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รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัยเรื่อง

บทบาทของฮอร์โมนเพศ ต่อสรีรวิทยาของกล้ามเนื้อหัวใจ (2)

โดย

รศ. จงกลณี วัฒนาเพิ่มพูล และคณะ

สิงหาคม 2554

สัญญาเลขที่ BRG 5180009

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย และ มหาวิทยาลัยมหิดล ที่ให้ทุนสนับสนุนโครงการวิจัยนี้ ขอขอบพระคุณ Prof. Dr. R. John Solaro (Department of Physiology & Biophysics University of Illinois at Chicago), Prof. Pieter de Tombe (Department of Cell and Molecular Physiology, Loyola University Chicago) และ ศ.ดร. นทีทิพย์ กฤษณามระ (ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล) ที่ให้คำปรึกษาอย่างต่อเนื่องตลอดโครงการ โดยเฉพาะ Prof. Pieter de Tombe ซึ่งกรุณามอบเครื่องมือและอุปกรณ์สำหรับการตรวจวัดแรงหดตัวให้แก่ห้องปฏิบัติการ พร้อมเดินทางมาให้ความช่วยเหลือในการประกอบชุดตรวจวัดด้วยตัวเอง และขอบพระคุณ ศ.ดร.ประพนธ์ วิไลรัตน์ ที่กรุณาให้คำแนะนำปรึกษา และวิจารณ์แก้ไข manuscript ให้เป็นอย่างดียิ่ง

สุดท้ายนี้ขอขอบคุณภาควิชาสรีรวิทยา นางโกสุม วงศ์ไทย และ นางสาวชลลดาย์ สาพียะ ที่ช่วยทำบัญชีเบิกจ่ายและพิมพ์รายงานต่างๆ และ นางสาวน้ำทิพย์ วิทยะวาณิชกุล และ นางสาวประภาวดี ภิรมย์พล นักศึกษาบัณฑิตภายใต้การควบคุมดูแลที่มีส่วนร่วมดำเนินงานวิจัยให้สำเร็จลุล่วง

บทคัดย่อ

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การศึกษาก่อนหน้าได้รายงานบทบาทสำคัญของฮอร์โมนเพศต่อการทำงานของหัวใจ จึงเน้นศึกษาต่อเนื่องถึงผลกระทบของฮอร์โมนเพศหญิงต่อ 1) การเปลี่ยนแปลงปฏิกิริยาการดูดกลับแคลเซียมโดย sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) และ 2) จลนศาสตร์การจับตัวของโปรตีนกล้ามเนื้อหัวใจในหนูตัดรังไข่ที่มีเบาหวานแทรกซ้อน อีกทั้งศึกษานัยสำคัญของฮอร์โมนเพศชายในด้าน 1) ผลกระทบต่อกลศาสตร์การหดตัวของกล้ามเนื้อหัวใจทั้งในหนูขาดฮอร์โมนเพศหรือได้รับฮอร์โมนสูง 2) ความสัมพันธ์ของภาวะกล้ามเนื้อหัวใจโตกับการทำงานของ SERCA และ 3) สัญญาณในการกระตุ้นการเกิดภาวะกล้ามเนื้อหัวใจโตเมื่อมีฮอร์โมนเพศชายสูง

การทำงานของ SERCA ที่ลดลงเท่ากันในหัวใจหนูที่ขาดฮอร์โมนเพศหญิงอย่างเดียวและเมื่อเป็นเบาหวานร่วมด้วย แสดงถึงการทำงานร่วมกันของทั้งฮอร์โมนเพศหญิงและอินซูลินในการควบคุมปริมาณแคลเซียมภายในเซลล์กล้ามเนื้อหัวใจด้วยปฏิกิริยาของ SERCA และเมื่อเปรียบเทียบผลของฮอร์โมนเพศหญิงและอินซูลินต่อจลนศาสตร์ของปฏิกิริยาการจับตัวของโปรตีนกล้ามเนื้อในหัวใจ พบผลกระทบที่แตกต่างกัน ซึ่งชี้ให้เห็นถึงอิทธิพลของฮอร์โมนเพศหญิงต่อการทำงานของเส้นใยกล้ามเนื้อหัวใจที่นอกเหนือจากการควบคุมปริมาณของโปรตีน myosin heavy chain (MHC)

การศึกษาผลกระทบของการขาดฮอร์โมนเพศชายในหนูตัดอัณฑะ พบว่าการขาดฮอร์โมนเพศชายส่งผลให้แรงการหดตัวของกล้ามเนื้อหัวใจลดลงพร้อมมีการเปลี่ยนแปลงของ MHC ซึ่งส่อถึงสภาวะพยาธิสภาพของหัวใจ ในทางตรงข้าม สภาวะที่มีฮอร์โมนเพศชายสูงจะเหนี่ยวนำให้เกิดภาวะกล้ามเนื้อหัวใจโตที่เป็นปกติหรือมีพยาธิสภาพขึ้นกับทั้งปริมาณและช่วงเวลาของการได้รับฮอร์โมน โดยช่วงเวลาที่สั้นจะส่งผลให้เกิดภาวะกล้ามเนื้อหัวใจโตที่มีการทำงานปกติแต่เมื่อได้รับฮอร์โมนเป็นระยะเวลานานจะส่งผลให้เกิดภาวะกล้ามเนื้อหัวใจโตที่มีพยาธิสภาพ ซึ่งสามารถยืนยันได้จากการมีปริมาณคอลลาเจนเพิ่มขึ้นในหัวใจ

เมื่อศึกษาความสัมพันธ์ของภาวะกล้ามเนื้อหัวใจโตจากการมีฮอร์โมนเพศชายสูงกับปฏิกิริยาของ SERCA พบว่าไม่มีความเกี่ยวเนื่องกัน แต่ในทางตรงกันข้าม การเกิดภาวะกล้ามเนื้อหัวใจโตกลับมีผลกระทบให้เกิดการเปลี่ยนแปลงการทำงานของปฏิกิริยาเอ็นไซม์ SERCA ซึ่งบ่งบอกถึงผลของฮอร์โมนเพศชายในการเหนี่ยวนำให้ SERCA ปรับการทำงานให้มากขึ้น แต่ในที่สุดการปรับตัวจะล้มเหลวเมื่อเข้าสู่สภาวะมีพยาธิสภาพหลังได้รับฮอร์โมนเพศเป็นเวลานาน ทั้งนี้ สัญญาณในการกระตุ้นให้เกิดภาวะกล้ามเนื้อหัวใจโตเมื่อมีฮอร์โมนเพศชายสูงนั้น อาจผ่านกระบวนการของวิถี Calcineurin-NFAT

คำหลัก: ฮอร์โมนเพศ การดูดกลับแคลเซี่ยมที่ซาร์โคพลาสมิคเรติคูลั่ม จลศาสตร์การจับตัวดตัวของโปรตีนกล้ามเนื้อ ภาวะหัวใจโต

ABSTRACT

Project Code : BRG5180009
Project Title : Role of Sex Hormones in Cardiac Muscle Physiology (II)
Investigator : Assoc. Prof. Dr. Jonggonnee Wattanapermpool
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We have reported the significant role of female sex hormones in cardiac contractile activity in a series of experiment. The present study was further investigated the significance of female sex hormones in two aspects of 1) the adaptive alterations in SR Ca^{2+} uptake activity and 2) the significance of cross-bridge kinetics in regulating cardiac myofilament activity in OVX rats complicated with diabetes. The significance of male sex hormones on the cardiac activation was also evaluated in three aspects, including 1) the mechanical contractile properties of skinned fiber preparation from castrated and testosterone-treated rats, 2) the relations of cardiac hypertrophy induced by testosterone to the SR Ca^{2+} uptake function, and 3) the signaling component involves in testosterone-induced cardiac hypertrophy.

Similar suppression of cardiac SR Ca^{2+} uptake activity between estrogen- and insulin-deficient conditions without additive effect provides clear evidence on the interactive effect of the two hormones in regulating the intracellular Ca^{2+} handling by changes both the activity and the content of SERCA2a protein in cardiomyocytes. The significant role of estrogen in the cross-bridge kinetics was further tested by evaluating the correlation of maximum myofibrillar ATPase activity to various levels of α-MHC in the heart of OVX rats compared to that of diabetic rats. Different expression levels of α-MHC in the heart of OVX and diabetic rats will be varied by manipulating the plasma thyroid hormone by tri-iodothyronin injection or 6-n-propyl-2-thiouracil administration. Our results indicate that estrogen, in contrast to insulin, plays an additional regulatory role on the cross-bridge cycling kinetics through changes in the myofilament proteins besides its effect on the expression of MHC isoforms. Our results also confirm that insulin indirectly affects the cross-bridge cycling kinetics through the direct effect of thyroid hormones on the α-MHC expression.

Concerning effects of male sex hormones, testosterone deprivation after orchidectomy (ORX) induced a reduction in maximum force contraction with isoform shift of MHC toward pathological development. In contrast, testosterone administration induced either physiologic or pathologic cardiac hypertrophy depending on both the dose and the duration of hormone treatment. Physiologic cardiac hypertrophy was observed only with short term treatment but pathologic hypertrophy will be the case when the treatment is prolonged. The induction of pathological cardiac hypertrophy was further confirmed by an increase in the content of collagen.

Association of cardiac hypertrophy to changes in SERCA activity after high testosterone treatment was further analyzed. Interestingly, our results show no association between SERCA activity and testosterone-induced cardiac hypertrophy. It is, however, the cardiac hypertrophy reversely affected the Ca^{2+} pumping activity of SERCA indicating an adaptive change of SERCA after prolonged exposure to high testosterone which may eventually lead to deterioration of the heart if the high testosterone persists. The signaling components in testosterone-induced pathologic hypertrophy of the heart possibly involve the process of calcineurin-NFAT pathway but results are still inconclusive.

Keywords: Sex hormones, SR Ca^{2+} uptake, cross-bridge cycling kinetics, cardiac hypertrophy

CONTENTS

INTRODUCTION

Heart failure is the most common outcome of many cardiovascular diseases, such as ischemic heart, hypertension, valvular diseases, and idiopathic cardiomyopathy, and also the leading cause of death in both Western and Eastern countries including Thailand. Many risk factors of heart failure including age, sex, nationality, smoking, hypertension, hyperlipidemia, and diabetic mellitus have been indicated from various epidemiological studies [1-6]. Despite many known risk factors, the basic knowledge, the mechanisms, and the treatment regimen of the disease are still not clearly understood. Based on these well recognizable sex differences in the incidence of cardiovascular diseases, the present study is therefore aimed to understand the nature, sites, and mechanisms of how sex hormones regulate cardiac myofilament activity.

An important question related to the well recognizable sex difference in the incidence of cardiovascular diseases is the significance of sex hormones on cardiac function. A better survival of women with advanced heart failure than men indicates a sex-specific adaptation to cardiac stress [7]. Epidemiological data have clearly shown a lower cardiac mortality in women compared to age-matched men until the age of menopause [6, 8]. The cardiovascular disease incidence is highly elevated in menopausal female resulting in a decreased male to female ratio of cardiac mortality. An increased risk of coronary heart disease was also observed in young women undergone bilateral oophorectomy in which appears to be prevented by estrogen-replacement therapy [9]. The information thus point to two potential effects of sex hormones on the heart including a protective effect of female sex hormones but a risky effect of male sex hormones. Based on the two main targets of the disease, coronary vessels and myocardium, effects of sex hormones on coronary heart diseases are better defined [6, 10-14]. On the other hand, effects of sex hormones on cardiac muscle physiology have been relatively discounted despite the significant function as the machinery tools for the pump of life as well as the presence of sex hormone receptors on the myocardium [15-17]. The long-term objective of this study is therefore focused on understanding the significant role of sex hormones in cardiac muscle physiology especially the cardiac adaptations induced after hormone deprivation or exogenous loading.

Our current understanding of the significance of female sex hormones on cardiac muscle activation has been achieved in a series of experiment using a variety of techniques [18-25]. Both subcellular and molecular adaptations of cardiac muscle activity have been demonstrated after female sex hormone deprivation. Using whole heart preparations, a reduction in cardiac performance as measured by stroke work, ejection fraction, and fractional shortening after ovarian sex hormone deprivation have been reported implicating a primary defect of cardiac function in developing heart diseases after menopause [26-28]. Using subcellular preparations of the ventricular tissue, we have further shown a suppressed maximum myofibrillar ATPase activity with a significant shift in myosin heavy chain (MHC) expression towards β-MHC isoform in ovariectomized rat hearts [18, 19]. An increase in Ca^{2+} responsiveness of cardiac myofilament activation in ovariectomized rats was uniquely indicated

from both biochemical and mechanical measurements [18, 19]. Theses changes in ovariectomized rat hearts could be prevented by estrogen supplementation [20].

One striking issue related to these findings is the similar Ca^{2+} hypersensitive adaptation detected in ovariectomized rat hearts to that of cardiomyopathy with almost the same magnitude of changes [29-31] in addition to the suppressed maximum ATPase activity. Thus, a potential of cardiomyopathic development in ovarian sex hormone-deprived condition is absolutely indicated. We therefore tested whether there are significant changes in the intracellular Ca^{2+} mobilization which may underlie the Ca^{2+} hypersensitivity of the cardiac myofilament after ovarian sex hormone deprivation. Our data showed for the first time that female sex hormones play an important role in regulating the Ca^{2+} uptake activity of cardiac sarcoplasmic reticulum (SR) by inducing an adaptive response of the SR Ca^{2+} ATPase activity (SERCA) that escaped the regulatory effect of phospholamban [22]. We have also reported that deprivation of female sex hormones modulates the intracellular Ca^{2+} concentration in cardiac myocytes, possibly via an increased Na^{+}-H^{+}-exchanger-1 activity, which may act in concert with Ca^{2+} hypersensitivity of myofilament activation as a determinant of sex differences in cardiac function [23]. All information thus point to the significance of female sex hormones in cardio-protective function. Interestingly, with the combined introduction of diabetic cardiomyopathy, we found that the hypersensitivity of cardiac myofilament to Ca^{2+} is specifically induced in ovariectomized rats which may determine the gender difference in cardiac muscle activation [24]. Further investigations on mechanisms underlying the Ca^{2+} hypersensitivity and the suppressed maximum ATPase activity of cardiac myofilament in ovariectomized rats challenged with diabetes will certainly provide more insights on the role of sex hormones in the contractile function. We, therefore, propose experiments guided by a question how diabetes complicates the functional significance of female sex hormones in the intracellular Ca^{2+} mobilization and the myofilament crossbridge kinetics of the heart.

The proposal that modification of the intracellular Ca^{2+} mobilization by ovarian sex hormones is an important modulating device for myofibrillar response to Ca^{2+} was nicely proved from our previous project. We concluded that Ca^{2+} hypersensitivity of cardiac myofilament is an adaptative response to changes in intracellular free Ca^{2+} availability induced after ovariectomy. Change in intracellular free Ca^{2+} is one potential cause of cardiac myofilament alteration and adaptation [32-36]. We have recently reported a decrease in Ca^{2+} transient amplitude implying a reduced Ca^{2+} activation on the myofilament during the cardiac contraction/relaxation cycle in the cardiomyocyte of ovariectomized rats [23]. It is interesting that changes in Ca^{2+} transients and the myofilament Ca^{2+} sensitivity detected in ovariectomized hearts are all in the same direction as those changes reported in many heart failure models [18-25, 29-31, 37-41]. Deficit in myocyte Ca^{2+} storage due to either mutation of SERCA or over-activity of phospholamban has been shown to also induce the impairment leading to heart failure [33, 36]. On the other hand, stimulation of the SR Ca^{2+} uptake activity by transgenic improvement of SERCA provided better cardiac function and life span in dilated hypertrophy-induced mouse [32, 34]. We have shown that deprivation of ovarian sex hormones induced a decrease in the cardiac SR Ca^{2+} uptake activity through suppression of both the activity and the expression of the SERCA pump [22]. The suppressed uptake activity was due, in part, to

an increased interaction of SERCA with the nonphosphorylated phospholamban proteins. In addition, estrogen and progesterone equally exerted a regulatory effect on SR Ca^{2+} uptake function. As mentioned, we have also investigated the role of female sex hormones in cardiac activation under diabetes-induced dysfunction to the heart and found that hypersensitivity of cardiac myofilament to Ca^{2+} is specifically induced in ovariectomized rats even under diabetes complication which may determine the gender difference in cardiac activation [24]. It is, therefore, interesting to study whether the altered SR Ca^{2+} uptake activity may also, in part, play a mechanistic role underlying the cardioprotective effects of female sex hormones in determining the gender difference in cardiac activation.

Besides inducing myofilament Ca^{2+} hypersensitivity, a suppression of maximum myofilament ATPase activity was also shown in the heart of ovariectomized rats. The interactive effect of female sex hormones and insulin on the maximum myofibrillar ATPase activity reported recently [24] indicates a final common pathway of the hormones 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 hearts of ovariectomized and diabetic rats [19, 42]. 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 ovariectomized group [24]. The information thus suggests an additional mechanism besides MHC isoform shift in responsible for the regulatory effects of estrogen on the cardiac contractile activation.

Generally, differential responses of cardiac contractile activation could be molecularly induced by alterations in cross-bridge interactions either at the number of cross-bridge interactions or at the amount of cross-bridge single force [43, 44]. A significant reduction in the active cross-bridge number without changes in the cross-bridge single force has been reported in diabetic rats [45]. Contrastly, there is no data concerning modifications of cross-bridge interactions in the hearts under estrogen-deficient condition. Evidence of myofilament Ca^{2+} hypersensitivity in the hearts of ovariectomized rats [18] suggests a possibility that changes in cross-bridge kinetics may account in part for the suppression of cardiac contractile activity induced by estrogen deficiency. Moreover, evidence of higher cardiac MHC content in ovariactomized rats with the same level of maximum myofibrillar ATPase activity as those of diabetic rats[24] further implies a significant reduced cross-bridge cycling rate in the hearts of ovariectomized rats. Whether estrogen deficiency induces changes in cardiac contractile responses through modifications in both the quantity and quality of cross-bridge interactions remains to be elucidated. Results from this study may be applied to the effect of male sex hormones, which has been found to also induce a suppressed maximum myofibrillar ATPase activity in the hearts of castrated rats.

