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โครงการวิจัยเรื่อง

บทบาทของฮอร์โมนเพศ ต่อสรีรวิทยาของกล้ามเนื้อหัวใจ

โดย

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สัญญาเลขที่ BRG 4880005

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย และ มหาวิทยาลัยมหิดล ที่ให้ทุนสนับสนุนโครงการวิจัยนี้ ขอขอบพระคุณอาจารย์ที่ปรึกษาของโครงการทั้ง 3 ท่าน คือ Prof. Dr. R. John Solaro และ Prof. Pieter de Tombe (Department of Physiology & Biophysics University of Illinois at Chicago) และ ศ.ดร. นทีทิพย์ กฤษณามระ (ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล) ที่ให้คำปรึกษาอย่างต่อเนื่องตลอดโครงการ โดยเฉพาะ Prof. Dr. R. John Solaro ซึ่งกรุณาเป็นอาจารย์ที่ปรึกษาร่วมในการดำเนินงานวิทยานิพนธ์ ของนักศึกษาภายใต้โครงการปริญญาเอกกาญจนาภิเษกในส่วนการตรวจวัดปริมาณของแคลเซียมอิสระภายในเซลล์ อีกทั้งขอขอบคุณ ผศ.พญ. อรุณี ธิติธัญญานนท์ (ภาควิชาจุลชีววิทยา คระวิทยาศาสตร์ มหาวิทยาลัยมหิดล) ที่ช่วยสอนเทคนิคการตรวจวัด mRNA ด้วย Real-Time PCR แก่นักศึกษาภายใต้การควบคุมวิทยานิพนธ์ และขอบพระคุณ ศ.ดร. ประพนธ์ วิไลรัตน์ ที่กรุณาให้คำแนะนำปรึกษา และวิจารณ์แก้ไข manuscript ให้เป็นอย่างดียิ่ง

สุดท้ายนี้ขอขอบคุณภาควิชาสรีรวิทยา นางโกสุม วงศ์ไทย และ นางสาวชลลดาย์ สาพียะ ที่ช่วยทำบัญชีเบิกจ่ายและพิมพ์รายงานต่างๆ และ นายเทพมนัส บุปผาอินทร์ นางสาวอริยพร ถาวรไกรวงศ์ และ นางสาวจิตานันท์ เหล่าศิริไพศาล นักศึกษาบัณฑิตภายใต้การควบคุมดูแลที่มีส่วนร่วมดำเนินงานวิจัยให้สำเร็จลุล่วงเป็นอย่างดี

บทคัดย่อ

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การศึกษานี้เน้นศึกษาบทบาทสำคัญของฮอร์โมนเพศหญิงต่อการทำงานของหัวใจใน 5 หัวข้อย่อย คือ 1) การเปลี่ยนแปลงปริมาณแคลเซียมในเซลล์กล้ามเนื้อหัวใจหนูตัดรังไข่ที่สภาวะเป็นกรด 2) การเปลี่ยนแปลงปริมาณ mRNA ของ sarco (endo) plasmic reticulum (SR) calcium ATPase (SERCA) 3) ความเป็นไปได้ของการออกกำลังกายเป็นประจำในการป้องกันการเปลี่ยนแปลงปฏิกิริยาการดูดกลับแคลเซียมที่ SR 4) การแทรกแซงของเบาหวานต่อการตอบสนองของเส้นใยกล้ามเนื้อต่อแคลเซียม และ 5) การแทรกแซงของเบาหวานต่อความสัมพันธ์ระหว่างการเปลี่ยนแปลง isoform ของ myosin heavy chain กับการเปลี่ยนแปลงปฏิกิริยาการหดตัวของเส้นใยกล้ามเนื้อหัวใจ

เมื่อเปรียบเทียบกับหนูปกติ สภาวะเป็นกรดมีผลลดการหดตัวของเซลล์กล้ามเนื้อหัวใจหนูตัดรังไข่น้อยกว่า แต่เมื่ออยู่ในสภาวะเป็นกรดเป็นเวลานาน จะเกิดการปรับเปลี่ยนให้หดตัวเพิ่มขึ้นซึ่งสัมพันธ์กับการเพิ่มปริมาณแคลเซียมในเซลล์ เมื่อทดสอบสภาวะความเป็นกรดโดยมีสารยับยั้งตัวแลกเปลี่ยนโซเดียม-ไฮโดรเจนอยู่ด้วย จะไม่มีผลเปลี่ยนแปลงทั้งปริมาณแคลเซียมและการหดตัวของเซลล์ ชี้ให้เห็นว่าการขาดฮอร์โมนเพศหญิงมีผลปรับเปลี่ยนปริมาณแคลเซียมในเซลล์กล้ามเนื้อหัวใจ โดยอาจเกิดจากการแลกเปลี่ยนโซเดียม-ไฮโดรเจนที่เพิ่มขึ้น จึงสามารถใช้บ่งชี้อิทธิพลของเพศต่อการทำงานของหัวใจเมื่อรวมกับผลการเพิ่มความไวของเส้นใยกล้ามเนื้อต่อการกระตุ้นของแคลเซียมหลังขาดฮอร์โมนเพศ

เราได้พบเป็นครั้งแรกเช่นกันว่า ฮอร์โมนเพศหญิงมีบทบาทสำคัญในการควบคุมการดูดกลับแคลเซียมในเซลล์กล้ามเนื้อหัวใจ โดยในหัวใจหนูตัดรังไข่พบการลดทั้งปริมาณและปฏิกิริยาการดูดกลับแคลเซียมของ SR แต่เพิ่มการตอบสนองต่อแคลเซียมในการดูดกลับ ทั้งนี้พบร่วมกับการลดลงของปริมาณโปรตีน SERCA ซึ่งการเปลี่ยนแปลงจะกลับเป็นปกติเมื่อให้ฮอร์โมนเอสโตรเจนหรือโปรเจสเตอโรนทดแทน นอกจากนี้ยังพบว่า ระดับ SERCA mRNA ลดลงตามการลดของปฏิกิริยาการดูดกลับแคลเซียม และการออกกำลังกายเป็นประจำสามารถป้องกันการเปลี่ยนแปลงการดูดกลับแคลเซียมของหัวใจหนูตัดรังไข่ได้เช่นเดียวกัน

เมื่อศึกษาหนูตัดรังไข่ที่เหนี่ยวนำให้เป็นโรคเบาหวาน เพื่อทดสอบฤทธิ์ร่วมของเอสโตรเจนและอินซูลินต่อการทำงานของเส้นใยกล้ามเนื้อหัวใจ พบว่าการลดลงของปฏิกิริยาการหดตัวสูงสุดของเส้นใยกล้ามเนื้อหัวใจ ไม่แตกต่างจากทั้งหนูตัดรังไข่และหนูเบาหวาน ในทางตรงข้าม การเพิ่มความไวของเส้นใยกล้ามเนื้อต่อการกระตุ้นของแคลเซียม ซึ่งพบจำเพาะในหัวใจหนูตัดรังไข่แต่ไม่พบในหนูเบาหวานนั้น สามารถพบเช่นกันในหัวใจหนูตัดรังไข่ที่เป็นเบาหวานและป้องกันได้เมื่อให้เอสโตรเจนทดแทน ในขณะที่ปริมาณ β_1-adrenergic receptors เพิ่มขึ้นตามการเพิ่มความไวของเส้นใยกล้ามเนื้อต่อการกระตุ้นด้วยแคลเซี่ยม ปริมาณของ heat shock protein 72 จะพบเปลี่ยนแปลงตามค่าปฏิกิริยาการหดตัวสูงสุดของเส้นใยกล้ามเนื้อ แสดงให้เห็นว่าการเพิ่มความไวของเส้นใยกล้ามเนื้อต่อการกระตุ้นของแคลเซียมเป็นการเปลี่ยนแปลงที่พบจำเพาะต่อภาวะขาดฮอร์โมนเพศหญิงแม้ในภาวะที่มีเบาหวานร่วมด้วย โดยการเปลี่ยนแปลงปริมาณ β_1-adrenergic receptors อาจเป็นกลไกหนึ่งในการออกฤทธิ์ป้องกันหัวใจของเอสโตรเจน และร่วมกำหนดความแตกต่างของเพศต่อการทำงานของหัวใจ

คำหลัก: ฮอร์โมนเพศหญิง การออกกำลังกายเป็นประจำ แคลเซียมในเซลล์ การดูดกลับแคลเซี่ยมที่ซาร์โคพลาสมิคเรติคูลั่ม

ABSTRACT

Project Code : RSA/11/2540
Project Title : Role of Sex Hormones in Cardiac Muscle Physiology
Investigator : Assoc. Prof. Dr. Jonggonnee Wattanapermpool
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The significant role of sex hormones in cardiac contractile activity has been reported in a series of experiment. The present study was further focused on investigating the significance of female sex hormones in five specific aspects, including 1) the relative contribution of acidosis on the Ca^{2+} transients of cardiomyocytes isolated from ovariectomized (OVX) rats, 2) alterations in the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) mRNA level, 3) the preventive effect of exercise training on changes in the SR Ca^{2+} uptake activity, 4) the complication of diabetes on changes in the cardiac myofibrillar response to Ca^{2+}, and 5) the complication of diabetes on the relationship between shifts in myosin heavy chain (MHC) isoform and changes in myofibrillar ATPase activity.

Hypercapnic acidosis suppressed the shortening of OVX myocytes to a lesser extent than that in shams. A larger compensatory increase in %cell shortening was also obtained in OVX myocytes during prolonged acidosis. The elevated compensation in cell shortening was related to a higher amount of increase in the amplitude of the Ca^{2+} transient. However, these differences in Ca^{2+} transients and %cell shortening were no longer evident in the presence of 1 μM cariporide, a specific inhibitor of Na^+/H^+ exchanger type 1 (NHE_1). Our results indicate that deprivation of female sex hormones modulates the intracellular Ca^{2+} in cardiac myocytes, possibly via an increased NHE_1 activity, which may act in concert with myofilament Ca^{2+} hypersensitivity as a determinant of sex differences in cardiac function.

