-OVX rats with estrogen and/or insulin supplementation.

No	% monomer of phospholamban Protein Expression (Relative Intensity)							
	SHAM	OVX	DM		DM-OVX			
	+ Oil	+ Oil	+ Oil	+ INS	+ Oil	+ E_2	+ INS	+ E_2 + INS
1	9.00	18.35	26.97	14.43	28.78	28.17	19.50	9.18
2	8.90	17.16	22.84	9.99	28.13	28.28	17.64	8.84
3	9.43	22.13	27.81	9.12	23.66	22.79	13.54	9.96
4	11.00	14.98	28.00	10.85	23.99	24.48	24.70	9.89
5	7.60	16.90	24.07	10.98	28.91	33.00	20.71	9.28
6	9.39	27.97	35.16	12.81	24.02	25.63	19.73	12.25
MEAN	10.85	19.8*	26.68*$^{\#}$	11.20	27.48*$^{\#}$	27.49*$^{\#}$	20.01*	11.09
SE	0.93	1.32	1.44	0.54	1.05	1.03	1.35	0.85
n	6	6	6	6	6	6	6	6

Results are means ± SE from each group. n = number of rats. $P < 0.05$, significant difference from SHAM (*) and OVX ($^{\#}$) groups, respectively, using Student Newman-Keuls test after ANOVA.

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