In considering the influence of male sex hormones on cardiac function, most of the information are still controversy and far less studied than those of female. Previous evidence from both pre-pubertal and pubertal castrated rats demonstrated the same direction of changes in cardiac performance as those detected in female sex hormone deficient group [46]. The information thus weaken the possible risky effect for heart disease induction but instead point

to a significant function of male sex hormones for maintaining normal contractile activity. Our preliminary studies in castrated and high plasma testosterone rats indicate significant effects of male sex hormones on cardiac myofilament activation with suggested differential effects under different levels of the hormone. An important finding related to the effects of male sex hormones is a significant induction of cardiac hypertrophy in high plasma testosterone rats without changes in myofilament activation. Despite the consideration of hypertrophy as an adaptive response required to sustain cardiac output in the face of biomechanical stress, prolonged hypertrophy is associated with a significant progression to heart failure [47]. This finding raises question whether male sex hormones-induced hypertrophy does any good or whether it may initially adaptive but finally lead to cardiac demise. Strategies to stimulate or inhibit the induction of cardiac hypertrophy in the case may provide preventive and/or therapeutic value to the setting of heart failure. We, therefore, proposed experiments to investigate the mechanical changes in cardiac performance impacted by testosterone in male rats. The signaling component imparted in testosterone-induced cardiac hypertrophy will also be concentrated using both the functional indicator linked to SR function and the signaling transduction pathway.

Therefore, the hypothesis of this study is that sex hormones play significant role in modulating cardiac contraction and relaxation. The specific objectives for the study have been focused on the following aspects:

I. To determine the adaptive alterations in SR Ca^{2+} uptake activity in the heart of ovariectomized rats complicated with diabetes

II. To evaluate the significance of cross-bridge kinetics in regulating cardiac myofilament activity in ovariectomized rats complicated with diabetes

III. To analyze the mechanical contractile properties of cardiac muscle fiber prepared from castrated and high testosterone rats

IV. To elucidate the relations of cardiac hypertrophy induced in high plasma testosterone rats to the SR Ca^{2+} uptake function

and **V.** To determine the signaling component, physiologic or pathologic pathway, that involves in testosterone-induced cardiac hypertrophy

RESEARCH APPROACHES AND METHODS

In section I, we describe the general approach to the specific aims. In section II, we describe the preparations and procedures as well as general methods and statistical analysis.

Section I: Research Approaches to Specific Aims.

Approach to Aim #1: To determine the adaptive alterations in SR Ca^{2+} uptake activity in the heart of ovariectomized rats complicated with diabetes

We have recently reported a dominant effect of ovarian sex hormone deficiency in inducing Ca^{2+} hypersensitivity of cardiac myofilament, a determinant of the gender difference in cardiac activation, under diabetes complication, emphasizing the significance of enhanced myofilament response to Ca^{2+} in the pathophysiology of the heart in ovariectomy [24]. The mechanistic adaptation underlying the cardioprotective effects of estrogen involves elevated expression of β_1-adrenoceptors and loss of protective factor, heat shock protein 72. We have also demonstrated that ovarian sex hormones exert an important cardio-regulatory role in inducing adaptive alterations of the SR Ca^{2+} uptake [22].

Using diabetes complication, the study here is therefore designed to evaluate whether changes in SR Ca^{2+} uptake activity could serve another specific mechanistic adaptation underlying the cardioprotective effects of estrogen. Measurements of both SR Ca^{2+} uptake and SERCA activity will be performed in various groups of animal including sham, ovariectomized rats with and without estrogen supplementation, diabetic rats with and without insulin supplementation, and diabetic-ovariectomized rats with and without estrogen, insulin, or estrogen plus insulin supplementation for the 10-weeks period of study. The SR protein contents including SERCA, calsequestin, and RYR-CRC will also be quantified using Western Blot analysis.

Approach to Aim #2: To determine the significance of cross-bridge kinetics in regulating cardiac myofilament activity in ovariectomized rats complicated with diabetes

As recently reported from our laboratory, the interactive effect of female sex hormones and insulin on the maximum myofibrillar ATPase activity indicates a final common pathway of the hormones in regulating the cardiac contractile activity. It is well recognized that the shift in the isoform population of MHC underlies the suppressed maximum myofilament ATPase activity in ovariectomized and diabetic rat hearts. Supportively, possible direct effects of estrogen and insulin on MHC expression have clearly been demonstrated. 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 ovariectomized rat hearts. The information thus suggests an additional mechanism besides the shift in MHC isoforms in responsible for the regulatory effects of estrogen on the cardiac contractile activation. We have therefore set the last objective in our first Basic Research Grant to investigate the

potential regulatory effects of estrogen on the cross-bridge interactions with diabetes challenge. The relationships of % α-MHC and maximum myofibrillar ATPase activities from various experimental groups similar to those of Aim #1 were compared.

Results from our preliminary study indicate differential effects of estrogen and insulin in regulating maximum myofibrillar ATPase activity. In contrast to insulin, estrogen possibly exerts an additional regulatory effect on variables of the cross-bridge kinetics besides its effect on the number of cross-bridge. In this proposal, we then further test the significant role of estrogen in the cross-bridge kinetics by evaluating the correlation of maximum myofibrillar ATPase activity to various levels of the % α-MHC in the heart of ovariectomized rats compared to that of diabetic rats. Different levels of α-MHC expression in the hearts of ovariectomized and diabetic rats will be varied by manipulating the plasma thyroid hormone status using tri-iodothyronin (T_3) injection or 6-n-propyl-2-thiouracil (PTU) administration.

Approach to Aim #3: To determine the mechanical contractile properties of cardiac muscle fiber prepared from castrated and high plasma testosterone hearts

Our preliminary results have demonstrated significant biochemical changes in cardiac myofilament Ca^{2+} activation in 10-weeks castrated rats. On the other hand, a significant cardiac hypertrophy was detected in 10-weeks high testosterone-treated male rats without affecting the maximum myofibrillar actomyosin ATPase activity. It is, however, not known how the mechanical changes in which will provide more insight on physical properties of the heart would be apparent in the castrated and high plasma testosterone hearts. The mechanical properties of the cardiac fibers prepared from both castrated and high plasma testosterone hearts will be measured with the parallel experiments of sham hearts as control using skinned fiber technique. The shift in the MHC isoforms of individual fiber used for force measurement will also be analyzed to clarify the correlation of developed force to the MHC isoforms in each experimental group.

Approach to Aim #4: To determine the relations of cardiac hypertrophy induced in high plasma testosterone male hearts to the SR Ca^{2+} uptake function

As previously mentioned, a significant cardiac hypertrophy as indicated by an increased heart to body weight ratio was clearly demonstrated in high testosterone-treated male rats. Although it is known that androgen could modulate the cardiac phenotype and produce hypertrophy [17], the functional consequences and the mechanism of hypertrophic induction are still unclear. It has also been suggested that impaired SR function could present a potential mechanism of disease leading to contractile dysfunction, heart failure, and hypertrophy [48]. It is then questioned whether high plasma testosterone induces changes in SR function and consequently induces hypertrophy of the hearts?

Our approach will be first focused on determining the relations of cardiac hypertrophy to both the SR Ca^{2+} uptake and SERCA activity in different plasma testosterone male rats from

different doses of hormone injection. The cross sectional area of cardiomyocytes and the amount of interstitial collagen deposition will be measured in the heart of high testosterone-treated rats by histochemical approach. An expression of gap junction protein connexin43, which indicates a possibility of arrhythmic induction in cardiac hypertrophy, will also be analyzed together with N-cadherin as a loading control. The expression and the activity of SERCA will be determined using immunoblot and enzyme kinetic assay, respectively. Moreover, the expression and the phosphorylation level of phospholamban, a SERCA regulatory protein, will be examined using immunoblot analysis.

Approach to Aim #5: To determine the signaling component, physiologic or pathologic pathway, that involves in testosterone-induced cardiac hypertrophy

Cardiac hypertrophy is a remodeling response of the heart to many insults that lead to a better cardiac performance in case of physiological hypertrophy or a sustained/decreased performance in pathological hypertrophy depending on type, degree, and exposed frequency of stress. It is well accepted that both remodeling processes share some signaling processes besides the different signaling mechanisms. It is presently unknown whether the hypertrophy of the heart induced by testosterone is physiological or pathological. The study is therefore aimed to determine the specific signaling process of action in high testosterone-treated rats. Changes in the activation, expression, and/or phosphorylation of potential pathological signals, including calcineurin, mitogen-activated protein kinase (MAPK: p38, JNK, $ERK_{1/2}$), and nuclear factor of activated T cell (NFAT) will be analyzed. On the other hand, phosphorylation and total levels of Akt (also known as protein kinase B, PKB) and the mammalian-target of rapamycin (mTOR) will also be measured to represent the physiological signal of cardiac hypertrophy.

Based on the well-recognized effect of exercise training in inducing physiological hypertrophy of the heart, the experimental rats will also be introduced with the combined treatment of high testosterone and nine-weeks running program. Results from this set of study will provide information concerning the preventive or additive effect of exercise training on inducing cardiac hypertrophy.

Section II. Preparations and Procedures

1. Maximum Cardiac Myofibrillar Actomyosin Mg^{2+}-ATPase Activity

Cardiac myofibrils will be prepared from the left ventricles as described by Pagani and Solaro [49]. Maximum Ca^{2+}-dependent Mg^{2+}-ATPase activity of isolated myofibrils will be assayed by determination of inorganic phosphate released in a 10-min linear reaction at 30°C in 2 mM Mg^{2+}, 60 mM imidazole, 5 mM $MgATP^{2-}$, pH 6.5 and pH 7.0, and ionic strength of 120 mM. Assays are run in the free Ca^{2+} concentration of –log free Ca^{2+} concentration (pCa) 5.125 and 4.875. Total concentrations of $CaCl_2$, EGTA, KCl, $MgCl_2$, and ATP are calculated using a computer program generated from the dissociation constants given by Fabiato [50]. The concentration of inorganic phosphate will be measured by the method of Carter and Karl [51].

2. Gel electrophoresis of MHC isoforms

MHC isoforms of left ventricular trabeculae were preformed using SDS-PAGE as described with the following modification [52]. Trabeculae was isolated from left ventricle in saline at 4°C, frozen immediately in liquid nitrogen, pulverized while frozen, and the sample powder was added with Laemmli sample buffer. The samples were then sonicated for 1 hr at 4°C and finally centrifuged in a microcentrifuge at 10,000 rpm for 5 min. The supernatant was collected and measured the total protein concentration by the bicinchoninic acid method, and then the 2-mercaptoethnol was added in sample to a final concentration of 5% (v/v). The sample was aliquoted and stored at -80°C. Sample was loaded onto well of a discontinuous gel containing 5% acrylamide in the stacking gels and 6.5% acrylamide in the resolving gels. The acrylamide and biscrylamide ratio was 100:1. Gels were cast in SE600 and maintain at 2-4°C while subjected to a constant current until voltage reach to 350 V and changed into constant voltage until 9 hr. After the run, gels were stained overnight with a modified Coomasie Blue and destained with dH_2O until the background was clean. 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.

3. Cardiac Homogenate and SR Ca^{2+} Uptake Measurement

Whole left ventricle is homogenized in 20 mM imidazole with 30 passes in Teflon-glass homogenizer. Fifty mg of whole left ventricular homogenate is added to make a final concentration of 1 mg of protein per ml of the reaction mixture. The reaction is assayed with various concentrations of Ca^{2+} ranging from pCa (-log [Ca^{2+}]) 8.0 to 4.875 with 0.1% of radiolabel $^{45}CaCl_2$. Total concentrations of free Ca^{2+}, EGTA, KCl, $MgCl_2$, and ATP are calculated using a computer program generated from the dissociation constants given by Fabiato [50]. The Ca^{2+}-uptake assay is started by addition of ATP to a final concentration of 5 mM. The reaction mixture is then incubated for three minutes at 37^{o}C with 60 rpm shaking and stopped by rapid cooling in ice. A part of the reaction mixture is filtered through 0.45 μm Millipore filter (Millex HA). Equal amount of filtrated and non-filtrated solution are determined for the radioactivity. Subtraction of non-filtrated with filtrated portion represents the amount of Ca^{2+} uptake. Protein concentration will be confirmed by Bradford assay. The relations between pCa and Ca^{2+} uptake are plotted using non-linear least square regression based on Hill equation.

4. Determination of SERCA Activity

SERCA activity is determined by triple enzyme assay as previously described by Chu et al. [53]. The assay is run at various concentrations of Ca^{2+} ranging from pCa 8.0 to 5.0, pH 7.0 at 37^{o}C. The SR enriched membrane vesicles of 5 μg protein is incubated in 1 ml reaction (mM; MOPS 21, NaN_3 4.9, EGTA 0.06, KCl 100, $MgCl_2$ 3.0, NADH 0.2, phosphor(enol)-pyruvate 1.0, pyruvate kinase 8.4 unit/ml, lactate dehydrogenase 12 unit/ml). Kinetic reaction of NADH degradation is monitored with spectrophotometer for 350 second and the activity will be determined from the linear reaction during 250 to 350 seconds. The activity is then calculated from the optical density using extinction coefficient of NADH. Non-specific

SERCA activity is determined in the presence of 0.1 nM thapsigagin in the reaction. The relations between pCa and Ca^{2+}-ATPase activity are then plotted using non-linear least square regression based on Hill equation.

5. Skinned Fiber Preparations and Mechanical Force Measurements

The procedure follows that described by Pan and Solaro [54]. Thin bundles of papillary muscle fiber (0.05-0.1 mm in diameter) are dissected and extracted at $4^{o}C$ for at least 24 h before force measurement in a relaxing solution containing 1% Triton X-100, 10 mM EGTA, 5.5 mM ATP, 12 mM creatine phosphate, 8.2 mM $MgCl_2$, 30 mM KCl, 60 mM imidazole, 60 mM phenylmethysulfonate fluoride, pH 7.0. For mechanical force measurements, the muscle strip is mounted horizontally between the isometric tension transducer and a thin glass rod in the chamber containing relaxing solution. The transducer and the glass rod are connected to two micromanipulators allowing manual adjustment of the muscle length. The fiber length will be set at the sarcomere length of 1.9 μm using Laser Light Diffraction technique. The force developments of the fiber at various concentrations of Ca^{2+} ranging from pCa 8.0 to 4.5 at $15^{o}C$ will be recorded on a linear chart recorder after the output signal of the transducer is amplified.

6. Histological Study of Myocyte and Collagen Deposition.

Rats will be anesthetized with thiopental sodium (100 mg/kg BW) and the heart will be rapidly excised and frozen in the optimum temperature compound (OTC, sakura tissue tek). Sections of 10 μm will be prepared using cryotome (Frozen section) and kept in $-20^{o}C$ until use. Hematoxylin and Eosin (H&E) and Sirius Red (Sigma) stains will be performed for quantifications of cell cross-sectional area and total collagen deposition, respectively. The myocardial section will be imaged under bright field microscope (400X) using CCD-based sensor camera. The cross-sectional area of the myocytes and the collagen deposition area per tissue area will be analyzed using Image J program (NIH based software).

7. Determinations of Protein Expression and Phosphorylation

General procedure of immunoblot analysis will be followed for measuring the expression and phosphorylation of the proteins. Left ventricular tissue from the sample will be homogenized with extraction buffer containing protease inhibitors and phosphatase inhibitor cocktail for serine and theronine (Sigma). Nitrocellulose membrane will be used for all procedure. Specific antibodies against SERCA (ABR), calsequestrin (ABR), total phosphor-lamban (ABR), phopho-Ser^{16} and phosphor-Thr^{17} phospholambans (Badrilla), connexin 43 (Chemicon), N-cadherin (Cell Signaling), total and phospho-Akt, total and phosphor-mTOR (Cell Signaling Technology), total and phospho-MAPKs (Cell Siganling Technology) will be incubated with the membrane overnight following by secondary antibody conjugated with HRP.

To analyze the phospho-NFAT, 500 μg of left ventricular protein extract will be immuno-precipitated with either anti-NFATc1 (Santa Cruz) or anti-NFATc3 (Santa Cruz). After that, Western blot analysis will be performed with an anti–phosphoserine antibody (Zymed). The protein bands on the membrane will be developed using ECL reagent and detected by

exposing to hyperfilm (Amersham Pharmacia Biotech). The amount of proteins and phosphorylation levels will be analyzed using Image Master Labscan version 3.01 and Image Master Totallab version 1.0 (Amersham Pharmacia Biotech).

8. General methods and statistical analyses

Curves relating pCa and SR Ca^{2+} uptake or SERCA activity were fit to the Hill equation using nonlinear least squares regression analysis (GraphPad Prism, version 4.00) to derive the ECa50 (half-maximally activating calcium concentration) and the Hill coefficient (*n*). Data were presented as means $\pm$ SE. The significance of differences among groups of animals was analyzed using one-way ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons. A *P* value of < 0.05 was set for the significant difference among groups. The significance between the two groups was determined by a Student's *t*-test.

9. Materials

All chemicals were purchased from Sigma Chemical (St. Louis, MO). Some electrophoretic reagents were purchased from Bio-Rad (Hercules, CA) or Amersham Pharmacia Biotech (Buckinghamshire, UK). Thapsigargin was acquired from Alomone (Jerusalem, Israel). Perioxidase-conjugated affinipure donkey anti-mouse IgG (H+L) was purchased from Research Diagnostics (Flanders, NJ), and horseradish peroxidase-goat anti-rabbit IgG (H+L) conjugate (ZyMax grade) was obtained from Zymed (San Francisco, CA). Human insulin was purchased from Eli Lilly (Indianapolis, IN), and glucose strips from Roche (Indianapolis, IN).

RESULTS AND DISCUSSION

Project I: To determine the adaptive alterations in SR Ca^{2+} uptake activity in the heart of ovariectomized rats complicated with diabetes

i) Measurements of SR Ca^{2+} uptake

ii) Measurements of SERCA activity

iii) Immunoblots of SR proteins

We have previously reported an important role of ovarian sex hormones in inducing adaptive alterations of the cardiac SR Ca^{2+} uptake. We then further evaluated whether changes in the SR Ca^{2+} uptake activity could serve another mechanistic adaptation underlying the cardio-protective effects of estrogen using an approach of diabetes complication. Measurements of both the SR Ca^{2+} uptake and the SR Ca^{2+}-ATPase activities were carried out in various groups of animal including sham, OVX rats with and without estrogen supplementation, diabetic rats with and without insulin supplementation, and DM-OVX rats with and without estrogen, insulin, or estrogen plus insulin supplementation for the 10-week period of study. Contents of SR proteins including SERCA and calsequestin were also quantified using Western Blot analysis.

General characteristic of the rats in each group was summarized in Table 1. Significant decrease in uterine weights was observed in OVX and diabetic-OVX rats in which could be completely restored by estrogen supplementation. A significant reduction in uterine weight of diabetic rats was also observed in a smaller magnitude compared to OVX rats which could be completely restored by insulin supplementation. Hypertrophy of the heart was still demonstrated in diabetic and diabetic-OVX rats which could be prevented by insulin and co-administration of both estrogen and insulin supplementation, respectively.

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#
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.