We have also shown for the first time that female sex hormones play an important role in the regulation of the cardiac SR Ca^{2+} uptake. Significant suppressions of both maximum SR Ca^{2+} uptake and SERCA activities, but with an increase in SR Ca^{2+} responsiveness, were detected in OVX hearts. In combination with a significant downregulation of SERCA proteins which could be reversed either by estrogen or progesterone supplementation, a significant decrease in the SERCA mRNA level in association with the suppressed maximum SERCA activity was further demnstrated. Moreover, the cardio-protective effect of exercise training on changes in the SR Ca^{2+} uptake activity in OVX rat hearts was nicely demonstrated.

The potential interaction of estrogen and insulin on cardiac myofilament activation was further investigated in various groups of OVX rat complicated with diabetes (DM). A similar magnitude of suppression in maximum myofibrillar ATPase activity was detected in OVX, DM, and DM-OVX rat hearts. Such suppressed activity and the relative reduced α-myosin heavy chain level in DM-OVX rat could all be completely restored by estrogen and insulin supplementation. In contrast, the unique myofilament Ca^{2+} hypersensitivity detected in the OVX but not DM rat was also found in DM-OVX rat heart in which could be restored upon estrogen supplementation. While the amount of β_1-adrenoceptors significantly increased concomitant with the Ca^{2+} hypersensitivity of the myofilament without differences in the receptor binding affinity, changes in the amount of heat shock protein 72 paralleled that of maximum myofibrillar ATPase activity. Thus, hypersensitivity of cardiac myofilament to Ca^{2+} is specifically induced in OVX rats even under diabetes complication and the altered expression of β_1-adrenoceptors may, in part, play a mechanistic role underlying the cardioprotective effects of estrogen that act together with the myofilament Ca^{2+} hypersensitivity in determining gender differences in cardiac activation.

Keywords: female sex hormones, exercise training, intracellular calcium, SR Ca^{2+} uptake activity, SR Ca^{2+}-ATPase activity

CONTENTS

INTRODUCTION

Heart failure is currently the most prevalent cause of death in many countries including Thailand. Many risk factors of heart failure including age, sex, nationality, smoking, hypertension, hyperlipidemia, and diabetic mellitus have been reported (1-5) with gender as the most recognizable and concentrated factor by most investigations. However, the basic knowledge, the mechanisms, and the treatment regimen of the disease are still not clearly understood. The well recognizable sex difference in the incidence of cardiovascular diseases indicates the significance of sex hormones on cardiac function. Data of epidemiological studies of heart disease have clearly shown a lower cardiac mortality in women as compared to age-matched men (5, 7). The incidence of cardiovascular diseases in postmenopausal women is highly elevated resulting in a decrease in male to female ratio of cardiac mortality. An increased risk of heart disease was also observed in young women undergone bilateral oophorectomy in which appears to be prevented by estrogen-replacement therapy (8). The information thus point to two potential effects of sex hormones on the heart, a protective effect of female sex hormones and a risky effect of male sex hormones. While effects of sex hormones on coronary heart diseases are better defined (5, 9-13), effects 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 (14-16).

The influence of female sex hormones on cardiac activity has been achieved in a series of experiment using a variety of techniques (17-21). A reduction in cardiac performance after ovarian sex hormone deprivation has been reported in studies using whole heart preparations (22-24). Using subcellular preparations of the ventricular tissue, we have further shown the suppressed myofibrillar ATPase activity with a significant shift in myosin heavy chain (MHC) expression towards β-MHC isoform in ovariectomized rat hearts (17, 18). An increased Ca^{2+} responsiveness of cardiac myofilament activation in ovariectomized rats was also indicated using both biochemical and mechanical measurements. All these changes in ovariectomized rat hearts could be prevented by estrogen supplementation (19). The similar Ca^{2+} hypersensitive adaptation detected in ovariectomized rat hearts to that of cardiomyopathic condition with almost the same magnitude of changes suggests a potential of cardiomyopathic development in ovarian sex hormone-deprived condition (25-27). We then further investigated on potential mechanisms in inducing cardiac myofilament Ca^{2+} hypersensitivity after the hormone deficiency based on a guiding question that is there any significant change in the intracellular Ca^{2+} mobilization in ovariectomized rat hearts.

Ca^{2+} hypersensitivity of cardiac myofilament may be an adaptive 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 (28-32). Deficit in Ca^{2+} storage in the myocytes has been shown to induce an impairment leading to heart failure (29, 30). On the other hand, stimulation of the sarcoplasmic reticulum (SR) Ca^{2+} uptake activity by transgenic improvement of SR Ca^{2+} ATPase (SERCA) provided better cardiac function and life span in dilated hypertrophy-induced mouse (31, 32). These bodies of evidence signify a possible mechanism in the modification of myofilament by intracellular free Ca^{2+} levels as an environmental modulator. This mechanism has been supported by a report of decreased intracellular Ca^{2+} in the mouse overexpressed with non-phosphorylatable phospho-lamban in which a change in myosin expression towards β-MHC was also observed (33). We therefore proposed that modification of the intracellular Ca^{2+} mobilization by ovarian sex hormones is an

important modulating device for myofibrillar response to Ca^{2+}. The proposal has been nicely supported by our preliminary data showing a decrease in Ca^{2+} transient amplitude in ovariectomized rat hearts, which implies a reduced amount of Ca^{2+} activation on the myofilament during the cardiac contraction/relaxation cycle. It is interesting that changes in Ca^{2+} transients as well as myofilament Ca^{2+} sensitivity detected in ovariectomized rat hearts are in the same direction of changes as those reported in many heart failure models (25-27, 34-38). Moreover, a modifying effect of ovarian sex hormones on the SR Ca^{2+} contents has been nicely indicated from our studies demonstrating a significant reduction in the SERCA activity in ovariectomized rat hearts in which could be prevented by estrogen supplementation.

Although hormone replacement therapy (HRT) theoretically provide the best risk-benefit profile for prevention of cardiovascular disease in postmenopausal women, results from the landmark clinical trial (39) has indicated that the treatment is generally not beneficial. Adverse effects of sex hormones on increasing incidence of breast cancer (40) also raise serious concerns on HRT and urge the search for alternative safe modes. With many beneficial effects of regular exercise on improving the quality of life in patients and animals with heart failure (41-43), we have found cardioprotective effects of exercise training on myofilament Ca^{2+} activation in ovariectomized rats (21). The potential importance of exercise training as a cost-effective alternative strategy to be used in place of or in addition to HRT was therefore suggested. It is then interesting to further investigate whether exercise training also exerts its cardioprotective effects on changes in other functional aspects like the SR Ca^{2+} uptake activity in ovariectomized rat hearts.

Interestingly, the cardioprotective effect of estrogen on myocardial function is overcome by diabetes (44-46). The morbidity and mortality of cardiovascular diseases in diabetic patients appear to be increased in females compared with age-matched males. These gender differences in the incidence of heart disease suggest that deprivation of estrogen and insulin induces interactive effects on the cardiac myofilament response to Ca^{2+}. This notion is indirectly supported by an additive effect of estrogen deficiency and diabetes on bone mineral density in diabetic-ovariectomized rat (47). Moreover, deficiency of estrogen increases the severity of renal disease in a diabetic rat model in which estrogen replacement is reno-protective by improving renal function and the associated pathology (48). Despite these combined effects of estrogen and insulin in various organs, their interactive effects on the cardiac myofilament response to Ca^{2+} and the underlying mechanism remain unknown.

Alterations in the regulatory effect of β_1-adrenergic stimulation and the protective effect of heat shock proteins (HSP) appear to play important roles in estrogen and insulin effects on the cardiac myofilament response to Ca^{2+}. A physiologic significance of β_1-adrenergic receptor stimulation is to enhance cardiac contractility through modification of Ca^{2+} flow during the process of excitation contraction coupling (49). However, chronic or over-stimulation of the adrenergic system to the heart induces harmful effects on the contractile function (50-54). We have previously reported an upregulation of β_1-adrenergic receptors in cardiac plasma membrane vesicles in 10-week ovariectomized rats in which could be completely restored by either E_2 supplementation (20) or exercise training (21). Despite controversial data in the expression of β_1-adrenoceptors, a sustained and elevated norepinephrine spillover resulting in chronic stimulation of the receptors has been demonstrated in diabetic hearts (55). Evidence of sex hormone-induced loss of cardiac protection through a reduction in HSP72 expression was also shown in ovariectomized rat hearts (21, 56). This loss of cardioprotective effect in ovariectomized rats could be reversed by either E_2 supplementation (56) or exercise training (21). Similarly, downregulation of HSP72 has been documented in diabetic hearts in which the

impaired HSP protection could also be offset by endurance exercise (57). Whether deficiency of estrogen and insulin interactively induces a synergistic effect through increased β_1-adrenergic stimulation and/or loss of protective effect via reduced HSP72 expression on cardiac contractile response to Ca^{2+} remains to be elucidated.

Therefore, this study has been mainly focused to understand the significance of female sex hormones in cardiac muscle physiology especially the adaptation induced after the hormone deprivation in the following aspects:

I. the relative contribution of acidosis on cardiac Ca^{2+} transients

II. alterations in the mRNA level of SERCA

III. the preventive effect of exercise training on changes in the SR Ca^{2+} uptake activity

IV. the complication of diabetes on changes in the myofibrillar response to Ca^{2+}

and V. the complication of diabetes on the relationship between shifts in MHC isoform and changes in cardiac myofilament ATPase activity.

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.