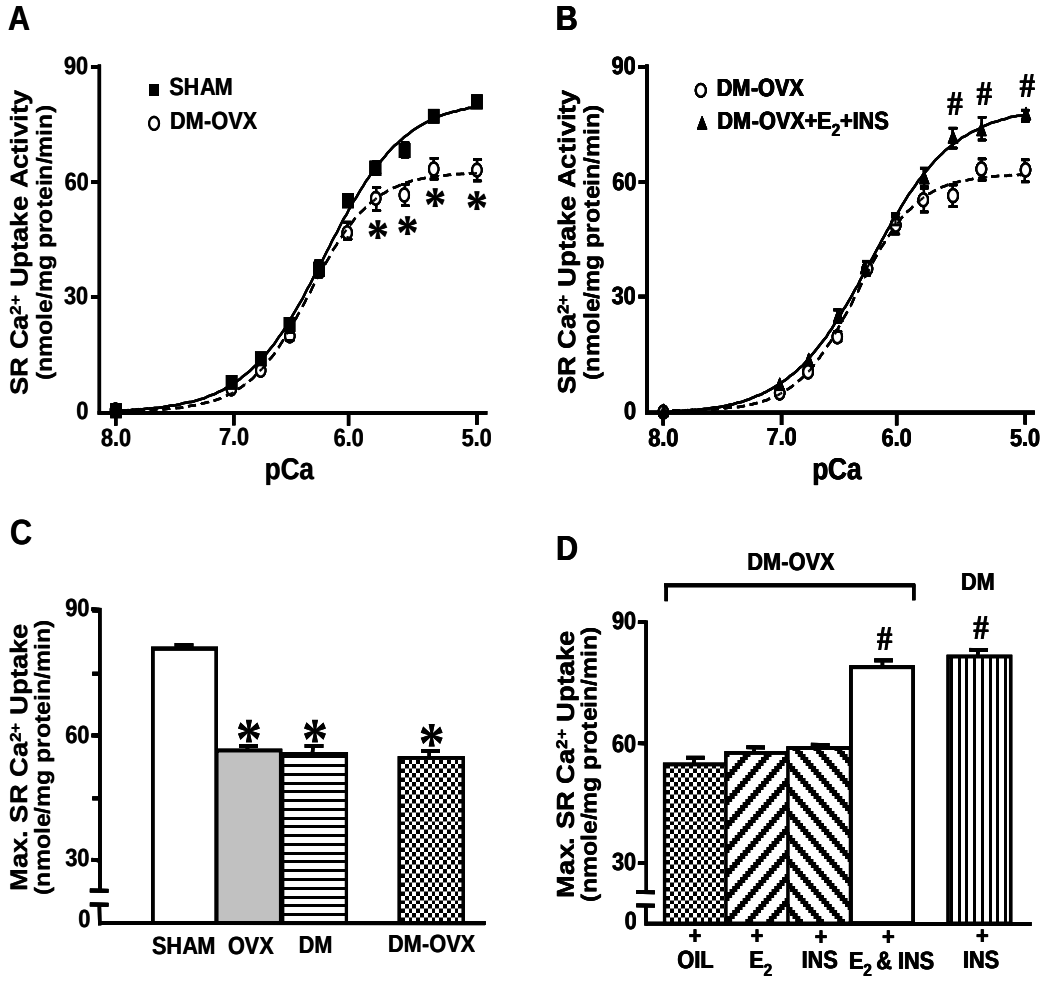

Figure 1. pCa-SR Ca^{2+}-uptake activity relation of left ventricular homogenates from SHAM and diabetic (DM)-OVX rats **(A)** and DM-OVX rats with and without estrogen and/or insulin supplementation **(B)**. Comparison of maximum SR Ca^{2+}-uptake activity from SHAM, OVX, DM, and DM-OVX rats **(C)** 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.

The SR Ca^{2+}-uptake activity was measured from left ventricular homogenates at various free Ca^{2+} concentrations. As shown in Figure 1B, 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 suppressed SR Ca^{2+}-uptake activity in DM-OVX and DM rats was completely restored by supplementation with estrogen plus insulin, and insulin, respectively (Figure 1D). In contrast to maximum activity, 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 2B). The increased SR Ca^{2+}-uptake sensitivity was also observed in DM-OVX rats (ECa_{50} = 0.48 ± 0.01). The increased uptake sensitivity could be prevented by supplementation with insulin and co-administration of estrogen and insulin in DM and DM-OVX groups, respectively (Figure 2D). Despite changes in the maximum SR Ca^{2+}-uptake rate and SR Ca^{2+}-uptake sensitivity in OVX rat hearts, the Hill slope (data not shown) was not different among the experimental groups. Taken together, results show no dominant effect of estrogen deficiency but estrogen and insulin may interactively affect the cardiac SR Ca^{2+}-uptake function.

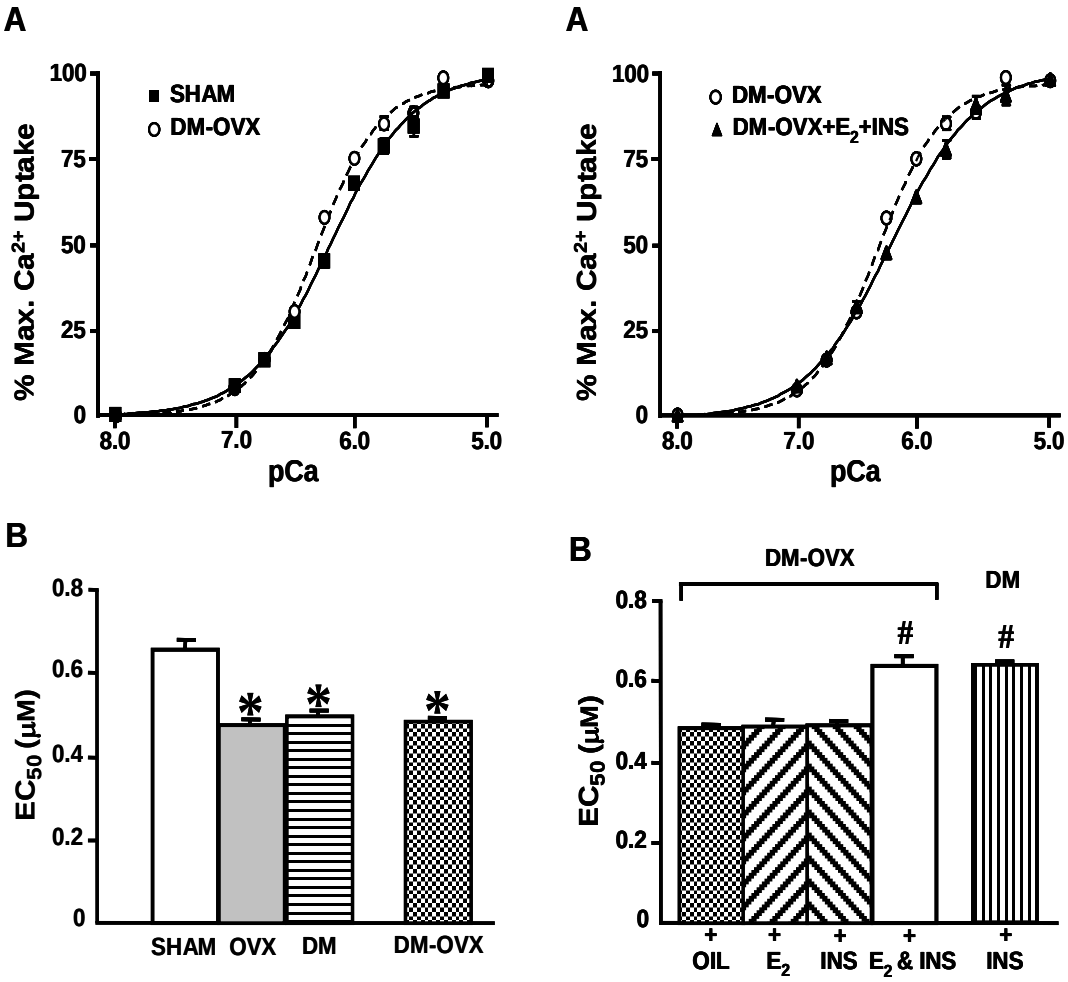

Figure 2. pCa-%maximum SR Ca^{2+}-uptake activity relation of left ventricular homogenates from SHAM and diabetic (DM)-OVX rats (A) and DM-OVX rats with and without estrogen and/or insulin supplementation (B). Comparison of Ca^{2+} concentration producing half-maximal activation (EC_{50}) from SHAM, 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.

We further specify the underlying mechanism of changes in the SR Ca^{2+} uptake activity by determining SR Ca^{2+}-ATPase or SERCA activity 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 decreased from sham control (1.34 ± 0.04 μmole Pi/mg protein/min) with almost the same magnitude of suppression (31.3% and 30.6%, respectively, Figure 3C). Maximum SERCA activity in diabetic-ovariectomized (DM-OVX) (0.9 ± 0.02 μmole Pi/mg protein/min) rats was also decreased without additive suppression compared to those of OVX and DM rats (Figure 3A). The reduced maximum SERCA activity in DM-OVX rat hearts was completely restored upon co-supplementation with estrogen and insulin (Figure 3B & 3D).

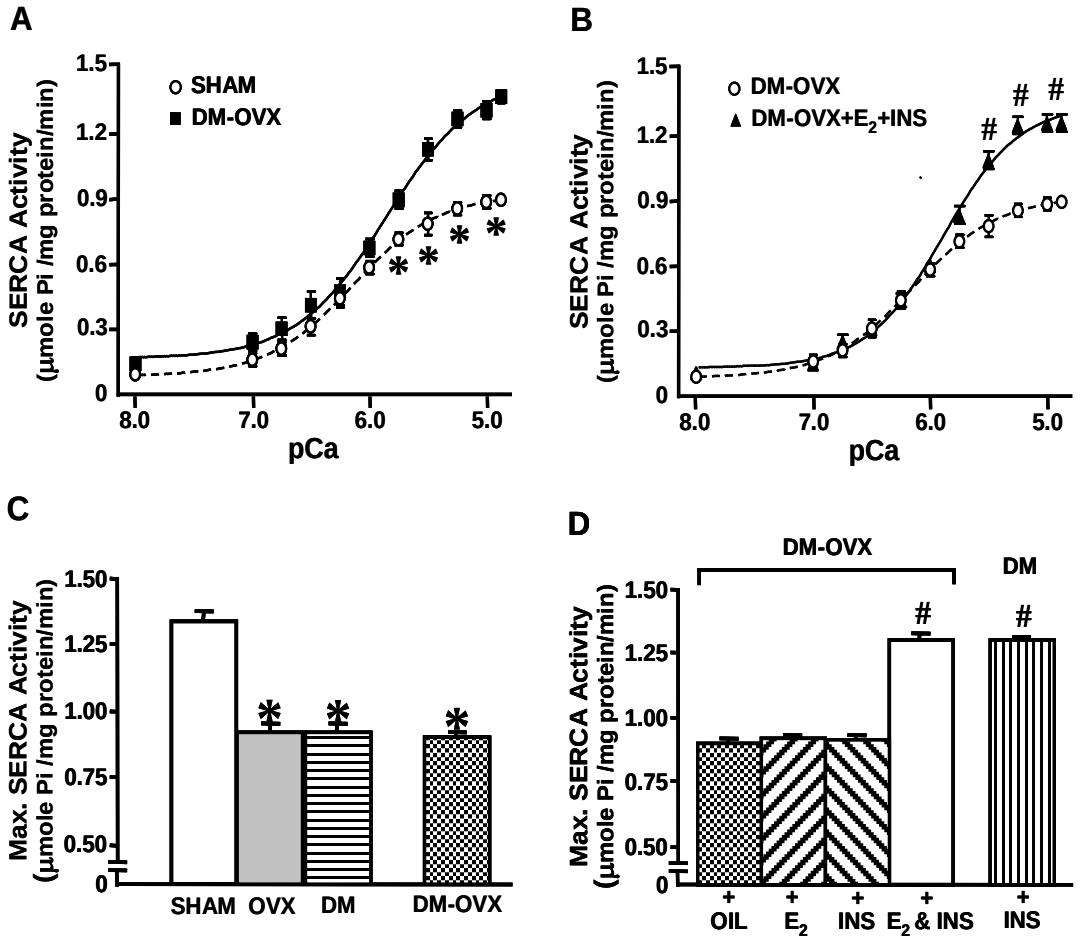

Figure 3. pCa-SR Ca^{2+}-ATPase (SERCA) activity relation of SR membrane vesicles from SHAM and DM-OVX rats (A) and DM-OVX rats with and without estrogen and/or insulin supplementation (B). Comparison of maximum SERCA activity from SHAM, OVX, DM, and DM-OVX rats (C) and DM-OVX rats with estrogen and/or 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.

As shown in Figure 4, sensitivity of SERCA to Ca^{2+} activation was also increased in OVX (EC_{50} = 0.74 ± 0.08) and DM (EC_{50} = 0.76 ± 0.10) rats compared to sham (EC_{50} = 1.44 ± 0.12). In the heart of DM-OVX rats the same degree of increase in SERCA sensitivity (EC_{50} = 0.73 ± 0.07) to that in OVX and DM rats was also observed. The increased cardiac SERCA sensitivity detected in DM and DM-OVX rats could be reversed by supplementation with insulin and co-administration of estrogen and insulin, respectively (Figure 4B & 4D). 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 estrogen- and insulin-deficient rat hearts.

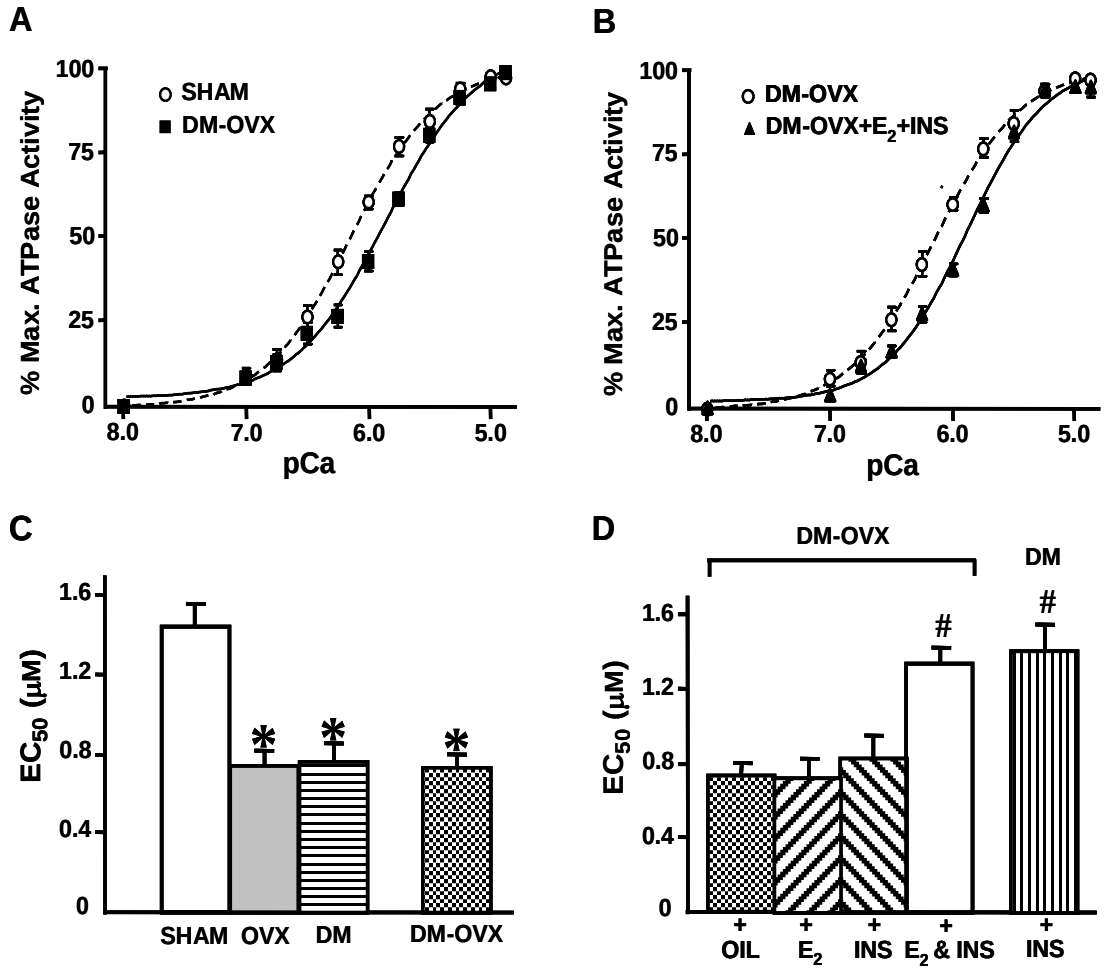

Figure 4. pCa-%maximum SERCA activity relation of SR membrane vesicles from SHAM and DM-OVX rats (A) and DM-OVX rats with and without estrogen and/or insulin supplementation (B). Comparison of Ca^{2+} concentration producing half-maximal activation (EC_{50}) from SHAM, OVX, DM, and DM-OVX rats (C) and 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 estrogen and insulin in regulating the cardiac SR Ca^{2+}-uptake activity was further analyzed on both qualitative and quantitative changes in SERCA proteins. Effects of estrogen and insulin deficiencies on the expression of SERCA and SR Ca^{2+}-uptake associated proteins were then evaluated. Figure 5 shows results of immunoblot analysis of SERCA, phospholamban, and calsequestrin (CSQ) in sham, OVX, DM, and DM-OVX rats. The protein bands of calsequestrin were used as loading control. Relative amounts of SERCA and phospholamban to calsequestrin in the experimental rats were summarized in figure 5C and 5E, respectively. As shown in figure 5C, 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, upregulation of phospholamban protein was detected only in DM (150%) and DM-OVX (153.8%) rat hearts but not in OVX rat (Figure 5E). 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 5G). All these changes in DM and DM-OVX rat hearts could be restored by insulin supplementation and estrogen plus insulin coadministration, respectively (Figure 5D, 5F and 5H). These results indicated that the suppression of maximum SR Ca^{2+}-uptake activity in estrogen- and insulin-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 estrogen- and insulin-deficient rat hearts, the level of the phosphorylated form of phospholamban at either Ser^{16} or Thr^{17} site was then 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 6 and 7, respectively. Downregulation of the phospho-Ser^{16} phospholamban was detected in DM (28.8 %) and DM-OVX (29.7%) groups (Figure 6A) in which could be reversed by insulin supplementation (Figure 6B). Despite the unchanged phospho-Ser^{16} phospholamban, a significant downregulation of the phospho-Thr^{17} phospholamban was detected in OVX (32.2%) rat hearts (Figure 7A). 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 of OVX group. Downregulation of the phospho-Thr^{17} phospholamban in DM and DM-OVX rats could be abolished by supplementation of insulin and coadministration of estrogen and insulin, respectively (Figure 7B). These results indicated that changes in the SERCA response to Ca^{2+} after estrogen and insulin deficiency were not modulated by alterations in phospholamban phosphorylation. Therefore, the increased Ca^{2+} sensitivity of SERCA in estrogen and insulin deficient groups might be due to other mechanisms.

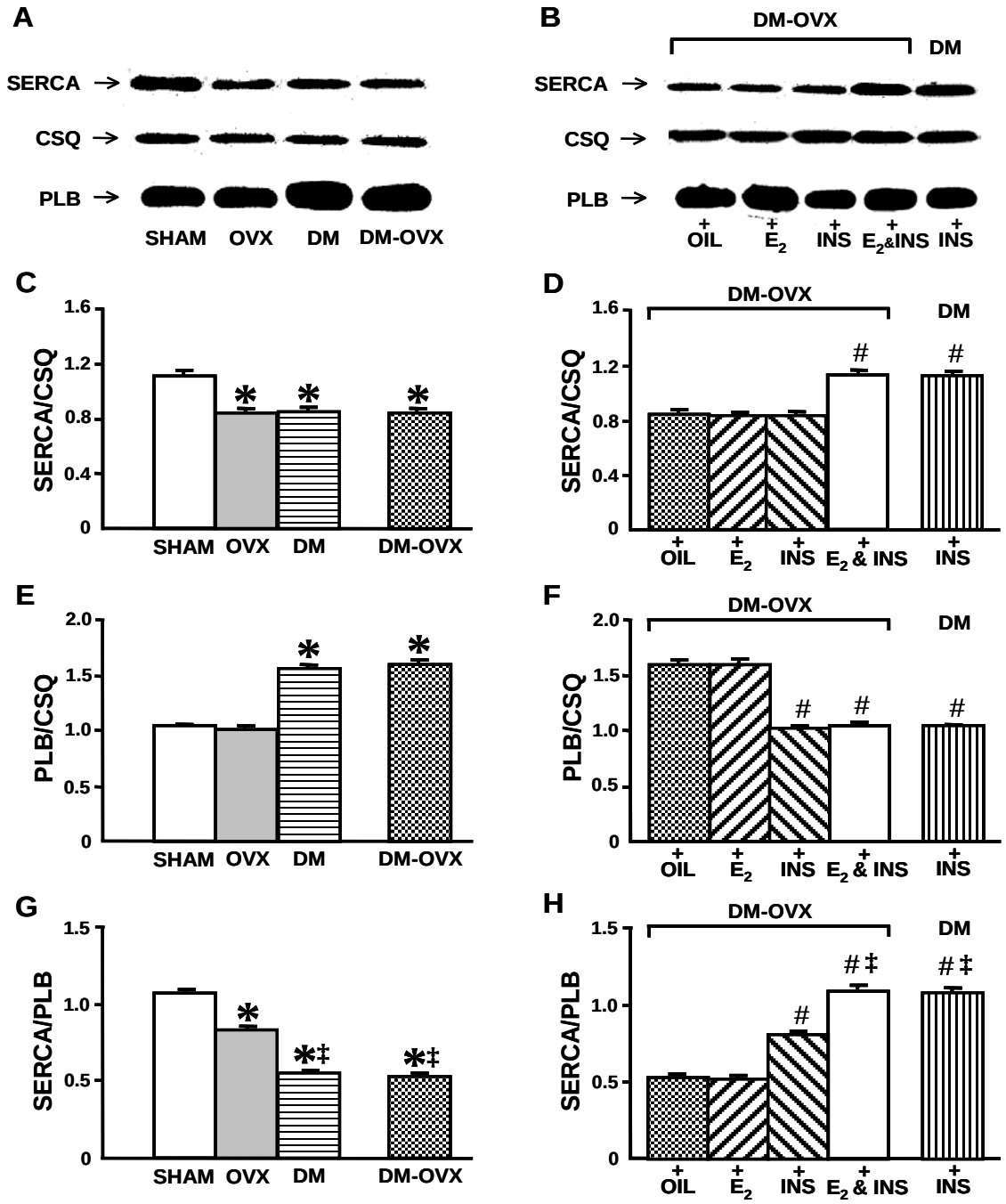

Figure 5. Immunoblot analyses of SERCA, calsequestrin (CSQ), and phospholamban (PLB) (A and B) and comparison of the band intensity expressed as a ratio of SERCA to CSQ (C and D), PLB to CSQ (E and F), and SERCA to PLB (G and H) of left ventricular homogenates from SHAM, OVX, DM, and DM-OVX rats and from DM-OVX rats with estrogen and/or insulin supplementation, and DM rat with insulin supplementation, respectively. Data are means $\pm$ SE from 6 hearts. $P < 0.05$, significant difference from SHAM (*), OVX (‡), and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

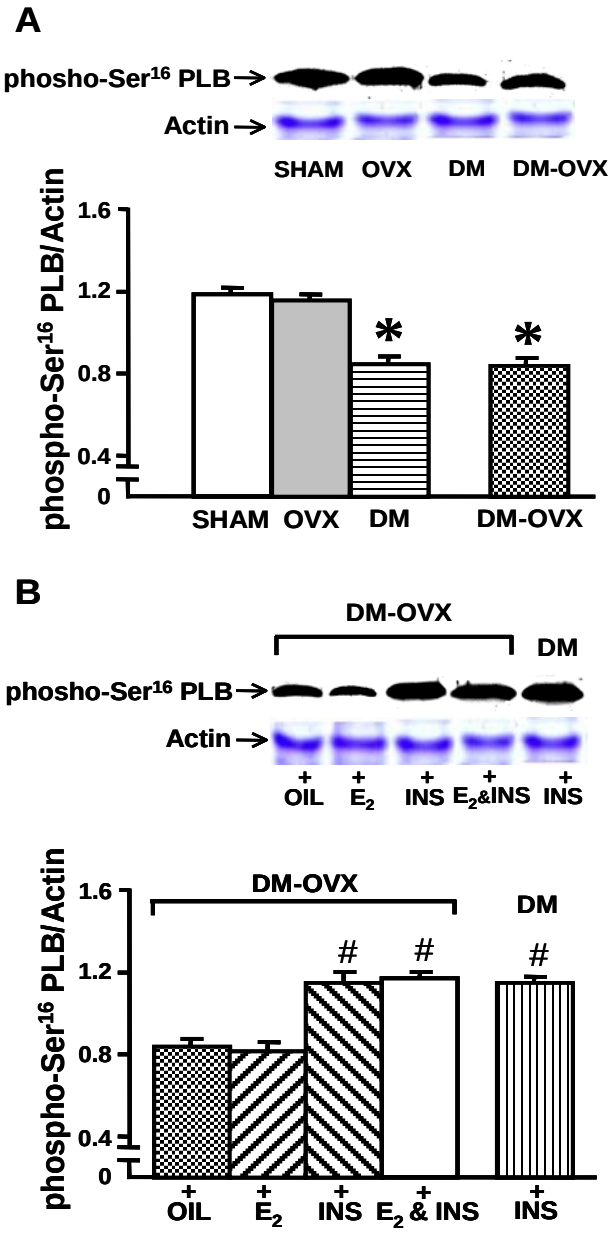

Figure 6. Immunoblot analysis of phosphorylated Serine-16 (phosphor-Ser^{16}) and actin and comparison of the band intensity expressed as a ratio of phosphor-Ser^{16}:actin of left ventricular homogenates from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with estrogen and/or insulin supplementation, 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.

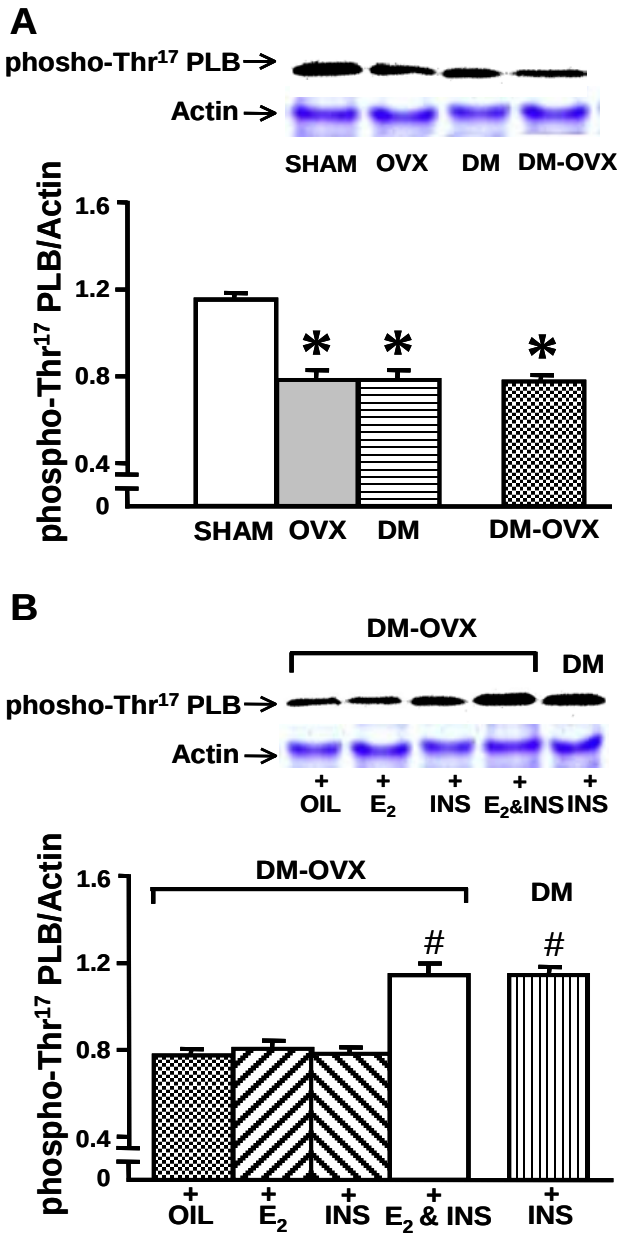

Figure 7. Immunoblot analysis of phosphorylated Threonin-17 (phosphor-Thr^{17}) and actin and comparison of the band intensity expressed as a ratio of phosphor-Thr^{17}:actin of left ventricular homogenates from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with estrogen and/or insulin supplementation, 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.

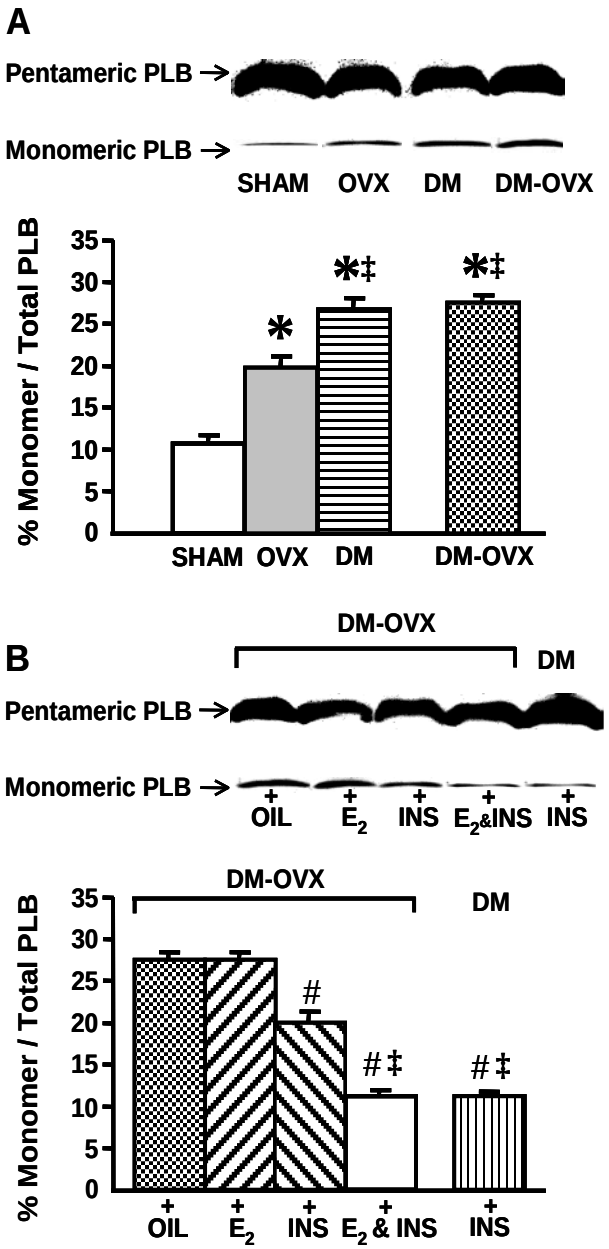

Figure 8. Amount of monomeric phospholamban expressed as percentage of total phospholamban of samples of left ventricular homogenates from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with estrogen and/or insulin supplementation, and DM rats with insulin supplementation (B). Data are means ± SE from 6 hearts. $P < 0.05$, significant difference from SHAM (*), OVX (‡), and DM-OVX (#) groups, respectively, using Student Newman-Keuls test after ANOVA.

Besides changes in the phospholamban phosphorylation, 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 phospholamban and the proportion of the monomeric form to the total amount of phospholamban were shown in Figure 8. An increase in the proportion of the monomeric phospholamban was observed in OVX rats (19.8 ± 1.3%) compared to sham. The increased monomeric phospholamban was also demonstrated 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 insulin supplementation and estrogen plus insulin coadministration, respectively. The increased proportion of the monomeric 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 toward pentamer. These data confirm that the Ca^{2+} hypersensitivity of SERCA activity induced by estrogen or insulin deficiency was not related to change in the quantity or the activity of phospholamban.

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 [23, 55, 56].

A suppressed SR Ca^{2+}-uptake activity is a common feature detected in most human and animal models of cardiomyopathy and heart failure [57, 58]. 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 [59, 60]. 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 [61, 62]. The parallel effects of E_2 and INS deficiencies like 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 [22, 63] 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 [64], 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 [65-69]. 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 [70], 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+} [59], leading to an enhancement of the rate of cardiac muscle relaxation [71, 72]. 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 [73]. Because of a reduction in the systolic Ca^{2+} concentration in E_2- and INS-deficient rat hearts [23, 55, 56], 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+} [74, 75]. 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 [76]. Whether sarcolipin contributes to the effect of E_2 and INS on the cardiac SR awaits further investigation.

Project II: To evaluate the significance of cross-bridge kinetics in regulating cardiac myofilament activity in ovariectomized rats complicated with diabetes

i) Measurements of myofibrillar ATPase activity

ii) Determinations of MHC isoforms

Our preliminary study from the last project has indicated differential effects of estrogen and insulin in regulating maximum myofibrillar ATPase activity. In contrast to insulin, estrogen possibly exerts an additional regulatory effect on variables of the cross-bridge kinetics besides its effect directly on the cross-bridge. In this project, we further test the significant role of estrogen in the cross-bridge kinetics of the cardiac contractile activity by evaluating the correlation of maximum myofibrillar ATPase activity to various levels of α-MHC expression in the heart of OVX rats compared to that of DM rats.

Maximum myofilament ATPase activity and % α-MHC isoform were measured and summarized in Figure 9 and 10, respectively. As shown in Figure 9, 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 estrogen and insulin supplementation in OVX and DM rats, respectively, but only by co-administration of estrogen and insulin 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 were detected compared to sham and could be completely reversed upon supplementation with estrogen and insulin, respectively (Figure 10A). 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 10B). Similar to myofibrillar ATPase activity, the isoform shift in MHC could be prevented by estrogen and insulin in OVX and DM rats, respectively, whereas co-administration of estrogen and insulin was needed for complete prevention in DM-OVX rats (Figure 10). Thus, differential effects of estrogen and insulin on regulating maximum myofibrillar ATPase activity were indicated.

To further prove the different effects of the estrogen and insulin on cross-bridge cycling kinetics, relationships of maximum ATPase activity and % α-MHC in experimental groups were 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 11A, different linear regression lines in OVX (dash line, slope = 2.60 $\pm$ 0.16) or DM rats (solid line, slope = 1.49 $\pm$ 0.09) to sham were observed. Regression analysis fitted by data of OVX+E_2 to that of OVX group gave rise to the same slope as that of OVX vs SHAM (Figure 11B). Similarly, regression analysis of DM+insulin data with DM group was the same as that of DM and SHAM groups. Interestingly, a similar slope of linear regression line from DM-OVX vs SHAM (semi-dash line, slope = 1.55 $\pm$ 0.08) to that of DM vs SHAM group was detected (Figure 11C). As expected, regression analysis from data of DM-OVX and DM-OVX+E_2+insulin groups was similar to that of DM and DM+insulin groups. The different linear relations between OVX and DM rat hearts indicated different regulatory effects of estrogen and insulin on the cross-bridge cycling kinetics.

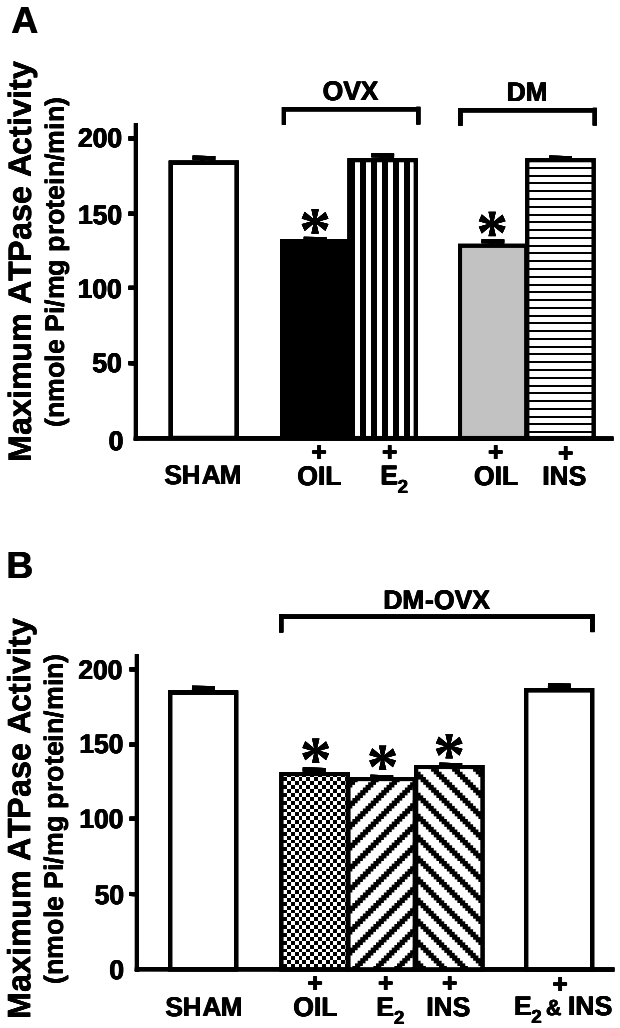

Figure 9. Comparison of maximum myofilament ATPase activity from SHAM, OVX rats with and without estrogen supplementation, diabetic (DM) rats with and without insulin supplementation (A), and DM-OVX rats with estrogen and/or insulin supplementation (B). Data are means ± SE from 10-12 preparations. *$P < 0.05$ = significantly different from SHAM, using Student Newman-Keuls test after ANOVA.