1. **Approach to Aim #1: To determine the relative contribution of acidosis in intracellular Ca^{2+} transients in ovariectomized hearts.**

It is well known that acidosis condition induced in failing cardiomyocytes affects cardiac myofilament activation as well as intracellular Ca^{2+} mobilization. Based on the similar effects detected in the heart of ovariectomized rat to the failing hearts, it is then possible that acidosis may also be a complication induced in ovarian sex hormone deficit hearts. We therefore raise two questions: 1) whether ovarian sex hormone deficiency induces acidosis in the hearts? and 2) if acidosis is the case, are there any changes in the function and expression of the major plasma membrane transporter, NHE_1, in ovariectomized rat hearts?

We approach the first question by measuring intracellular Ca^{2+} transients and cell shortening of ovariectomized cardiomyocytes comparing to sham under acidic pH of 6.8 using gas perfusion of CO_2 15%/O_2 85%. The relative contribution of acidosis in intracellular Ca^{2+} transients will be determined. If contribution of acidosis is detected, measurements of intracellular Ca^{2+} transients and cell shortening in the presence of specific NHE_1 inhibitor will be performed to approach the function of NHE_1 protein in the cardiomyocyte membranes. The amount of NHE_1 protein in the membrane is also measured using Western blot analysis.

2. **Approach to Aim #2: To determine alterations in the mRNA level of SERCA in ovariectomized rat hearts**

To determine whether downregulation of SERCA was associated with its altered transcription level, quantification of the SERCA mRNA was performed using real-time PCR. Briefly, the total RNA was isolated from the left ventricular tissue around the apex region of the hearts using TRIzol reagent and the total RNA (2 μg) was reverse transcribed using SuperScript™ III First stand Synthesis System with oligo-dT primer. PCR amplification was carried out in 20 μl reaction mixture containing cDNA, 0.2 μM of each specific primer, 0.2 μM of each dNTP, 2.5 U of HotStartTag, 0.5 mM $MgCl_2$, 2 μl of 10x PCR buffer, and 1:40,000 SYBR Green I. PCR was conducted using denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The SercaF and SercaR primers were synthesized according to Chu et al. (9). The ActinF and ActinR primers were used for amplification of β-actin. SERCA mRNA levels were obtained after normalization with β-actin mRNA.

3. **Approach to Aim #3: To determine the preventive effect of exercise training on changes in the SR Ca^{2+} uptake activity in ovariectomized rat hearts**

Although the cardioprotective effect of exercise training on myofilament Ca^{2+} activation in ovariectomized hearts was shown from our previous study (21), it is not known whether exercise training exerts any protective effects on intracellular Ca^{2+} mobilization especially the SR Ca^{2+} uptake function. Information derived from the study will provide more insights and an opportunity to clarify the benefit of exercise training on cardiac function, which may be applied to other cardiac defects. Regular running protocol with moderate intensity will be introduced to both sham and ovariectomized rats using the same protocol as previously

reported (21). Measurements of both SR Ca^{2+} uptake and SERCA activity will be performed to evaluate the protective effect. The SR protein contents including SERCA, calsequestin, and RYR-CRC will also be quantified using Western Blot analyses.

4. **Approach to Aim #4: To determine the complication of diabetes on changes in the myofibrillar response to Ca^{2+} in ovariectomized rat hearts**

The study was designed to evaluate the influence of diabetes on changes in response to Ca^{2+} of cardiac myofilaments preparations from various groups of experimental rats. The pCa (-log free Ca^{2+} concentration)-myofilament ATPase relationships of isolated myofibrillar preparations from sham, ovariectomized, diabetic, diabetic-ovariectomized, and diabetic-ovariectomized supplemented with estrogen, insulin, or estrogen plus insulin rat hearts were compared. The density and binding affinity of cardiac β_1-adrenoceptors in sarcolemmal preparations from these hearts were also compared to probe for changes in the effects induced by hormone deficiency. Contents of HSP72 in these hearts were analyzed and compared to probe for changes in the protective factor.

5. **Approach to Aim #5: To determine the complication of diabetes on the relationship between shifts in MHC isoform and changes in cardiac myofilament ATPase activity**

We designed our studies to evaluate the regulatory effects of estrogen on the cross-bridge kinetics. Based on the known effects of insulin, we therefore compared the relationships of % α-MHC and maximum myofibrillar ATPase activities from various experimental groups including sham-operated, ovariectomized, diabetic, diabetic-ovariectomized, and different hormone-supplemented groups. To probe for changes in the cross-bridge kinetics induced after estrogen deficiency, we compared responses at varied levels of cardiac α-MHC expression manipulated by thyroid status. We also confirmed changes in the cross-bridge kinetics using acidic condition in which both the number of cross-bridge and the amount of force per cross-bridge were suppressed.

Section II. Preparations and Procedures

1. Animal Preparations

Female Sprague-Dawley rats weighing between 180 and 200 g (8–9 wk old) were sham operated or ovariectomized as previously described (17). Ovariectomized rats were randomly divided into control and hormone-supplemented groups. The rats were individually housed in an 8 in. x 10 in. hanging cage and given rat chow and water *ad libitum*. Two days after the operation, hormone supplemented group was started by subcutaneous injection of estrogen (5 μg/rat), progesterone (1 mg/rat), or estrogen plus progesterone three times a week for 10 weeks as previously described (19). The sham-operated and ovariectomized control rats were injected with corn oil (vehicle) in the same manner as the hormone-treated rats. For the group with diabetic complication, both sham and OVX rats were further randomly divided into non-diabetic and diabetic groups two weeks after surgery. Diabetic rats were induced by intraperitoneal injection of freshly prepared streptozotocin (65 mg/kg body weight) while non-diabetic groups were injected with citrate buffer. Three days after diabetic induction insulin-supplemented rats were subcutaneously injected with 5 units of human insulin on a daily basis for the whole experimental period. We verified diabetic status by checking urinary glucose using glucose strip both on the following day after induction and on the day before sacrificing

the rats. Sufficiency of ovariectomy was verified by the decrease in uterine weight when the animals were killed. All the animal protocols were approved by the Experimental Animal Committee, Faculty of Science, Mahidol University, in accordance with National Laboratory Animal Centre, Thailand.

2. Isolation of Cardiac Myocytes and Measurements of Intracellular Ca^{2+} Transients and Cell Shortening

Ten-weeks ovariectomized rats were heparinized (1,000 unit/kg body wt), and left for 15 min before anesthetizing with pentobarbital sodium (25mg/kg body wt). Left ventricular myocytes were isolated as previously described (58). Briefly, the heart was rapidly excided and the aorta was then cannulated and perfused with oxygenated free Ca^{2+} Tyrode solution until the heart completely stopped beating. The perfusion solution was then switched to 25 μM $CaCl_2$ Tyrode solution containing 1 mg/ml BSA, 160 unit/ml collagenase type II (Boehringer Mannheim, IN), and 0.1 mg/ml protease type XIV (Sigma, MO). At appropriate perfusion time based on heart weight, ventricular myocytes were isolated using 50 μM $CaCl_2$ Tyrode-BSA buffer and then filtered through cell collector. Extracellular calcium concentration was gradually increased during each dispersing/precipitating intermission until the last concentration of 0.5 mM $CaCl_2$. Myocytes would be studied within six hours after isolation.

Myocytes were loaded with 0.5 μM Fura 2-AM in 0.5 mM $CaCl_2$ Tyrode buffer with 1 mg/ml BSA and 5% fetal bovine serum for 15 minutes. After washing off the excess Fura 2-AM, the myocyte was electrically stimulated at frequency of 0.5 for 10 to 15 minutes or until the shortening was stable with perfusion of 2 mM $CaCl_2$ Tyrode buffer at room temperature. Fura 2 fluorescence and cell shortening were monitored simultaneously as described in details by Wolska et al. (59). Fluorescence emission was measured using dual-excitation fluorescence & photomultiplier system at excitation wavelengths of 340 and 380 nm. Simultaneously, cell shortening was recorded with edge-to-edge imaging detector system on video monitor. Emission ratio of 340/380 nm excitation wavelengths was then calculated to represent the intracellular Ca^{2+} concentration.

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

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

4. Determination of SERCA Activity

SERCA activity was determined by triple enzyme assay as previously described by Chu et al. (61). The assay was run at various concentrations of Ca^{2+} ranging from pCa 8.0 to 5.0, pH 7.0 at 37°C. The SR enriched membrane vesicles of 5 μg protein was 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 was monitored with spectrophotometer for 350 second and the activity was determined from the linear reaction during 250 to 350 seconds. The activity was then calculated from the optical density using extinction coefficient of NADH. Non-specific SERCA activity was determined in the presence of 0.1 nM thapsigagin in the reaction. The relations between pCa and Ca^{2+}-ATPase activity were then plotted using non-linear least square regression based on Hill equation.

5. Semi-quantitative Determination of SERCA mRNA by Real-time PCR

Quantification of SERCA2a gene expression by real-time PCR was performed with the Rotor-Gene 3000 (Corbett, Sydney, Australia) using SYBR Green I nucleic acid stain (FMC BioProducts) and Rotor-Gene Analysis software version 5.0. Total RNA was isolated from the left ventricular tissue from the apex region of the hearts of each experimental group by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Total RNA (2 μg) was reverse-transcribed using SuperScript III First Strand Synthesis System (Invitrogen) with oligo-dT primer. PCR amplification was carried out in a 20-μl reaction mixture containing cDNA, 0.2 μM of each specific primer, 0.2 μM of each dNTP, 2.5 U of HotStartTag (Qiagen, Hilden, Germany), 0.5 mM $MgCl_2$, 2 μl of 10x PCR buffer, and 1:40,000 SYBR Green I. PCR was conducted using denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The SercaF (5'-GACATTGAAACATGCTTTCTAATGGGC-3') and SercaR (5'-TGAACTGACGATGAGCACTTTATTACG-3') primers were synthesized according to Chu et al. (9). The ActinF (5'-CCTGGCACCCAGCACAAT-3') and ActinR (5'-GGGCCGGAC-TCGTCATAC-3') primers were used for amplification of β-actin. SERCA mRNA levels were obtained after normalization with β-actin mRNA.