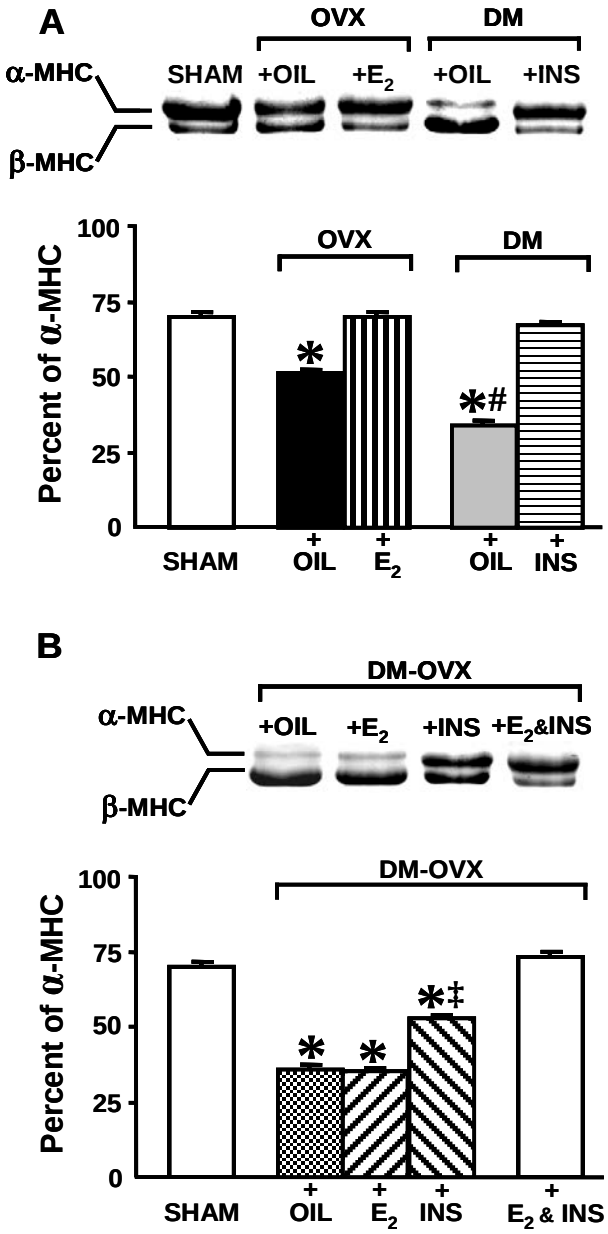

Figure 10. Myosin heavy chain (MHC) region on SDS gels and the relative amount of α-MHC (as a percentage of total MHC) of left ventricular papillary muscle from SHAM, OVX rats with and without estrogen supplementation, diabetic (DM) rats with and without insulin supplementation (A), and DM-OVX rats with 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.

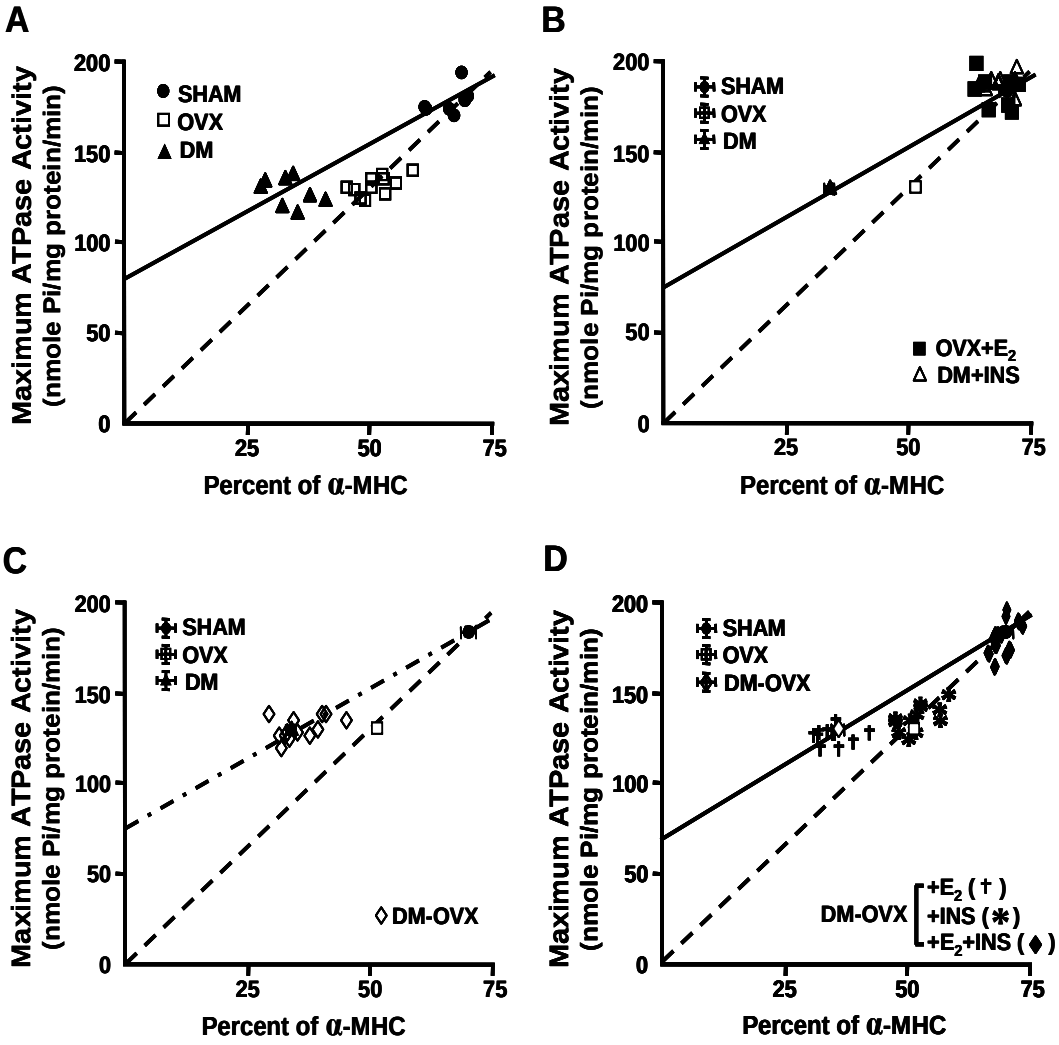

Figure 11. Relationships between maximum Ca^{2+}-dependent actomyosin MgATPase activity and % α-MHC isoform from (A) SHAM, OVX, and diabetic (DM) rats with linear plot of combined data of SHAM with OVX (dash line) or DM (solid line) group, (B) from OVX + estrogen (E_2) and DM + insulin (INS), (C) from 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.

To further specify the underlying mechanism of estrogen and insulin in regulating cardiac cross-bridge cycling kinetics, different levels of α-MHC expression in the heart of OVX and DM rats were varied by manipulating the plasma thyroid hormone status using tri-iodothyronin (T_3) injection or 6-n-propyl-2-thiouracil (PTU) administration as well known that thyroid response element is existed in the α-MHC gene. General characteristics of the experimental rats were summarized in Table 2. 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 group. 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.

Table 2. 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 *#†	1.31 ± 0.07 *#†	0.10 ± 0.03 *	0.40 ± 0.02*#	393.1 ± 3.5 *#
OVX + PTU	292 ± 5 *#†	0.88 ± 0.02	0.09 ± 0.02 *	0.30 ± 0.02	20.3 ± 1.8 *#†
DM	226 ± 5 *#†	0.81 ± 0.01 #	0.31 ± 0.03 *#	0.36 ± 0.01*#	45.2 ± 2.8 *#
DM + T_3	200 ± 9 *# †	0.92 ± 0.03	0.31 ± 0.03 *#	0.46 ± 0.02*#†	60.3 ± 1.6 †
DM + PTU	228 ± 7 *#	0.82 ± 0.01 *#†	0.31 ± 0.02 *#	0.37 ± 0.02*#	17.1 ± 0.9 *#†
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 *#†	1.01 ± 0.03 #†	0.09 ± 0.01 *	0.41 ± 0.02*#	62.5 ± 0.8 †
DM-OVX + PTU	275 ± 8*#†	0.98 ± 0.02 †	0.09 ± 0.01 *	0.36 ± 0.01*#	16.3 ± 0.7 *#†

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; PTU, propyl-2 thiouracil. $P < 0.05$, significant difference from SHAM (*), OVX (#), and DM (†) groups, respectively.

As expected, maximum myofibrillar ATPase activity and % α-MHC were further suppressed in the hypothyroid rats of every group (Figure 12). 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 13A and 13B, respectively) was demonstrated with the same slope value as that previously observed in the regression of DM vs SHAM (slope = 1.49 ± 0.09) and DM-OVX vs SHAM (slope = 1.55 ± 0.08) (Figure 11). Similarly, both maximum myofibrillar ATPase activity and % α-MHC of OVX rat were significantly increased after T_3 treatment. However, the correlation of maximum ATPase activity to % α-MHC expression at different thyroid levels in OVX rats demonstrated a concave shape relationship fitted by the second degree polynomial equation (Figure 13C). These results indicate that estrogen, in contrast to insulin, plays an additional regulatory role on the cross-bridge cycling kinetics through changes in the myofilament proteins besides its effect on the expression of MHC isoforms. Moreover, our results confirm that insulin indirectly affects the cross-bridge cycling kinetics through the direct effect of thyroid hormones on the expression of α-MHC.

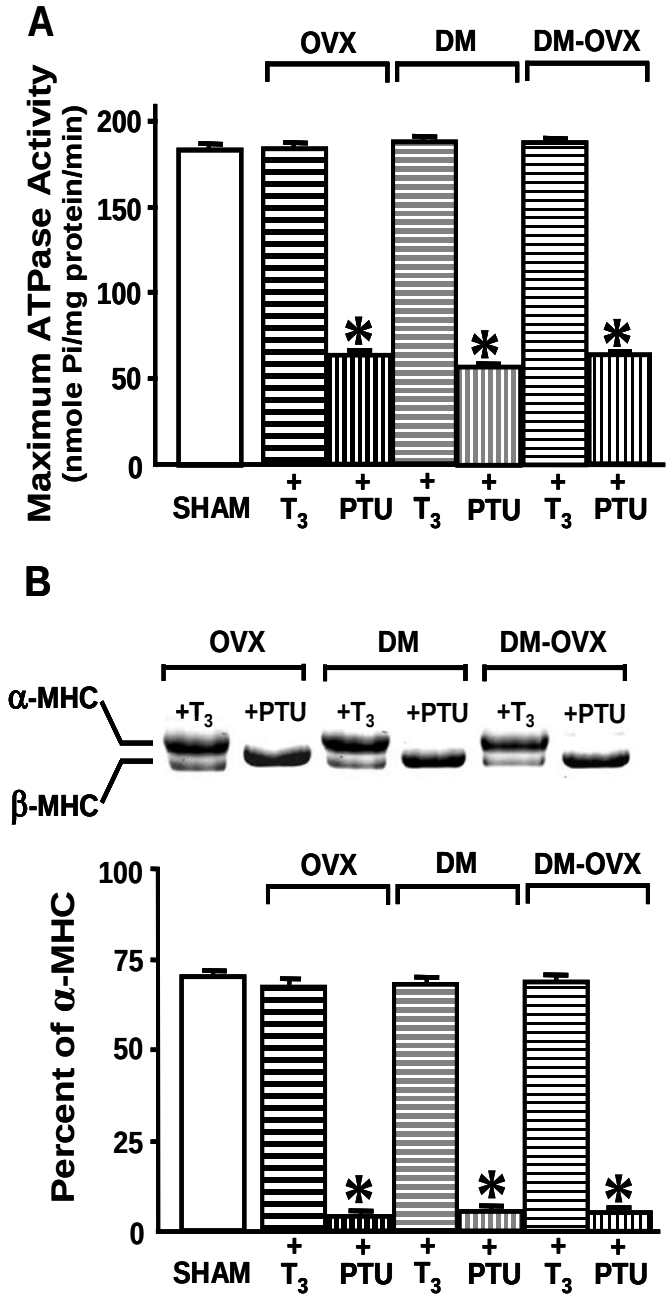

Figure 12.

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, diabetic (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 = signifycantly different from SHAM, using Student Newman-Keuls test after ANOVA.

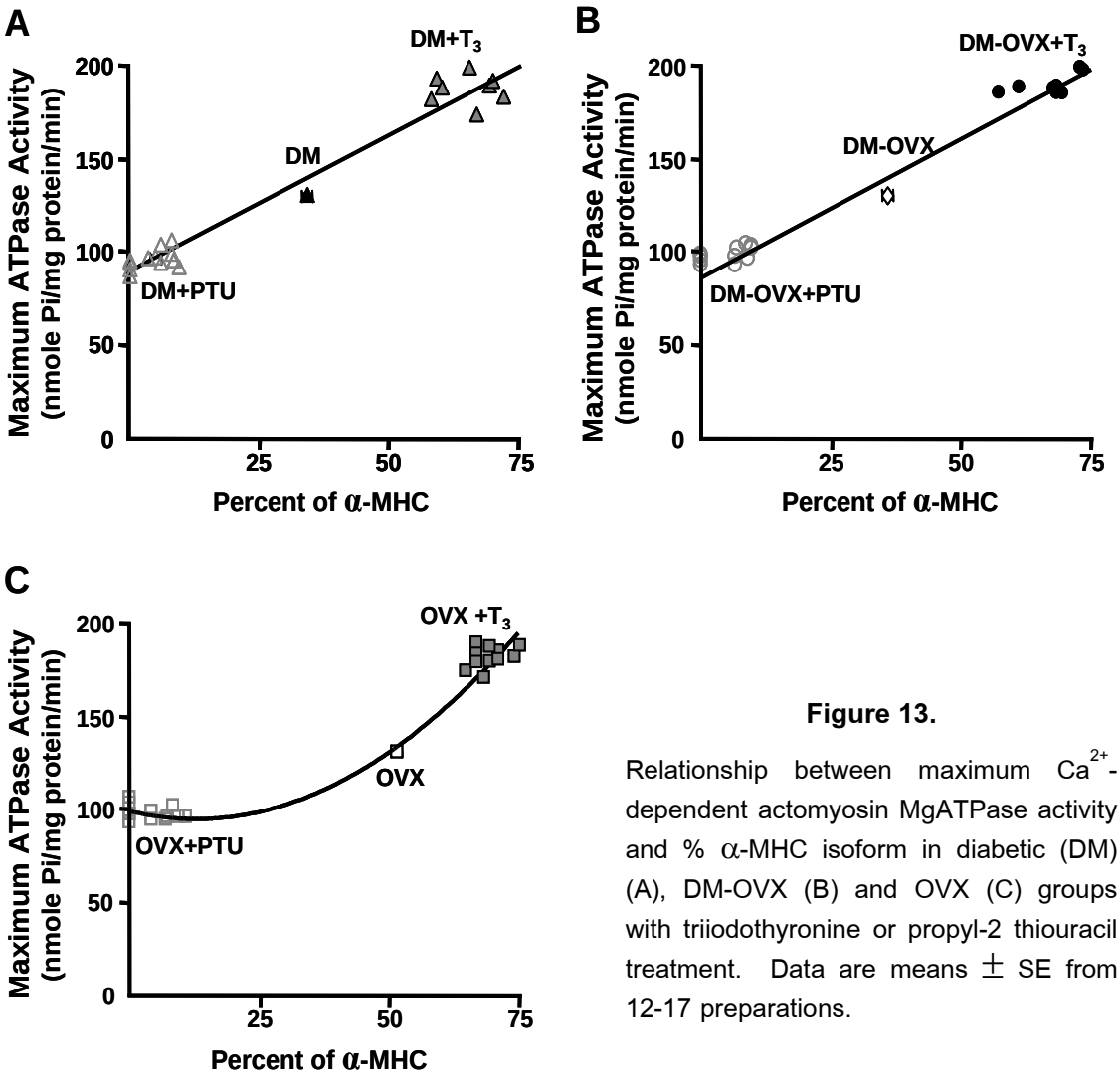

Figure 13.

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

Based on the determination of cross-bridge (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 [77], 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 [78]. 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 [79] 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 [80]. 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 tune of the CB cycling [81]. 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.[82, 83] 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 [84] 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.[85] 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 [86]. Another new mutation of human cardiac TnI detected in hypertrophic cardiomyopathy was also shown to result in deterioration of the CB cycling kinetics [87].

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 [88]. This finding was also supported by the work of Belin and his group [89]. 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 [90] 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.

Project III: **To determine the mechanical contractile properties of skinned cardiac muscle fiber in male rats**

i) Force measurements in ORX rat hearts

ii) Force measurements in high testosterone study

iii) MHC analysis

Our preliminary results have demonstrated a significant biochemical decrease in cardiac myofilament Ca^{2+} activation in 10-weeks orchiectomized (ORX) rats. On the other hand, a significant cardiac hypertrophy was detected in 10-weeks high testosterone-treated male rats without affecting the maximum myofibrillar actomyosin ATPase activity. It is, however, not known how the mechanical changes in which will provide more insight on physical properties of the heart would be apparent in the ORX and high plasma testosterone rat hearts. So, our studies have been designed to evaluate two main animal sets, including 1) 10-week orchiectomized set, and 2) high testosterone set. The mechanical properties of the cardiac fibers prepared from both ORX and high plasma testosterone rat hearts were measured with the parallel experiments of sham hearts as control using skinned fiber technique. Testosterone supplementation to the ORX rats was also performed to insure the significant effect of male sex hormones. While orchiectomized set was further divided into sham, ORX and supplementation groups, rats in high testosterone study were designed to answer both the time and dose-dependent effects of high testosterone. The time course study covers three time points including 4, 8 and 12 weeks of hormone administration. In addition, three doses of testosterone, including 5, 10 and 20 mg/Kg body weight of testosterone injection, were carried out. Lastly, the shift in the MHC isoforms of the left ventricular tissue of rats used for the force measurement was also analyzed to demonstrate the potential mechanism of change in the developed force of each experimental group.

The general characteristics of the rats were summarized in Table 3. While there was no change in heart and body weight among the groups, the seminal vesicular weight was significantly suppressed in ORX rat in which testosterone supplementation could prevent the change.

Table 3. Body, heart, and seminal vesicular weights of 10-week sham-operated (SHAM) and orchiectomized rats without/with testosterone supplementation

	SHAM	**ORX**	**ORX + T**
Body weight (g)	465 ± 14	459 ± 11	458 ± 12
Heart weight (g)	1.44 ± 0.06	1.42 ± 0.04	1.43 ± 0.04
Heart/body weight (%)	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.01
Seminal vesicular weight (g)	0.52 ± 0.03	0.05 ± 0.002*	0.50 ± 0.07

Values are presented as mean ± SEM from 8-10 rats in each group. *P<0.05 significantly different from sham group using Student-Newman-Kuel after ANOVA.