6. Cardiac Membrane Preparation and β_1-adrenergic Receptor Binding Assay

Cardiac membrane was prepared from the left ventricle by the method of Baker and Potter (62) with modifications. In brief, the left ventricle was homogenized in ice-cold 10 mM Tris-HCl, pH 8.0, and then incubated in 1 M KCl to dissolve the myofilament proteins. Subsequently, the incubated homogenate was filtered through several layers of cheesecloth and the filtrate was then centrifuged at 43,900 g, 4°C for 20 min. The pellet was re-suspended in Tris buffer, homogenized, and sedimented. The final pellet was dispersed in ice-cold 50 mM HEPES buffer, pH 8.0, in a Teflon glass homogenizer and was immediately used for receptor binding assay after determining the protein content by the Bradford protein assay kit (Bio-Rad). Binding assay for β_1-adrenergic receptor was performed under equilibrium condition in various concentrations of [^{3}H]-dihydroalprenolol (specific activity 92 Ci/mmol, Amersham Phamacia Biotech) as previously described (17). Nonspecific binding was analyzed in a parallel set of experiment with the addition of (-)-alprenolol, a specific antagonist of β_1-adrenergic receptor. The saturation binding was determined from the relationship between specific binding and free ligand using nonlinear least-square regression analysis. Binding parameters, including the density and dissociation constant of the receptors, were determined from a linear transformation of data to the Scatchard plot of bound/free to bound form.

7. Cardiac myofibrillar actomyosin MgATPase activity

Cardiac myofibrils were prepared from the left ventricles as described by Pagani and Solaro (63). Ca^{2+}-dependent MgATPase activity of isolated myofibrils was 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 7.0, ionic strength of 120 mM, and various

concentrations of Ca^{2+} ranging from pCa of 7.5 to 4.875. Total concentrations of $CaCl_2$, EGTA, KCl, $MgCl_2$, and ATP were calculated using a computer program generated from the dissociation constants given by Fabiato (60). The concentration of inorganic phosphate was measured by the method of Carter and Karl (64).

8. Gel electrophoresis of MHC isoforms

MHC isoforms of left ventricular trabeculae were preformed using SDS-PAGE as described with the following modification (65). 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.

9. Immunoblot Analysis

The frozen left ventricular tissue was homogenized in the extracting buffer containing 150 mM Tris·HCl, 150 mM NaCl, 5 mM EGTA, 0.1% SDS, 50 mM NaF, 40 mM β-glycero-phosphate, 2 mM Na_3VO_4, and protease inhibitors, including leupeptin, pepstatin-A, aprotinin, and PMSF. Protein concentration of the left ventricular homogenate was determined by bicinchoninic acid assay. The homogenate with freshly added dithiothreitol was subjected to SDS-PAGE and immunoblot analysis. The protein content of SERCA and phospholamban in 100 μg of the left ventricular homogenate was quantified with monoclonal antibodies of SERCA2 (1:1,000) and phospholamban (1:5,000) (Affinity Bioreagents, Golden, CO). The proportion of the monomer to the pentamer of phospholamban was also quantified. Amounts of phosphorylated Ser^{16} (phospho-Ser^{16}) and phospho-Thr^{17} phospholamban were determined in nondenatured preparations using polyclonal antibodies of phospho-Ser^{16} (1:20,000) and phospho-Thr^{17} phospholamban (1:5,000) (Badrilla, Leeds, UK). The relative amount of calsequestrin or actin was used for determining the total protein loading. Band density was analyzed by Image Master Labscan version 3.01 and Image Master Totallab version 1.0 (Amersham Pharmacia Biotech). The amount of β_1-adrenergic receptor and HSP72 were also determined by Western blot analysis of left ventricular homogenates using polyclonal antibodies against β_1-adrenergic receptor (Affinity Bioreagents, Golden, CO) and HSP72 (Stressgen, Victoria, BC) and HRP-labeled secondary antibody, with visualization by ECL (Amersham Pharmacia).

10. 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.

11. 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), and radioactive 45CaCl2 was obtained from PerkinElmer (Boston, MA). 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: The relative contribution of acidosis on Ca^{2+} transients in ovariectomized rat hearts

Based on the well-known effect of acidosis on the cardiac contractile activation and the intracellular Ca^{2+} mobilization, we then determined the relative contribution of acidosis on the contractile activity and the Ca^{2+} transients in ovariectomized cardiomyocytes. Intracellular acidosis in cardiomyocytes was induced by gas perfusion of CO_2 15% / O_2 85%. Results in Figure 1 show a significant decrease in the intracellular pH from 7.25 ± 0.08 to 6.65 ± 0.07 within three minutes after gas perfusion. As a result, the shortening of cardiomyocytes was rapidly suppressed to the maximal suppression level within five minutes. Subsequently, an adaptive increment of cell shortening was demonstrated in both sham and ovariectomized cardiomyocytes. Interestingly, the adaptive increase in cell shortening of ovariectomized cardiomyocytes was more pronounced than that of sham. This adaptive increase in cardiomyocyte shortening was partially restored by estrogen supplementation.

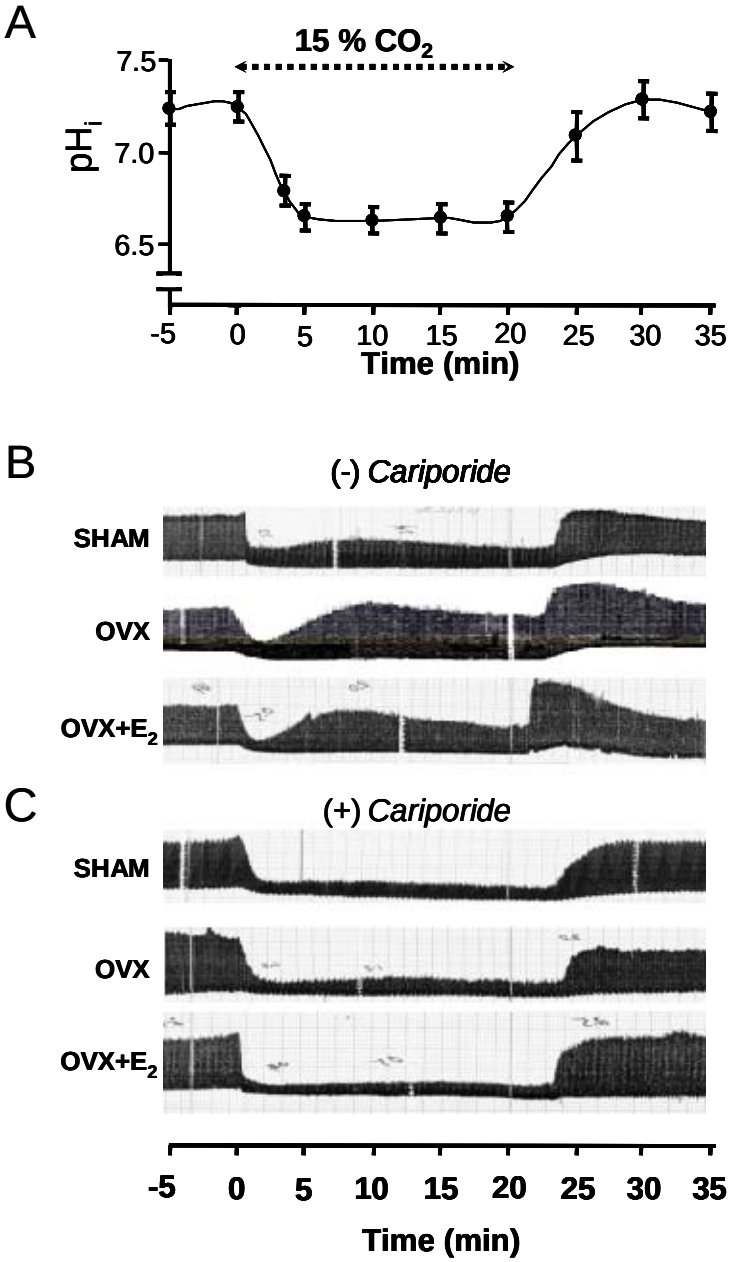

Figure 1. Changes in the intracellular pH with time after 15% CO_2 perfusion (A) and effects of hypercapnic condition on the cell shortening of cardiomyocytes isolated from SHAM and ovariectomized (OVX) rats with and without estrogen (E_2) supplementation in the absence (B) and presence (C) of cariporide.

The extents of cell shortening at different time points after acidotic induction were calculated and summarized in Figure 2. As shown in Figure 2A, at maximal suppression time point (5 min) the extent of shortening suppression in ovariectomized cardiomyocytes was significantly less than that of sham hearts. In contrast to the suppressed shortening of cardiomyocytes, measurements of intracellular Ca^{2+} transients during acidic induction demonstrated no changes in the Ca^{2+} transient amplitude at five minutes after gas perfusion (Figure 3A). The higher shortening of cardiomyocytes at the same level of intracellular Ca^{2+} concentration further supports the existence of Ca^{2+}-hypersensitivity for cell shortening in ovariectomized hearts even under acidotic condition.