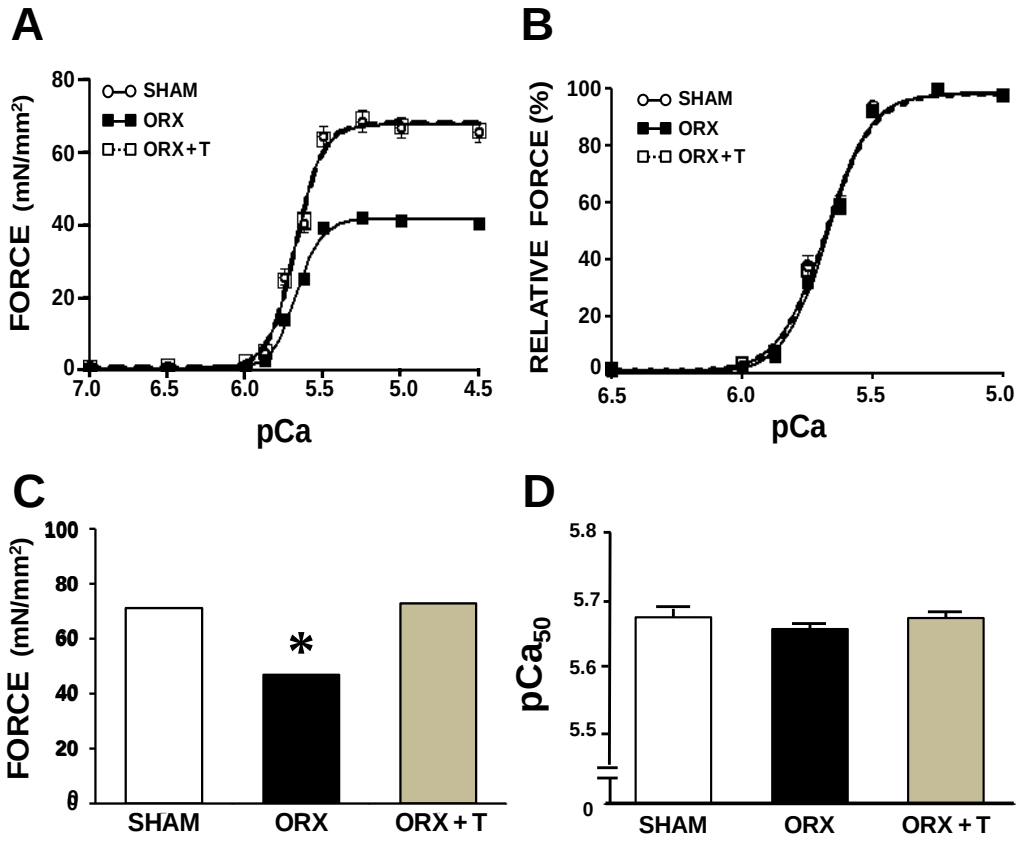

Figure 14. **A:** Relationship of pCa–Force from left ventricular skinned papillary fibers of sham (SHAM), orchiectomized rat without (ORX) and with testosterone supplementation (ORX+T), **B:** Relationship of pCa-% relative force, **C:** Comparison of maximum force contraction (mN/mm^2), and **D:** Comparison of pCa_{50} among the groups. Data are mean ± SEM from 5-8 preparations. **P*<0.05 reprsents significantly different from other groups using Student-Newman-Keuls test after ANOVA

Table 4. Mechanical activation of skinned papillary fiber from sham-operated (SHAM) and orchiectomized (ORX) rats

	SHAM	ORX	ORX + T
Maximum tension (mN/mm^2)	71.2 ± 3.4	47.3 ± 1.3*	72.5 ± 2.1
pCa_{50}	5.69 ± 0.01	5.66 ± 0.01	5.68 ± 0.01
Hill number	5.45 ± 0.15	5.81 ± 0.30	5.45 ± 0.07

Values are presented as mean ± SEM from 13-14 fibers from 5-6 hearts in each group. *P<0.05 significantly different from Sham using Student-Newman-Kuel after ANOVA. †P<0.05 significantly different from data of pH 7.0 using Student paired t-test.

Similar to biochemical activity of the myofilaments, maximum force generation of the skinned papillary muscle preparation from 10-week ORX rat heart was significantly decreased (46.9 ± 1.3 mN/mm^2) compared to that of SHAM control (71.2 ± 3.4 mN/mm^2) (Figure 14A & 14C and Table 4). The decrease in maximum force generation could be perfectly prevented by testosterone supplementation. On the other hand, testosterone deprivation induced no change in the myofilament sensitivity to Ca^{2+} activation (Figure 14B & 14D, Table 4). These results clearly indicate a significant role of male sex hormone in the cardiac contractile activity.

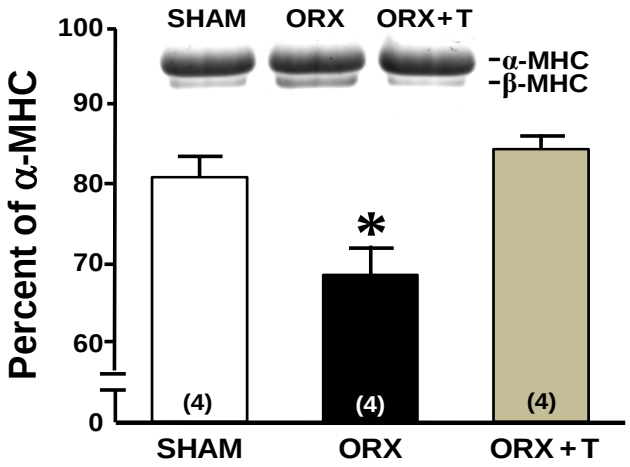

Figure 15. Gel representative and percentage of α-MHC isoform in the left ventricular tissue of sham control (SHAM) and orchiectomized rats without (ORX) and with testosterone (ORX+T) supplementation. Data are mean ± SEM from 4-5 preparations. **P*<0.05 represents significantly different from other groups using Student-Newman-Keuls test after one way ANOVA.

As shown in Figure 15, deficiency of testosterone after orchiectomy for 10 weeks significantly induced a shift in the MHC isoforms toward β-MHC. This shift in MHC isoforms could be normalized by supplementation with testosterone. Taken together with results of mechanical study, the suppressed maximum contractile activity induced in the heart of ORX rats is due to the shift of MHC from the fast α to the slow β isoform. The force-generating capacity of cardiac myosin isoenzymes has been previously documented that myosin V_3 (ββ) produces double the average cross-bridge force more than V_1 (αα) [91]. Therefore, the suppressed maximum force contraction detected in ORX rat heart in which contains more V_3 found in the present study suggests a potential alteration in force developed per single cross-bridge interaction.

Plasma testosterone level of the various groups of rats was summarized in Table 5. As expected, a dose-dependent design was established with different doses of testosterone injection. Table 6 summarized the body weight of rats from sham control and various testosterone-treated groups for duration of 4, 8, and 12 weeks. Body weight of the testosterone-treated rats was significantly lower than the sham control of the same age

without dose dependency. As expected along the duration of study, a significant stepwise increase in the body weight was clearly observed in sham groups. However, there was no significant change in the body weight among the testosterone-treated rats along the duration of study.

Table 5: Testosterone level of sham rat and testosterone-injected groups with various doses (5, 10, and 20 mg/kg body weight) for 4, 8, and 12 weeks

Duration (weeks)	TESTOSTERONE LEVEL (ng/dL)			
	SHAM	Testosterone-injected Groups (mg/kg BW)		
		5	10	20
4	260 ± 46.9	1,654 ± 174	4,499 ± 1,345$^{*\#}$	4,56 ± 1,101$^{*\#}$
8	261 ± 35.3	1,940 ± 404	3,574 ± 1,361*	5,33 ± 676$^{*\#}$
12	282 ± 31.5	1,930 ± 232*	3,774 ± 572$^{*\#}$	5,17 ± 649$^{*\#\dagger}$

Data are means ± SEM from 5-9 rats per group. *P<0.05, $^{\#}$P<0.05 and †P<0.05 indicate dose-dependent difference from sham control, 5, and 10 mg/kg BW testosterone-injected groups of the same duration of study, respectively, using Student-Newman-Keuls test after two-ways ANOVA.

Table 6: Body weight of sham rat and testosterone-injected groups with various doses (5, 10, and 20 mg/kg body weight) for 4, 8, and 12 weeks

Duration (weeks)	BODY WEIGHT (g)			
	SHAM	Testosterone-injected Groups (mg/kg BW)		
		5	10	20
4	409 ± 5.8	371 ± 5.6*	361 ± 5.9*	359 ± 5.5*
8	450 ± 8.7$^{\#}$	398 ± 6.3*	386 ± 8.8*	378 ± 5.6*
12	481 ± 6.9$^{\#\dagger}$	417 ± 6.1*	392 ± 4.8*	378 ± 6.7*

Data are means ± SEM from 15-21 rats per group. *P<0.001 indicates dose-dependent difference from sham control of the same duration of treatment. $^{\#}$P<0.001 and †P<0.001 indicate time-dependent difference from sham control of 4 and 8 weeks, respectively, using Student-Newman-Keuls test after two-ways ANOVA.

Measurements of active tension development using skinned papillary fiber isolated from the left ventricle of various experiment groups demonstrated both the time- and dose-dependent effect of testosterone treatment as shown in Figure 16. While cardiac hypertrophy was induced in every group of testosterone treatment (as shown in Figure 18), there was no change in the tension development of cardiac fibers from every testosterone-treated group for four weeks. Prolonged duration of testosterone treatment to eight weeks

significant induced a suppression of maximum active tension to the same degree in both 10 and 20 mg/kg of testosterone-treated groups but not the group with 5 mg/kg of the hormone. With the prolonged duration of treatment up to 12 weeks, decreases in the maximum active tension were demonstrated in every dose of the hormone treatment. On the other hand, there was no difference in the fiber sensitivity to Ca^{2+} activation for tension development among the experimental groups (Figure 16B). Thus, these results indicate that the testosterone-induced cardiac hypertrophy will be either physiologic or pathologic depending on both the dose and the duration of hormone treatment. Induction of physiologic hypertrophy of the heart will be observed only with short term treatment of the hormone without dose effect but pathologic cardiac hypertrophy will be the case when the treatment is prolonged.

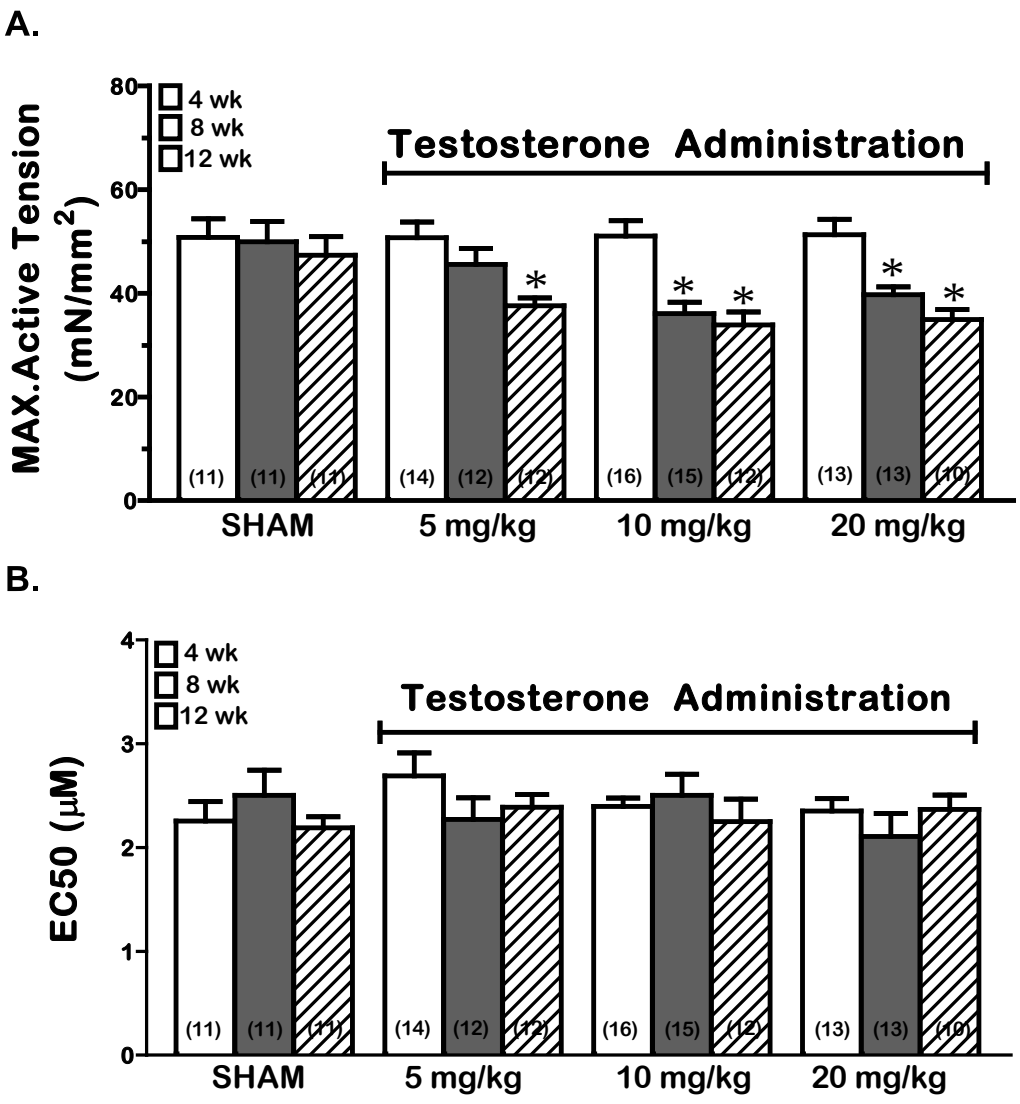

Figure 16. **(A)** Comparison of maximum active tension (mN/mm^2) and **(B)** EC50 (μM) of force generation of skinned papillary fiber preparations from sham control and testosterone-injected rats with various doses (5, 10, and 20 mg/kg body weight) for 4, 8, 12 weeks. Data are means ± SEM from number of fibers in the parenthesis. *P<0.05 represents significantly time-dependent difference from 4-week group of the same dose and significantly dose-dependent difference from sham control using using Student-Newman-Keuls test after two-ways ANOVA.

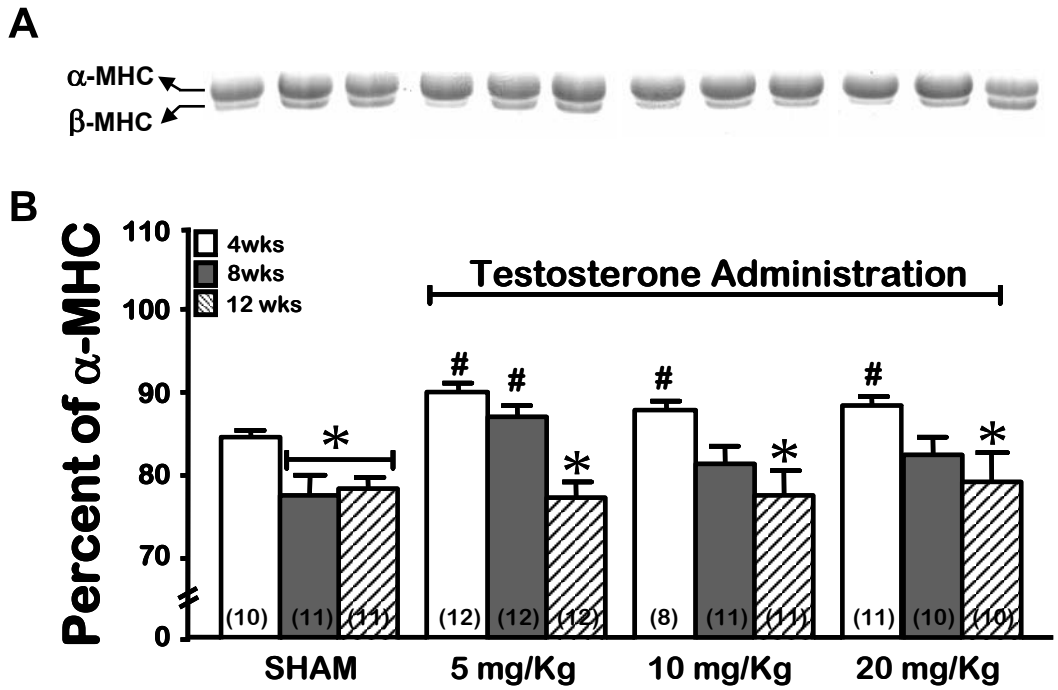

Figure 17. Percentage of α-MHC and gel representative of MHC isoforms in left ventricular tissue of sham control (SHAM) and testosterone-injected groups with various doses (5, 10, and 20 mg/kg body weight) for 4, 8, 12 week. Data are means ± SEM from number of fibers in the parenthesis. *P<0.05 represents significantly time-dependent difference from 4 week duration in the same dose and significantly dose-dependent difference from sham control in the same duration using Student-Newman-Keuls test after two-ways ANOVA.

Further analysis of MHC isoforms was performed to evaluate the potential underlying mechanism for the suppressed maximum tension of the contractile activity. As shown in Figure 17, there was both time- and dose-dependent effect of testosterone treatment on the relative amount of α-MHC. While the percentage of α-MHC was significant reduced with animal age as previously documented [92], significant increases in the percentage of α-MHC were observed in heart of rats treated with every dose of testosterone for 4 weeks compared to that of sham controls. Age-dependent decrease in the α-MHC expression was still present in the testosterone-treated groups. This age-dependent change in the isoform expression of α-MHC has been suggested to provide a greater economy of contraction which may help maintaining the cardiac contractile activity in aging as confirmed by results of maximum tension shown in the present study. Interestingly, the increased expression of α-MHC in testosterone-treated rats was observed without alterations in maximum active tension of the cardiac fibers. Moreover, with the same magnitude of α-MHC in the heart to that of sham, maximum tension development was significantly suppressed in 12-week testosterone-treated rat. These results thus imply that the compensatory response of the heart to testosterone treatment induced changes in the economy of cross-bridge cycling toward a lower effectiveness which may result in a possibility of pathological development.