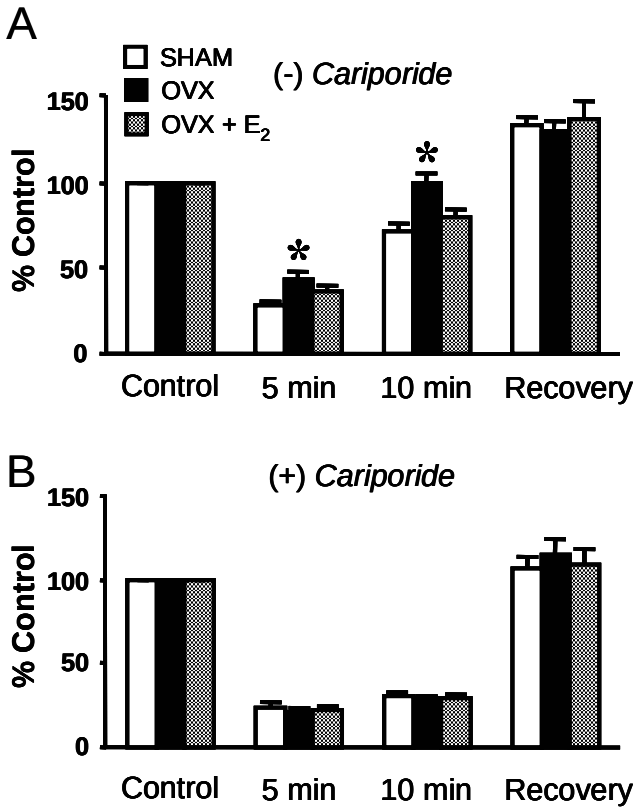

Figure 2. Changes in the cell shortening of cardiomyocytes isolated from SHAM and ovariectomized (OVX) with and without estrogen (E_2) supplementation in the absence (-) and presence (+) of cariporide at different time points of acidotic induction.

One speculation for the adaptive shortening of cardiomyocytes detected at 10 min after gas perfusion in both sham and ovariectomized hearts could be due to a significant increase in the intracellular Ca^{2+}. As shown in Figure 3A, there was a small increase in the intracellular Ca^{2+} in sham hearts at adaptive time point (10 min) as compared to the time point at five minutes after hypercapnic induction. In contrast, an almost 50% increase in the intracellular Ca^{2+} was clearly demonstrated in ovariectomized hearts at the adaptive time point as compared to that at maximal suppressive shortening time point. Moreover, the adaptive shortening of ovariectomized myocytes measured at 10 min after acidotic induction was significantly induced to the same extent as that of non-acidic control. The latter increases in intracellular Ca^{2+} transients imply an adaptive response of an acid-induced intracellular calcium accumulation in ovariectomized cardiomyocytes. It is then possible that deprivation of ovarian

sex hormones induces acidosis in cardiomyocytes and consequently an increased activity of the NHE. The possible involvement of NHE in the acid-induced intracellular calcium accumulation in ovariectomized cardiomyocytes was tested using cariporide, a NHE_1-inhibitor. As shown in Figure 1C, Figure 2B, and Figure 3B, shortenings of the cell were suppressed to the same magnitude throughout the acidic condition in every experimental group without changes in the intracellular Ca^{2+} transients. These results indicate a differential contribution of NHE in the adaptive responses of ovariectomized and sham cardiomyocytes to acidosis.

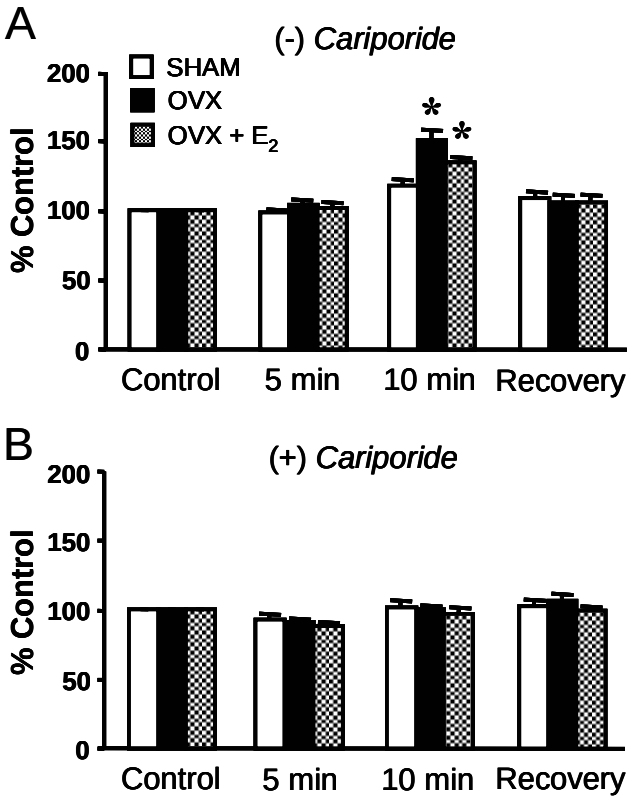

Figure 3. Changes in the intracellular Ca^{2+} transients of cardiomyocytes isolated from SHAM and ovariectomized (OVX) with and without estrogen (E_2) supplementation in the absence (-) and presence (+) of cariporide at different time points of acidotic induction.

Project II: The mRNA expression level of SERCA in ovariectomized rat hearts

To further determine whether downregulation of SERCA was associated with its altered transcription level, quantification of the SERCA mRNA was performed using real-time PCR. As shown in Figure 4, an approximately 70% reduction in the SERCA mRNA level was observed in ovariectomized hearts and the reduction could be restored by estrogen or progesterone supplementation. The suppression of maximum SR Ca^{2+}-uptake activity in ovarian sex hormone deficient-hearts was thus due to the decreased expression level of SERCA mRNA and hence its protein, together with the greater inhibitory effect of phospholamban previously demonstrated.

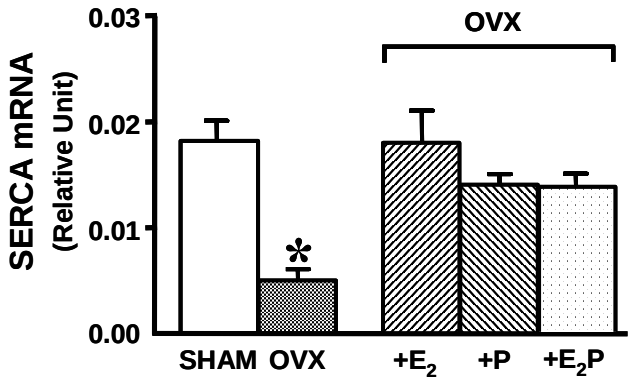

Figure 4. Quantification of SR Ca^{2+} ATPase (SERCA) mRNA levels expressed as a ratio of SERCA:β-actin of various ventricular homogenates from SHAM and ovariectomized (OVX) rats with and without estrogen (E_2) and/or progesterone (P) supplementation. Data are means ± SE from 6 hearts. *Significantly different from SHAM (*P<0.05)* using Student Newman-Keuls test after ANOVA.

Project III: The preventive effect of exercise training on changes in the SR Ca^{2+}-uptake activity in ovariectomized hearts

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

The cardioprotective effects of exercise training on changes in SR Ca^{2+}-uptake activity were investigated in four groups of rats including sham and ovariectomized rats with and without exercise training. The general characteristics of the rats were summarized in Table 1. The condition of ovarian sex hormone deficiency in ovariectomized rats was verified by a significant reduction in the uterine weight as compared to that of sham controls. Ovarian sex hormone-deficient rats have greater body and heart weights than those of sham. While exercise induced a significant increase in body weight of sham rats, exercise induced no effect on the body weight of ovariectomized groups. As expected, hypertrophy of the heart as indicated by an increase in the heart to body weight ratio was clearly observed in the exercised groups. In contrast, there was no hypertrophy of the soleus muscle in all experimental groups. Besides the hypertrophy of the hearts, efficiency of the running program was also confirmed by a significant increase in the citrate synthase activity of plantaris muscles in exercised rats.

Left ventricular homogenates from sham and ovariectomized rats, with and without exercise training were prepared for measurements of the SR Ca^{2+}-uptake activity at various free Ca^{2+} concentrations. As depicted in Figure 5, maximum SR Ca^{2+}- uptake activity was significantly suppressed to 72.4 nmole/mg protein/min in sedentary ovariectomized hearts compared to 88.2 nmole/mg protein/min in sedentary sham. While exercise training had no effect on the SR Ca^{2+}-uptake activity of sham control, it completely restored the suppressed activity in ovariectomized hearts (Figure 5B). Furthermore, the increase in SR Ca^{2+}-uptake sensitivity from EC_{50} of 0.575 ± 0.015 μM in sham controls to 0.460 ± 0.010 μM in ovariectomized hearts was abolished by exercise training as shown in Figure 6. Despite changes in the maximum SR Ca^{2+}-uptake rate and SR Ca^{2+}-uptake sensitivity in ovariectomized hearts, the Hill slope was not different among the experimental groups (data not shown). These results thus indicate the protective effect of exercise training on changes in SR Ca^{2+}-uptake activity in ovarian sex hormone-deficient hearts.

Table 1. Body, heart, soleus, and uterine weights, and plantaris citrate synthase activity of sedentary and exercise groups of sham-operated and ovariectomized rats.

	SEDENTARY		EXERCISE	
	SHAM	OVX	SHAM	OVX
Body Weight, g	276 ± 6	352 ± 9 *	296 ± 4 *	362 ± 6
Heart Weight, g	0.86 ± 0.01	1.00 ± 0.01*	0.98 ± 0.02*	1.09 ± 0.02
Uterine Weight, g	0.45 ± 0.03	0.08 ± 0.01*	0.49 ± 0.03	0.09 ± 0.01*
Soleus Weight, g	0.12 ± 0.003	0.15 ± 0.003*	0.13 ± 0.004	0.16 ± 0.004
% Heart/ BW	0.31 ± 0.006	0.28 ± 0.005*	0.33 ± 0.007*	0.30 ± 0.005#
% Soleus/ BW	0.042 ± 0.001	0.042 ± 0.001	0.043 ± 0.001	0.042 ± 0.001
CS Activity (μmole/g/min)	41.7 ± 1.4	42.0 ± 1.5	61.5 ± 1.3*	59.6 ± 1.4 #

Values are means ± SE of 16 rats. SHAM, sham-operated controls; OVX, ovariectomized rats; CS, citrate synthase. *P<0.05, #P<0.05, significantly different from sedentary-sham and sedentary-ovariectomized rats, respectively.