Project IV: **To elucidate the relations of cardiac hypertrophy induced in high plasma testosterone rats to the SR Ca^{2+} uptake function**

i) Histochemical study

ii) Measurements of SERCA activity

iii) Immunoblots of connexin43 and SR proteins

Due to a potential of impaired SR function in underlying the mechanistic dysfunction of the contractile activity leading to hypertrophy of the heart, we then tested whether high plasma testosterone induces changes in SR function and consequently induces hypertrophy of the hearts? We have focused on determining the relations of cardiac hypertrophy to the SERCA activity in the testosterone-injected rats. Both the time- and dose-dependent impacts of testosterone overload on SERCA activity were determined at various Ca^{2+} concentrations using SR membrane preparations. The cross-sectional area of cardiomyocytes and the amount of interstitial collagen deposition were analyzed in the heart of treated rats using histochemical approach. Expression of gap junction protein connexin43, which indicates a possibility of arrhythmic induction in cardiac hypertrophy, was also determined.

Figure 18 shows the cross-sectional area of cardiomyocytes from each experimental group. While there was no change in the cardiomyocyte cross-sectional area of in sham control rats along the duration of study, enlargement of the cardiomyocytes was significantly shown in every group of rats with high-testosterone administration without time- and dose-dependent manner. These results indicate the dose-independent effect of supra-physiological dose of testosterone in inducing hypertrophy of cardiac myocytes. In conjunction with results of active tension development, the induced cardiac hypertrophy is more likely pathological end.

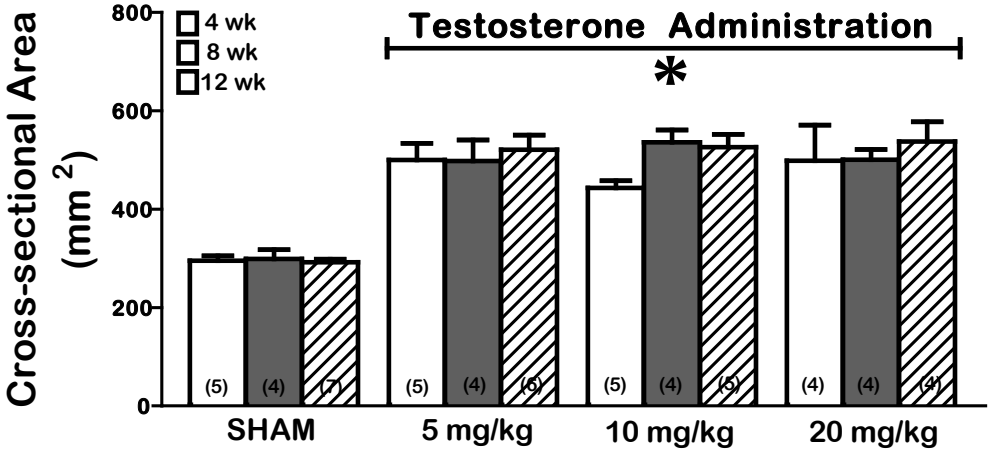

Figure 18. Cross–sectional area of cardiac myocytes from sham control and testosterone-injected rats with various doses (5, 10, and 20 mg/kg body weight) for 4, 8, 12 weeks. Data are means ± SEM from 100-150 myocytes/heart and of 4-7 hearts/group. *P<0.05 represents significant difference from sham control using Student-Newman-Keuls test after two-ways ANOVA.

Structural abnormality of the heart was further examined by measuring amount of collagen deposition. As shown in Figure 19, significant increases in the amount of collagen deposition were observed in the cardiac section of rats treated with high doses of 10 and 20 mg/kg BW for 8 and 12 weeks compared to the sham control. The results confirm the pathological induction of hypertrophy in testosterone-treated rats.

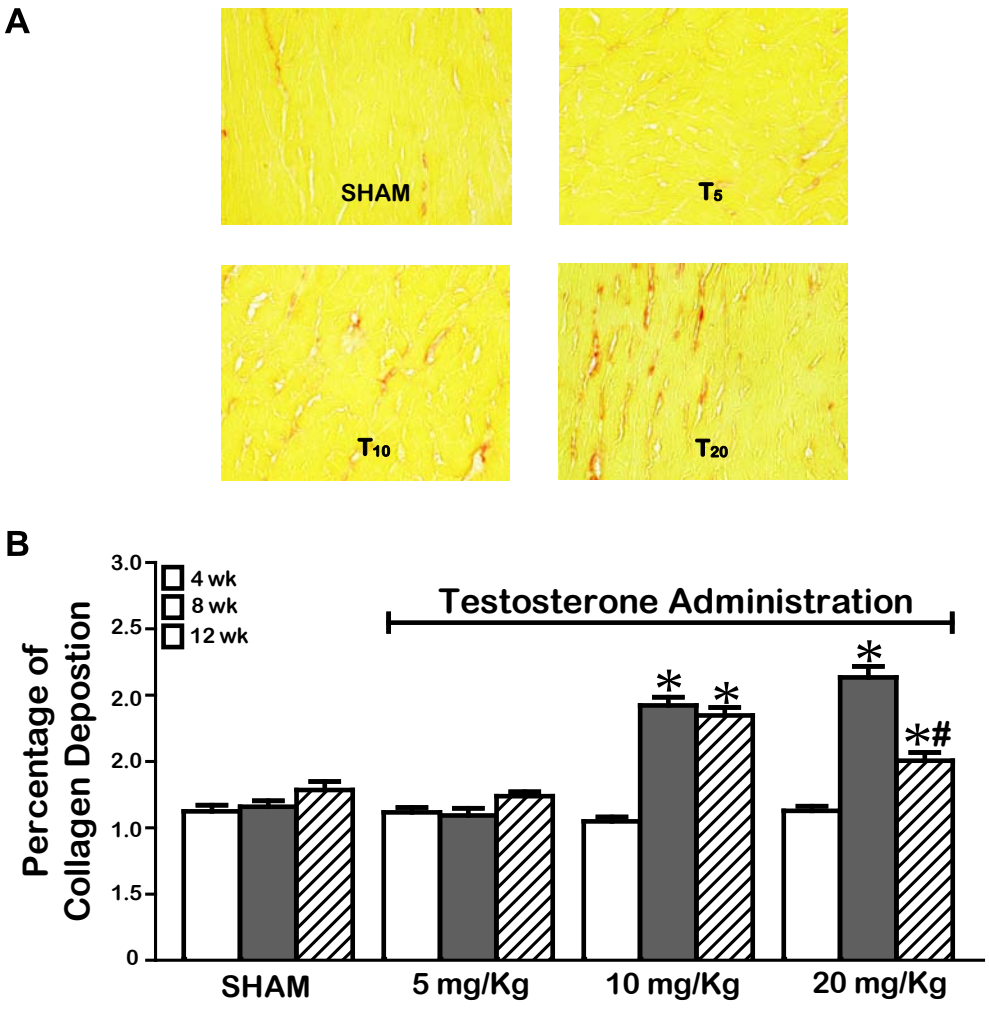

Figure 19. **A:** Cardiac section stained with Pico-Sirius red and viewed under light microscope (X400 magnification). **B:** Comparison of the percentage of collagen deposition in the cardiac section from sham and testosterone-injected rats with various doses (5, 10 and 20 mg/kg body weight) for 4, 8 and 12 weeks. Data are means $\pm$ SEM from 300-400 myocytes in 3-5 hearts per group. *P<0.05 represents significantly dose-dependent difference from sham control in the same duration and $^{\#}$P<0.05 represents significant difference from the 8-week duration group using Student-Newman-Keuls test after two-ways ANOVA.

Whether the pathological hypertrophy induced by testosterone treatment further affect the electrical conduction of the heart or not was demonstrated by evaluating the expression of connexin43 and its phosphorylated level. As shown in figure 20, there was no change the expression ratio of phospho-Ser^{368} CX43 to the total CX43 among the different doses of

testosterone treatment. Thus, treatment of supra-physiological dose of testosterone induced hypertrophy of the heart toward pathological end by suppressing the contractile activity without affecting the electrical conduction through the gap-junction between cardiac myocytes.

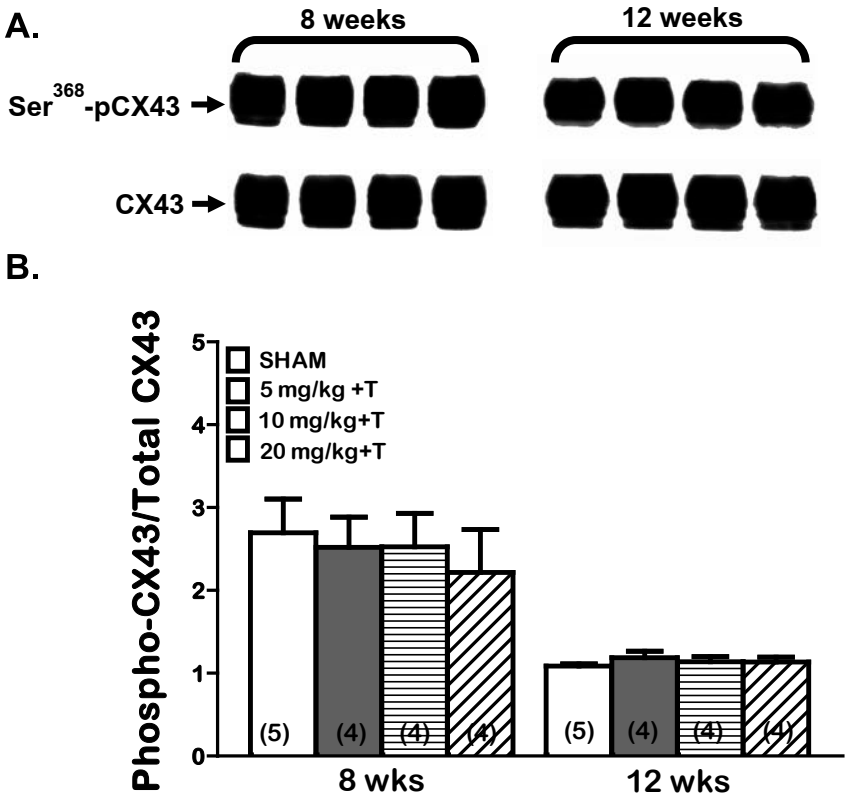

Figure 20. **(A)** Immunoblot analysis of phosphorylated Serine368 (phosphor-Ser^{368}) connexin 43 (CX43) and total CX43 and **(B)** comparison of the band intensity expressed as a ratio of phosphor-Ser^{368} CX43 to the total CX43 protein in the left ventricular homogenate of sham control and testosterone-injected rats (+T) with various doses (5, 10, and 20 mg/kg body weight) for 4, 8, 12 weeks. Data are means ± SEM from 3-5 hearts.

Measurements of SERCA expression and pump activity were performed using immunoblot and enzyme kinetic assay, respectively. Activity of SERCA was determined at various Ca^{2+} concentrations using SR membrane preparations. Figure 21 shows comparison of cardiac hypertrophy data derived from the percentage of heart to body weight ratio, the maximum SERCA activity, and the EC_{50} of SERCA activity among the experimental groups from the study. Maximum and the Ca^{2+} sensitivity of SERCA activity were summarized in Table 7 and 8, respectivity. While there was no dose effect after 4 weeks of testosterone treatment despite the appearance of cardiac hypertrophy, dose-dependent increases in maximum SERCA activity were observed in testosterone-treated rats for both 8 and 12 weeks. However, with the highest dose of testosterone (20 mg/kg BW) administration, maximum SERCA activity was returned to be the same as that of vehicle in both 8 and 12 weeks of treatment. The Ca^{2+} sensitivity of SERCA activity seems to have no change among the

groups except in one group with 10 mg/kg testosterone treatment for 12 weeks. These results indicate that supra-physiological dose of testosterone induces cardiac hypertrophy before any changes in SERCA activity. The parallel increase in SERCA activity with cardiac hypertrophy suggests a physiological adaptation of the hypertrophic heart. The reversed change in SERCA activity at high dose may imply the turning status from physiological to pathological adaptation of the heart to hypertrophy induction.

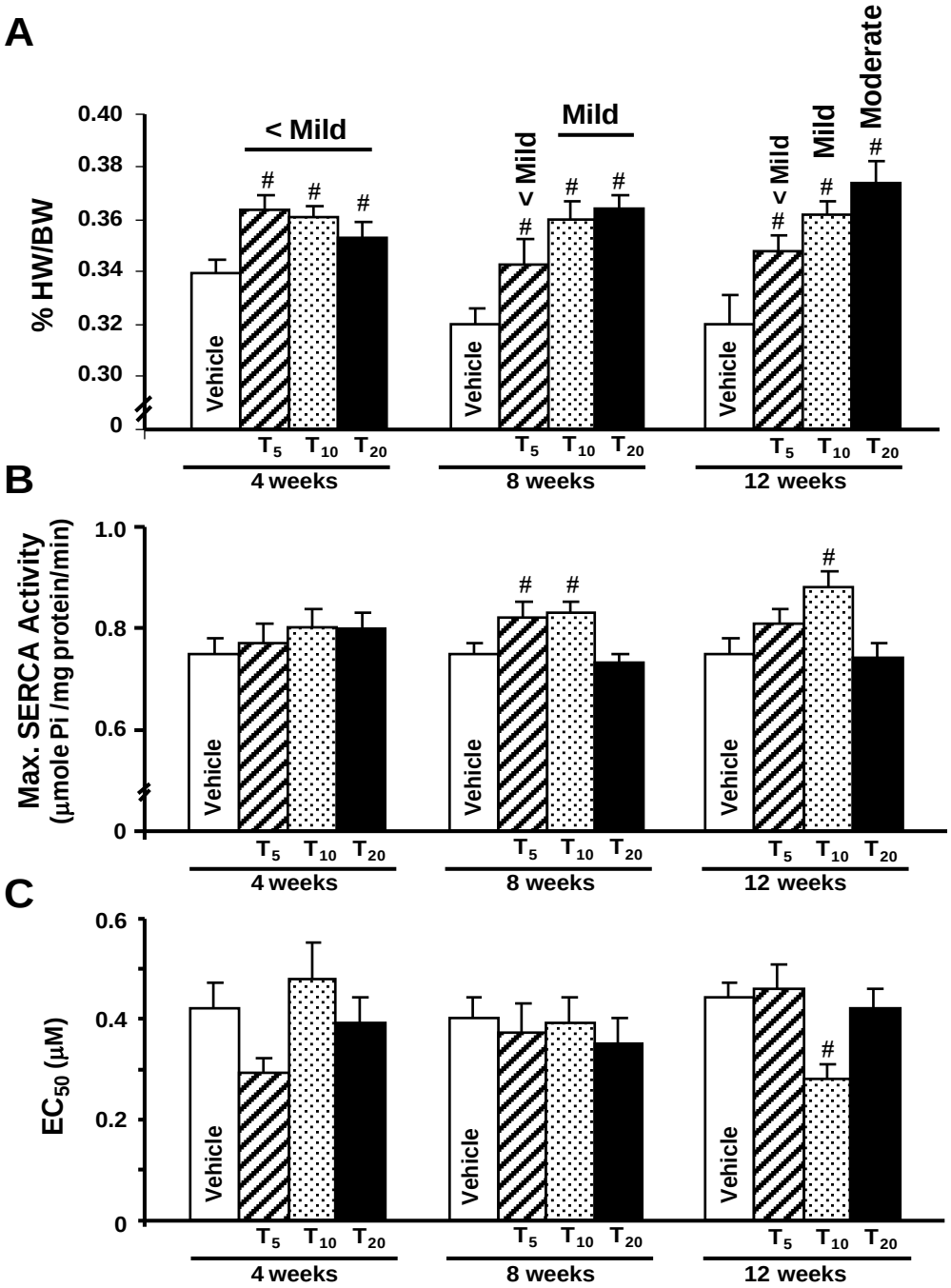

Figure 21. ***A*:** Comparison of maximum SERCA activity (µmole Pi/mg protein/min) among vehicle- and various high doses (5, 10, and 20 mg/kg BW) testosterone-injected rats for 4, 8 and 12 weeks and EC_{50} (*B*). Data are means ± SEM from 8-12 preparations. $^{\#}P$ 0.05 represents significantly dose-dependent difference from vehicle control in the same duration of study using Student-Newman-Keuls test after two-way ANOVA.

Table 7. The maximum SERCA activity (μmole Pi/mg protein/min) of cardiac SR membrane vesicles from vehicle- and high testosterone-injected rats (5, 10, and 20 mg/kg BW) for 4, 8 and 12 weeks of treatment

GROUP	Maximum SERCA activity (μmole Pi/mg protein/min)		
	4 weeks	8 weeks	12 weeks
Vehicle	0.75 ± 0.03 (10)	0.75 ± 0.02 (10)	0.75 ± 0.03 (8)
T5	0.77 ± 0.04 (11)	0.82 ± 0.03$^{\#}$ (10)	0.81 ± 0.03 (11)
T10	0.80 ± 0.04 (10)	0.83 ± 0.02$^{\#}$ (11)	0.88 ± 0.03$^{\#}$ (11)
T20	0.80 ± 0.03 (11)	0.73 ± 0.02 (11)	0.74 ± 0.03 (12)

Data are means ± SEM for rat numbers in the parenthesis. $^{\#}P<0.05$ represents significantly dose-dependent difference from vehicle control in the same duration of study using Student-Newman-Keuls test after two way ANOVA.

Table 8. The effective Ca^{2+} concentration producing half-maximal activation (EC_{50}) of SERCA activity of the cardiac SR membrane vesicles from vehicle- and high testosterone-injected rats (5, 10, and 20 mg/kg BW) for 4, 8 and 12 weeks

GROUP	EC_{50} (μM)		
	4 weeks	8 weeks	12 weeks
Vehicle	0.42 ± 0.05 (10)	0.40 ± 0.04 (10)	0.44 ± 0.03 (8)
T5	0.29 ± 0.03 (11)	0.37 ± 0.06 (10)	0.46 ± 0.05 (11)
T10	0.48 ± 0.07 (10)	0.39 ± 0.05 (11)	0.28 ± 0.03$^{\#}$ (11)
T20	0.39 ± 0.05 (11)	0.35 ± 0.05 (11)	0.42 ± 0.04 (12)

Data are means ± SEM for rat numbers in the parenthesis. $^{\#}P<0.05$ represents significantly dose-dependent difference from vehicle control in the same duration of study using Student-Newman-Keuls test after two way ANOVA.

Expression of SERCA and its regulatory protein, phospholamban, was further evaluated and results were demonstrated in Figure 22. While there was no change in the phospholamban levels among the groups, expression of $SERCA_{2a}$ was increased only in the group with 10 mg/kg testosterone administration for 8 weeks (Figure 22B). However, results of $SERCA_{2a}$ to phospholamban ratio then demonstrated no change among the groups (Figure 22C).

Taken together, we tested whether supra-physiological dose of testosterone induces changes in the Ca^{2+}-handling activities leading to hypertrophy of the heart and/or testosterone-induced cardiac hypertrophy that reversely impacts on the Ca^{2+}-handling proteins. Our results show no association between Ca^{2+}-handling activities and testosterone level in cardiac hypertrophy induction. It is, however, the cardiac hypertrophy reversely changes the Ca^{2+} pumping activity of SERCA. These data indicate an adaptive change of the cardiac Ca^{2+}-

handling proteins evoked after a prolonged exposure to high testosterone. However, this adaptive response may eventually degenerate to maladaptive response which may lead to deterioration of the heart if the high testosterone persists. The conclusion is partially supported by previous reports suggesting that an increase in the intracellular Ca^{2+} mobilization may precede cardiovascular decompensation in some models of cardiac hypertrophy.[93-95]

Administration of testosterone or synthetic anabolic steroids has been well recognized to be related to increase the risk of hypertension, alteration in lipid profile and coronary artery disease [96]. With the identification of androgen receptors in isolated cardiac myocytes [17, 97], the finding may explain how testosterone promotes the arterial stiffness, ventricular remodeling and compliance, inflammation and apoptosis, and cardiac hypertrophy and fibrosis of the heart [98-101].

Generally, evaluation of hypertrophic induction pattern of cardiomyocytes involves three parameters, including 1) changes in the expression of β-MHC and SKA, anti-skeletal actin, which are well known cardiac hypertrophy marker, 2) cardiomyocyte size, and 3) protein synthesis assessed by amino acid incorporation. Testosterone has been shown to significantly increase all three parameters that were blocked by inhibiting mTORC1, mammalian target of rapamycin complex 1 [102]. A hallmark event for anabolic action of testosterone is through protein synthesis, which is highly regulated by the mTORC1. Testosterone has recently been demonstrated to activate the mTORC1 pathway in cardiomyocytess through inositol 1,4,5-triphosphate (IP_3)-mediated Ca^{2+} release and MEK/$ERK_{1/2}$ [102]. Based on this pathway of action, our results of 4 weeks testosterone-treated group showing that differences doses of testosterone have no effects on SERCA activity but induce cardiac hypertrophy to the same degree further indicate the dissociation of cardiac hypertrophy to changes in SERCA activity. Furthermore, the association was observed in prolonged testosterone treatment to 8 and 12 weeks. The results further confirm the reversed impact of cardiac hypertrophy on the SERCA function.

How testosterone-induced cardiac hypertrophy could reversely change the SERCA activity is presently unknown? Effects of testosterone on the SERCA activity seems to be maximized under physiological condition as demonstrated from our results in castrated rat study. Moreover, no pharmacological effect of testosterone on SERCA function was observed. However, other Ca^{2+} mediating proteins like L-type Ca^{2+} channels and ryanodine receptors still leave possibility for effect of testosterone-induced cardiac hypertrophy to indirectly activate or deteriorate the SERCA activity. As shown in two previous studies in neonatal [103] and adult [104] rat ventricular myocytes, chronic incubation of testosterone enhances Ca^{2+} influx via the L-type Ca^{2+}channel. Golden et al. [105] also demonstrated that testosterone also increases the expression of L-type Ca^{2+} channels. In addition, a spontaneously arising in Ca^{2+} spark in chronic testosterone-treated cardiomyocytes was clearly detected by Line-scan images [104]. In the adult ventricular myocyte, testosterone also increases the Ca^{2+} spark, indicating an increase the Ca^{2+} releases from the SR. These effects are abolished upon androgen receptor blockade, indicating receptor-mediated events. In any case, the information is still inconclusive and more direct studies need to be done in details.

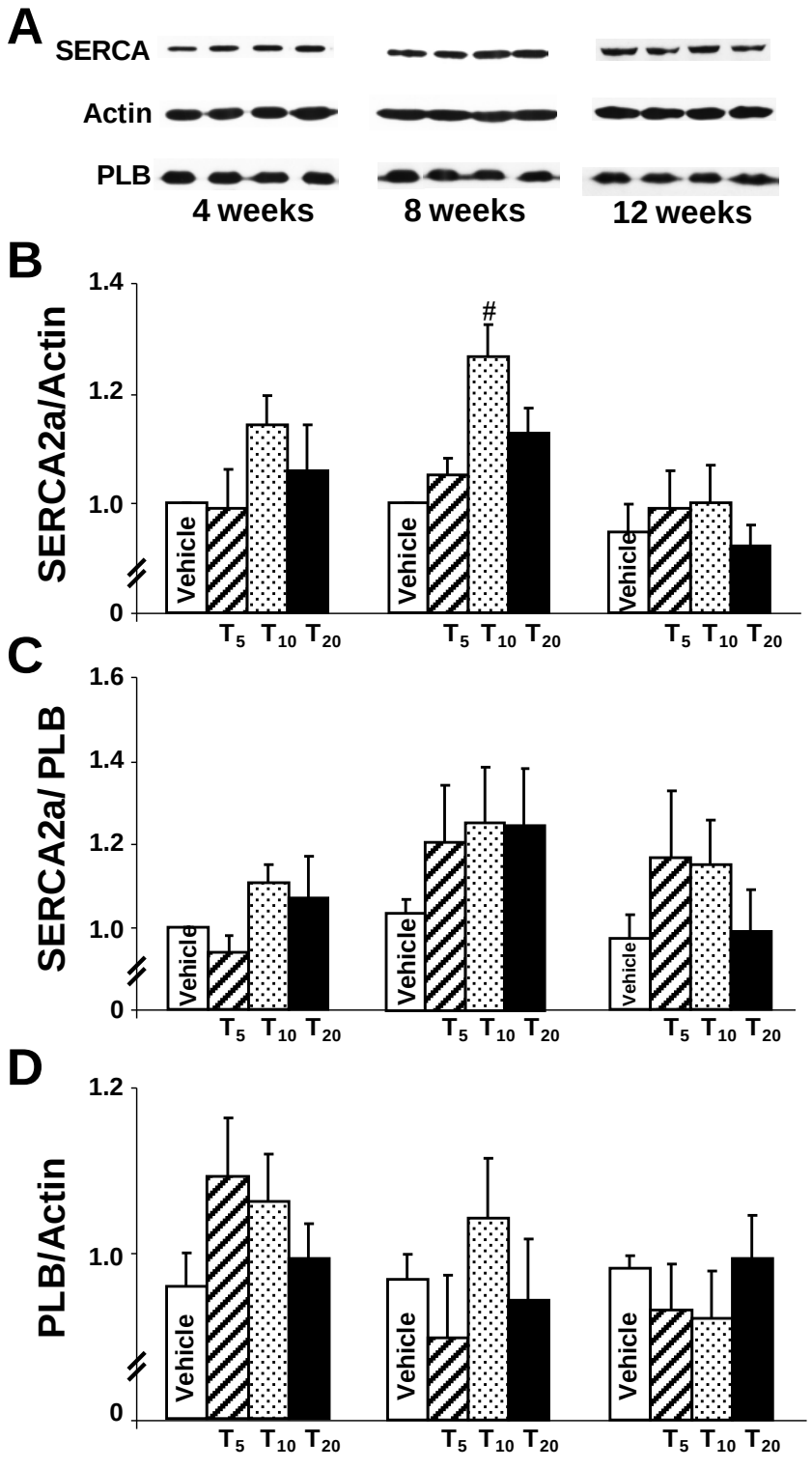

Figure 22. ***A***: Immunoblot analysis of SERCA2a, phospholamban (PLB) and actin, and comparison of the band intensity expressed as a ratio of SERCA2a to actin (*B*), PLB to actin (*C*) and SERCA2a to PLB (*D*) of left ventricular homogenates from vehicle control and high testosterone-injected groups (5, 10, and 20 mg/kg BW) after 4, 8 and 12 weeks of treatment. Data are means ± SEM from 4-6 hearts. $^{\#}P<0.05$ represents significantly dose-dependent difference from vehicle control in the same duration of study using Student-Newman-Keuls test after two way ANOVA.

Project V: **To determine the signaling component that involves in testosterone-induced cardiac hypertrophy**

i) **Determinations of pathological signals in high testosterone rat hearts**
ii) **Determinations of physiological signals in high testosterone rat hearts**
iii) **Determinations of pathological and physiological signals in high testosterone rats with exercise training (preliminary study in this project)**

Cardiac hypertrophy is a remodeling response of the heart to many insults that lead to a better cardiac performance in case of physiological hypertrophy or a sustained/decreased performance in pathological hypertrophy depending on type, degree, and exposed frequency of stress. It is well accepted that both remodeling processes share some signaling processes besides the different signaling mechanisms. It is presently unknown whether the hypertrophy of the heart induced by testosterone is physiological or pathological. The study is therefore aimed to determine the specific signaling process of action in high testosterone-treated rats. Changes in the activation, expression, and/or phosphorylation of potential pathological signals, including calcineurin, mitogen-activated protein kinase (MAPK: p38, JNK, $ERK_{1/2}$), and nuclear factor of activated T cell (NFAT3c) will be analyzed. On the other hand, level of the mammalian-target of rapamycin (mTOR) was also planned to be measured to represent the physiological signal of cardiac hypertrophy. Based on the well-recognized effect of exercise training in inducing physiological hypertrophy of the heart, the experimental rats with combined treatment of high testosterone and running-trained program has also been included in this project as preliminary study for the future research. Results from this set of study will provide information concerning the preventive or additive effect of exercise training on inducing cardiac hypertrophy.

Based on results in project III & IV indicating the potential of pathological cardiac hypertrophy in 8 and 12 weeks after testosterone treatment, the signaling components determined in this part was then concentrated on pathological signals like calcinurin and NFAT. Unexpectedly, both calcineurin (Figure 23) and NFAT (Figure 24) show a tendency to be increased in the heart of testosterone-treated rats compared to sham control using the blotting technique. Activation of calcineurin will usually dephosphorylate the NFAT to allow the translocation of NFAT into the nucleus and therefore turn on many gene transcriptions in underlying cardiac hypertrophy. A tendency increase in calcineurin should then cope with a tendency decrease in NFAT level. These unexpected results of NFAT may be due to the measurement of both the phosphorylated form in the cytosol and the dephosphorylated form in the nucleaus. So, the histoimmunochemical technique may be a better approach of determination which would need to wait for further study.

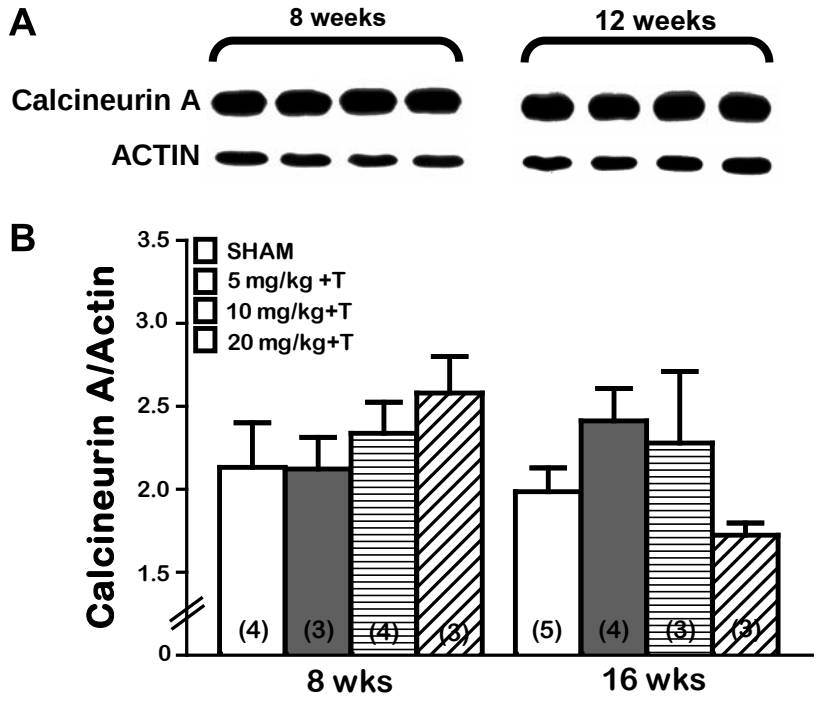

Figure 23. **A:** Immunoplot analysis of calcineurin A, and **B:** comparison of the band intensity expressed as a ratio of calcineurin A to actin of left ventricular homogenates from sham control and testosterone-injected rats (+T) with various doses (5, 10 and 20 mg/kg BW) for 8 and 12 weeks. Data are means ± SEM from 3-5 hearts.

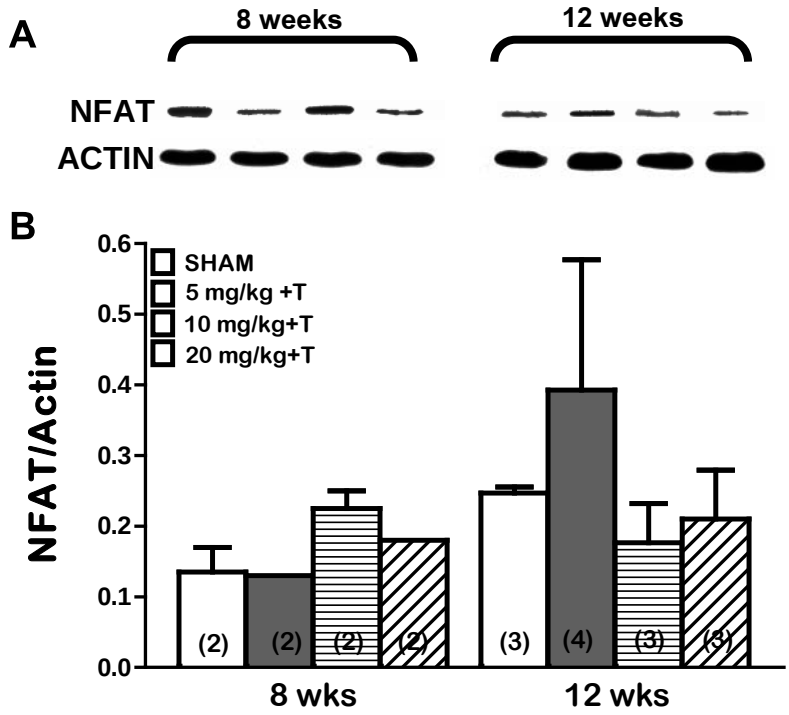

Figure 24. **A:** Immunoblot analysis of NFAT3 and **B:** comparison of the band intensity expressed as a ratio of NFAT3 to actin of left ventricular homogenates from sham control and testosterone-injected rats (+T) with various doses (5, 10 and 20 mg/kg BW) for 8 and 12 weeks. Data are means ± SEM from 2-4 hearts.

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RESEARCH OUTPUT

I. INTERNATIONAL PUBLICATIONS:

1. Bupha-Intr T., J Laosiripisan, and **J Wattanapermpool.**
   Moderate intensity of regular exercise improves cardiac SR Ca^{2+} uptake activity in ovariectomized rats.
   J. Appl. Physiol. 107: 1105-1112, 2009.
   Impact Factor = 3.732 (Journal Citation Reports 2009)
2. Two more manuscripts are on the process of preparation.

II. ABSTRACT & ORAL/POSTER PRESENTATION:

1. Pirompol P, T. Bupha-Intr, J. Wattanapermpool. Chronic administration of high testosterone tends to induce pathological cardiac hypertrophy. The XX World Congress Meeting of the International Society for Heart Research, May 13-16, 2010, Kyoto, Japan.
2. Witayavanitkul N, T. Bupha-Intr, J. Wattanapermpool. Suppressed activities of cardiac SR Ca^{2+}-ATPase and Na^{+}-Ca^{2+} exchanger in testosterone-deficient rat. The XX World Congress Meeting of the International Society for Heart Research, May 13-16, 2010, Kyoto, Japan.
3. Pirompol P, Bupha-Intr T and Wattanapermpool J. Pathological Hypertrophy of the Heart after Chronic Administration of Testosterone. ในการประชุม “นักวิจัยรุ่นใหม่ ..พบ.. เมธีวิจัยอาวุโส สกว." ครั้งที่ 10 , วันที่ 14-16 ตุลาคม 2553 ณ โรงแรมรีเจนท์ ชะอำ จังหวัดเพชรบุรี
4. Pirompol P, Bupha-Intr T and Wattanapermpool J. Chronic administration of high testosterone tends to induce pathological cardiac hypertrophy. ในการประชุมวิชาการประจำปี ของสรีรวิทยาสมาคมแห่งประเทศไทย วันที่ 5 -7 พฤษภาคม 2553 ณ โรงแรมอมารีออร์คิดรีสอร์ทแอนด์ทาวเวอร์ พัทยา จังหวัดชลบุรี
5. Witayavanitkul N, T. Bupha-Intr, J. Wattanapermpool. Differential effects of testosterone deficiency and overload on cardiac SR Ca^{2+}-ATPase and Na^{+}-Ca^{2+} exchanger activities. ในการประชุมวิชาการประจำปี ของสรีรวิทยาสมาคมแห่งประเทศไทย วันที่ 5 -7 พฤษภาคม 2553 ณ โรงแรมอมารีออร์คิดรีสอร์ทแอนด์ทาวเวอร์ พัทยา จังหวัดชลบุรี

   (ได้รับรางวัล BUNGORN CHOMDEJ AWARD ในการนำเสนอ โปสเตอร์ระดับนักศึกษาปริญญาโท ดีเด่น)
6. Pirompol P, Bupha-Intr T and Wattanapermpool J. Pathological cardiac hypertrophy is induced after long-term testosterone administration. ในการประชุม MUSC Graduate Research Exposition ณ. อาคารสตางค์มงคลสุข คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล วันที่ 26-27 ตุลาคม 2553

   (ได้รับรางวัลการนำเสนอ โปสเตอร์ระดับปริญญาเอก ดีเด่น)

III. AS INVITED SPEAKER

Oral presentation in two meetings and one special seminar

1. Wattanapermpool J. The Gender Matter with Cardiac Contractile Activation. Guest speaker for special seminar at Department of Cellular & Molecular Physiology, College of Medicine, Loyola University Chicago, Maywood, USA. June 3, 2009.
2. จงกลณี วัฒนาเพิ่มพูล Beautiful Heart in Healthy Women ณ. Rotary club of Bangkok-Ploenchit, Bangkok, Thailand on August 4, 2009.
3. จงกลณี วัฒนาเพิ่มพูล Sex Hormones and Your Heart สำหรับการบรรยายสัมนา ณ ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร จังหวัดพิษณุโลก วันที่ 22 กันยายน 2553

IV. การสร้างนักวิจัยรุ่นใหม่

สร้างนักวิจัยรุ่นใหม่ เป็นนักศึกษาระดับ**ปริญญาโท** 1 คน คือ

นางสาวน้ำทิพย์ วิทยะวาณิชกุล

สร้างนักวิจัยรุ่นใหม่ เป็นนักศึกษาระดับ **ปริญญาเอก** 1 คน คือ

นางสาวประภาวดี ภิรมย์พล