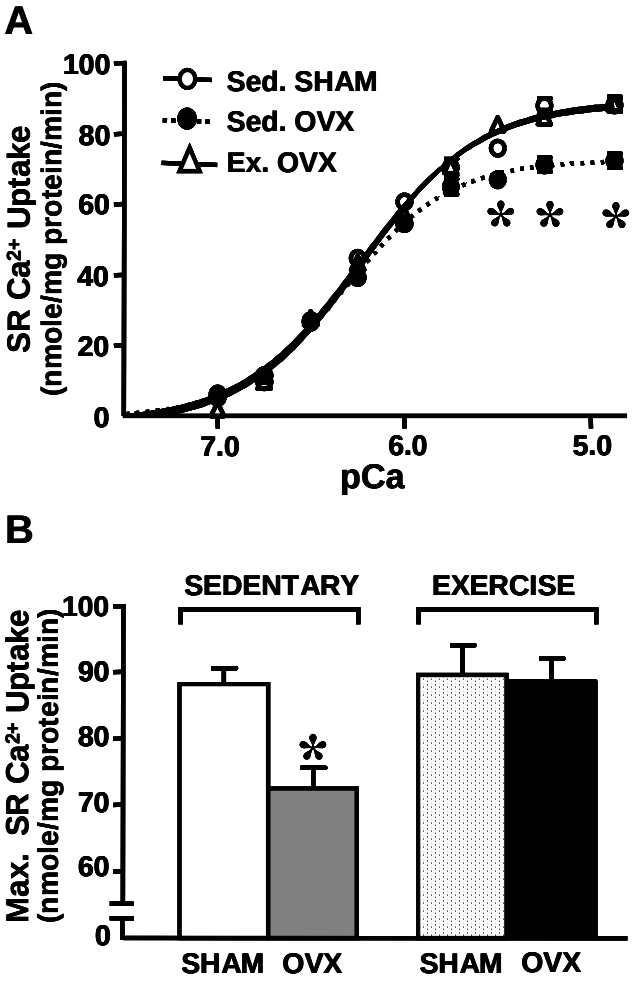

Figure 5. (A) Effects of ovariectomy and exercise training on the sarcoplasmic reticulum (SR) Ca^{2+}-uptake activity of left ventricular homogenates at various Ca^{2+} concentrations ranging from pCa 7.5 to 4.875, pH 7.0 without showing graph of exercise-sham group, which would super-impose on the graph of sedentary-sham. (B) Comparisons of the maximum SR Ca^{2+} uptake between sham controls and ovariectomized (OVX) rats of sedentary (Sed.) and exercise (Ex.) groups. Data are means ± SE from 14-16 preparations. *P < 0.05 = significantly different from sedentary sham controls using Student Newman-Keuls test after ANOVA.

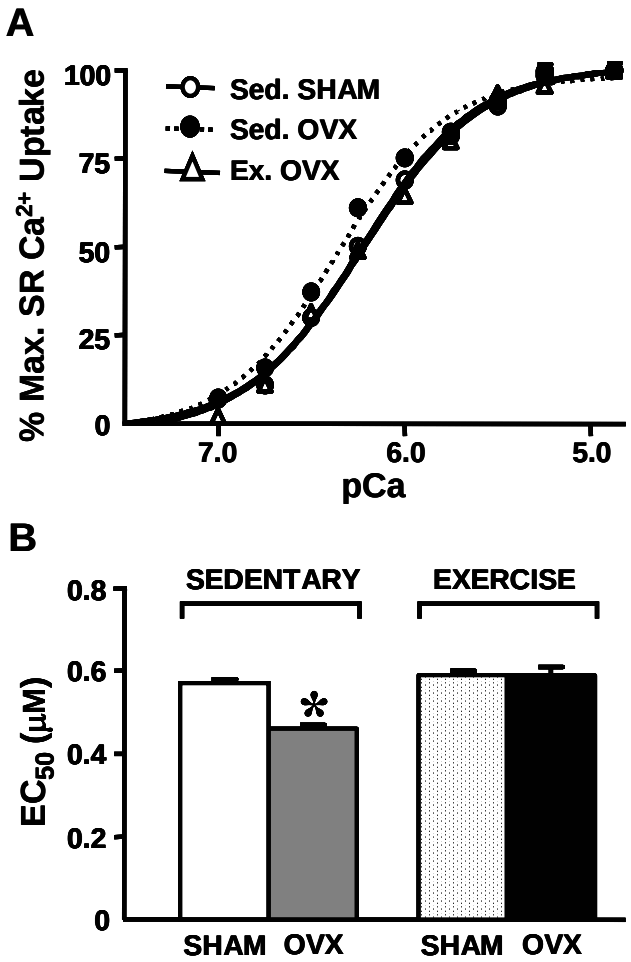

Figure 6. (A) Effects of ovariectomy and exercise training on the % maximum sarcoplasmic reticulum (SR) Ca^{2+} uptake of left ventricular homogenates at calcium concentrations ranging from pCa 7.5 to 4.875, pH 7.0 without showing graph of exercise-sham group, which would superimpose on the graph of sedentary-sham. (B) Comparisons of EC_{50} between sham controls (SHAM) and ovariectomized (OVX) rats of sedentary (Sed.) and exercise (Ex) groups. Data are means ± SE from 14-16 preparations. *P<0.05 = significantly different from sedentary sham controls using Student Newman-Keuls test after ANOVA.

ii) Measurements of SERCA Activity

The cardioprotective effects of exercise training on changes in SERCA activity were also investigated. Similar to those changes in the SR Ca^{2+}-uptake activity, the maximum SERCA activity of sedentary ovariectomized hearts was significantly suppressed to 1.04 ± 0.04 μmole Pi/mg protein/min from 1.29 ± 0.05 μmole Pi/mg protein/min of sham controls (Figure 7). While exercise training had no effect on the SERCA activity, it prevented the suppressed activity in ovariectomized hearts. The sensitivity of SERCA to Ca^{2+} activation was also increased in ovariectomized hearts to the EC_{50} value of 0.641 ± 0.049 μM from 0.999 ± 0.082 μM of sham controls (Figure 8). In addition, exercise training prevented the increased SERCA sensitivity to Ca^{2+} activation in ovariectomized hearts as expected (Figure 8B). The similar changes in the SR Ca^{2+}-uptake and SERCA activities thus suggest that changes in SERCA activity underlied changes in the SR Ca^{2+}-uptake function in ovariectomized hearts. Moreover, the protective role of exercise training in ovariectomized hearts at the level of SERCA activity was clearly indicated.

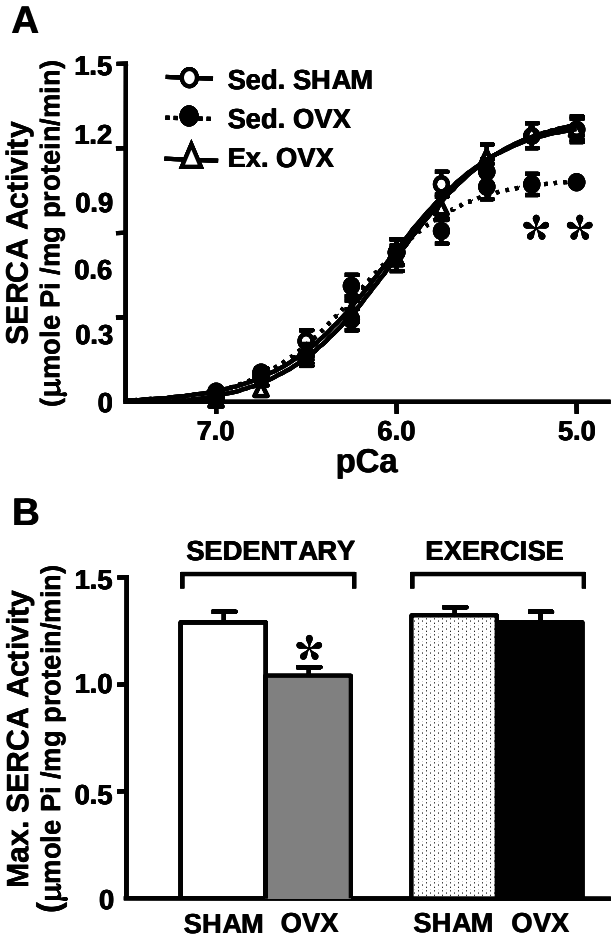

Figure 7. (A) Effects of ovariectomy and exercise training on sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) activity at various calcium concentrations from pCa 8.0 to 5.0, pH 7.0 without showing graph of exercise-sham group, which would superimpose on the graph of sedentary-sham. (B) Comparisons of maximum SERCA activity between sham controls (SHAM) and ovariecto-mized (OVX) rats of sedentary (Sed.) and exercise (Ex.) groups. Data are means ± SE from 11-13 preparations. **P*<0.05 = significantly different from sedentary-SHAM controls using Student Newman-Keuls test after ANOVA.

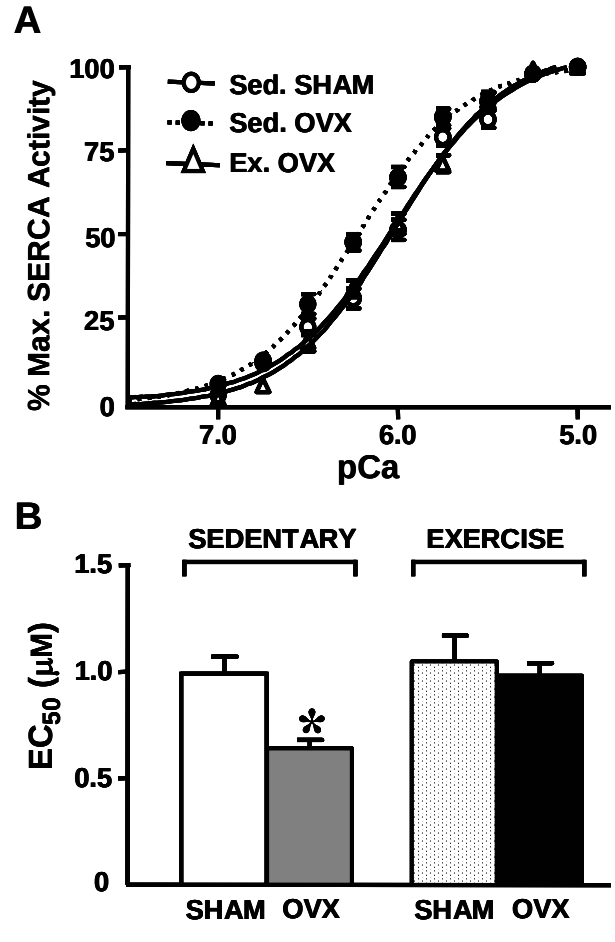

Figure 8. (A) Effects of ovariectomy and exercise training on the % maximum SERCA activity at various calcium concentrations ranging from pCa 8.0 to 5.0, pH 7.0 without showing graph of exercise-sham group, which would superimpose on the graph of sedentary-sham. (B) Comparisons of EC_{50} between sham controls (SHAM) and ovariectomized (OVX) rats of sedentary (Sed.) and exercise (Ex.) groups. Data are means ± SE from 11-13 preparations. **P*<0.05 = significantly different from sedentary-SHAM controls using student Newman-Keuls test after ANOVA.

iii) Immunoblots of SR proteins

A reduction in the SERCA activity can be responsible by quantitative and qualitative reductions in SERCA proteins. Figure 9A, shows results from immunoblot analysis of SERCA, phospholamban (PLB), and calsequestrin (CSQ). The protein bands of CSQ were used as control of protein loading. Relative amounts of SERCA and phospholamban to CSQ in the experimental rats were summarized in figure 9B and 9C, respectively. As shown in figure 9B, the quantity of SERCA proteins in ovariectomized hearts was significantly lower than that in sham controls and exercise training prevented the downregulation of SERCA proteins in ovariectomized hearts. Qualitatively, activity of SERCA is regulated by an inhibitory protein, phospholamban. Changes in either the level or the phosphorylated/dephosphorylated state of phospholamban could all affect SERCA activity. In contrast to the expression of SERCA, there was no change in the expression amount of phospholamban protein among the experimental groups (Figure 9C). As a result, a reduction in the SERCA to phospholamban ratio indicates a greater inhibitory effect of phospholamban on SERCA activity in ovariectomized hearts (Figure 9D). After exercise training, the SERCA to phospholamban ratio in ovariectomized hearts was restored to the same level as that in sham controls (Figure 9D). Thus, reductions in both the SERCA expression and SERCA to phospholamban ratio underlie the suppression of maximal SR Ca^{2+}-uptake activity (V_{max}), which could be prevented by exercise training, in ovarian sex hormone-deficient hearts.

To further elucidate the regulatory effect of phospholamban on SERCA activity, levels of phosphorylated-phospholamban were determined using individual specific antibody to the phospho-Ser^{16} phospholamban and the phospho-Thr^{17} phospholamban. Figure 10A shows the protein bands of phospho-Ser^{16} phospholamban, phospho-Thr^{17} phospholamban and actin. Actin bands on SDS-PAGE were used as loading control. The relative amount of the phospho-Ser^{16} phospholamban to actin and the phospho-Thr^{17} phospholamban to actin were summarized in Figure 10B and 10C, respectively. While there was no change in the phospho-Ser^{16} phospholamban among the experimental groups, a significant downregulation (28.42 ± 4.98 %) of phospho-Thr^{17} phospholamban was detected in sedentary ovariectomized hearts as compared to that of sedentary sham controls. This downregulation of the phospho-Thr^{17} phospholamban in ovariectomized hearts could be abolished by exercise training (Figure 10C). The reduction in phosphorylation level at Thr^{17} of phospholamban in ovariectomized hearts could not explain the Ca^{2+} hypersensitivity of SERCA activity in ovariectomized hearts. Therefore, the increase in Ca^{2+} sensitivity of SERCA in ovariectomized hearts might be underlied by other mechanisms.

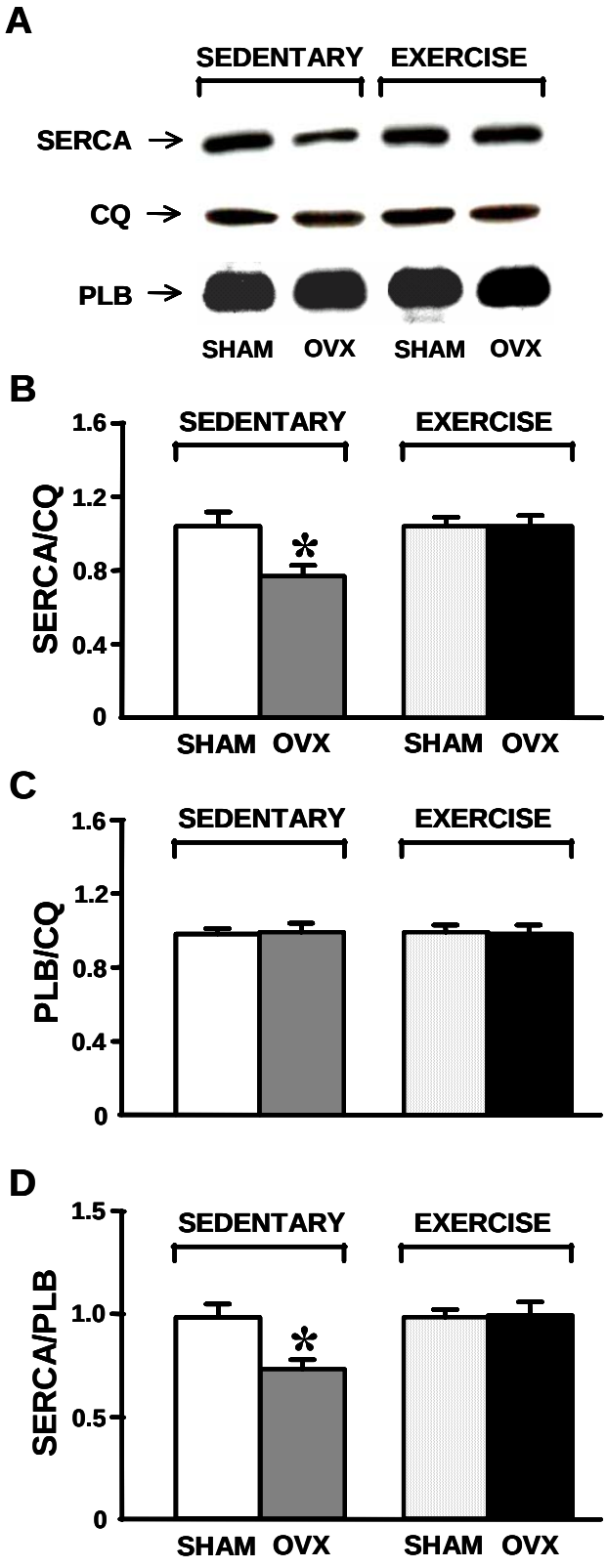

Figure 9. (A) Western blot analysis of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), phospholamban (PLB), and calsequestrin (CSQ). Comparisons of the relative intensity from the regions of (B) SERCA to CSQ, (C) PLB to CSQ and (D) SERCA to PLB on immunoblots on which were samples of left ventricular homogenates from sham-operated (SHAM) and ovariectomized (OVX) rats of sedentary and exercise groups. Data are means ± SE from 6 hearts. *P<0.05 = significantly different from sedentary SHAM controls using Student Newman-Keuls test after ANOVA.

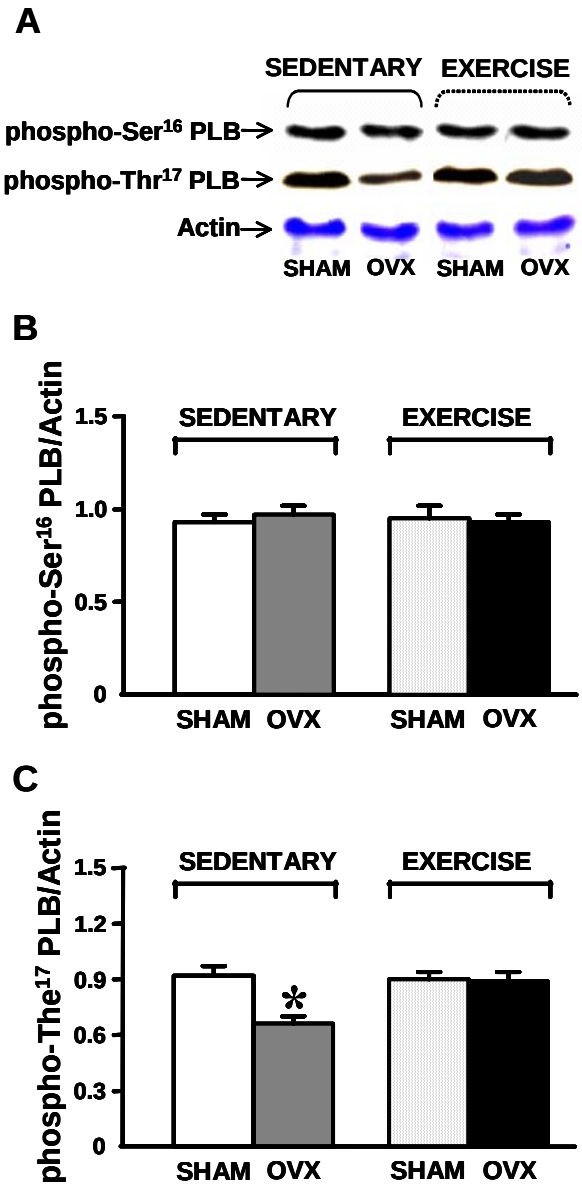

Figure 10. (A) Western blot analysis of the phospho-Ser[16] (p-Ser[16]), and phospho-Thr[17] phospholamban (p-Thr[17]). Comparisons of the relative intensity from the regions of (B) phospho-Ser[16] phospholamban to actin, (C) phospho-Thr[17] phospholamban to actin on immunoblots on which were samples of left ventricular homogenates from sham-operated (SHAM) and ovariectomized (OVX) rats of sedentary and exercise groups. Data are means ± SE from 6 hearts. *P<0.05 = significantly different from sedentary SHAM controls using Student Newman-Keuls test after ANOVA.

Besides changes in the phosphorylation level of phospholamban, changes in the structural stoichiometry of phospholamban between the monomer, an active inhibitory form and the pentamer, an inactive inhibitory form of phospholamban could also affect the SERCA sensitivity to Ca^{2+} activation. Immunoblot of the monomeric and pentameric forms of phospholamban were shown in Figure 11A. Proportions of the monomeric and the pentameric forms to the total amount of phospholamban were calculated and summarized in Figure 11B and 11C, respectively. Proportions of the monomeric and the pentameric phospholamban in sham controls were found to be 10.30 ± 0.65 % and 89.70 ± 0.65 %, respectively. These

proportions in ovariectomized hearts were changed to be 21.40 ± 0.83 % for the monomeric phospholamban and 78.60 ± 0.83 % for the pentameric phospholamban. While exercise training has no effect on the structural stoichiometry of phospholamban in sham hearts, it normalized those changes in the monomeric and pentameric proportions of phospholamban in ovariectomized hearts. The increased proportion of the monomeric form of phospholamban in ovariectomized hearts was paralleled with the reduction in the phosphorylation level of Thr^{17}-phospholamban. These similar changes indicate the involvement of phosphorylation status of phospholamban to shifting the equilibrium from monomer to pentamer. Results presented in this study clearly indicate the cardioprotective effect of exercise training on those changes in the SERCA activity induced after ovariectomy. The protective effect of exercise involved both the expression level of SERCA proteins and the interaction of SERCA to its regulatory protein.

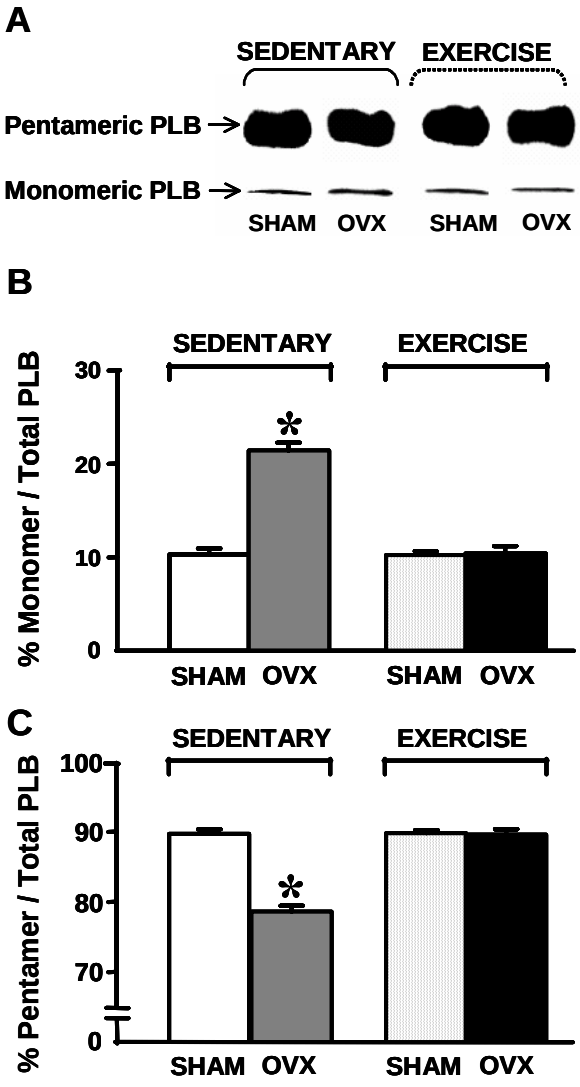

Figure 11. (A)Western blot analysis of monomeric and pentameric forms of PLB protein. Comparisons of the proportion of (B) monomeric and (C) pentameric PLB on immunoblots which were samples of left ventricular homogenates from sham-operated (SHAM) and ovariectomized (OVX) rats of sedentary and exercise groups. Data are means ± SE from 6 hearts. *P<0.05 = significantly different from sedentary SHAM rats using Student Newman-Keuls test after ANOVA.

Project IV: Complication of diabetes on changes in the myofibrillar response to Ca^{2+} in ovariectomized rat hearts

The information that diabetes seems to overcome the cardioprotective effect of estrogen on myocardial function suggests interactive effects of estrogen and insulin on cardiac myofilament response to Ca^{2+}. Despite many reports on the combined effects of estrogen and insulin on various organs, their interactive effects on the cardiac myofilament response to Ca^{2+} and the underlying mechanism remains unknown. We therefore evaluated the influence of diabetes on changes in the Ca^{2+} response of cardiac myofilaments in sex hormone-deprived condition. The pCa-myofilament ATPase relationships of isolated cardiac myofibrillar preparations from sham, ovariectomized, diabetic, diabetic-ovariectomized, and diabetic-ovariectomized supplemented with estrogen, insulin, or estrogen plus insulin rats were studied.

Table 2 summarizes body, heart, and uterine weight of all seven experimental groups, namely, sham, OVX, DM, DM-OVX, and DM-OVX supplemented with E_2, INS, or E_2 + INS. As expected, uterine weights were significantly decreased in OVX and DM-OVX groups compared to sham controls, and increased upon E_2 supplementation. Uterine weights of DM rats were also significantly lower than shams, but in a smaller magnitude compared to ovarian sex hormone-deficient groups. While a significant increase in both heart and body weight was demonstrated in OVX rats, a significant decrease in both heart and body weight was detected in DM rats. A decrease in body weight was still observed in the DM-OVX rats without insulin supplementation. Similarly, hypertrophy of the heart represented by an increased heart weight/body weight ratio was demonstrated in DM and DM-OVX hearts without insulin supplementation.

Table 2. Body weight (BW), heart weight (HW), uterine weight (UW) and % heart weight/ body weight (% HW/BW)

GROUPS	BW	HW	UW	% 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 ± 0.01*	0.37 ± 0.02*#
DM-OVX + E_2	233 ± 5*#	0.86 ± 0.02*#	0.37 ± 0.03#	0.37 ± 0.02*#
DM-OVX + INS	355 ± 8*	1.01 ± 0.01*	0.09 ± 0.01*	0.29 ± 0.01*
DM-OVX + E_2 + INS	263 ± 4#	0.90 ± 0.01#	0.40 ± 0.01#	0.34 ± 0.01#

Values are means ± SE of 8 -10 rats. OVX; ovariectomized, DM; diabetes, DM-OVX; diabetic-ovariectomized, E_2; estrogen, INS; insulin. *P<0.05 and #P<0.05 are significant difference from SHAM and OVX groups, respectively.

Relationships of pCa-myofilament ATPase were compared to evaluate the interactive effect of E_2 and INS deprivation on the cardiac myofilament response to Ca^{2+}. As summarized in Figure 12A, maximum myofibrillar ATPase activity of OVX and DM hearts were significantly

depressed from shams to the same magnitude of suppression (22.7% and 32.9% respectively). Maximum myofibrillar ATPase activity was also depressed in DM-OVX hearts (29.43%) from shams without additive suppression when compared to OVX and DM rats, and completely restored upon supplementation with both E_2 and INS (Figure 12B).

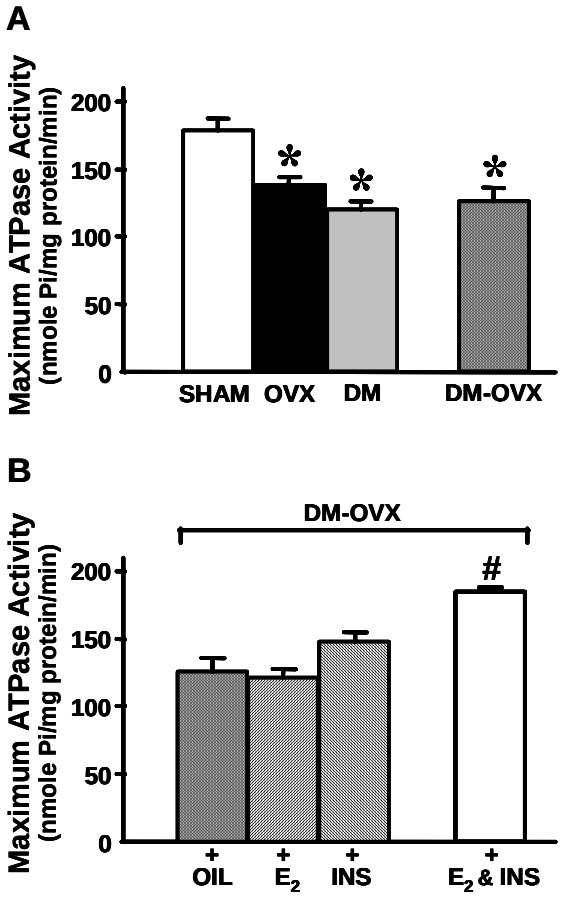

Figure 12. *A*: Comparisons of the maximum Ca^{2+}-dependent actomyosin MgATPase activity of cardiac myofibrillar preparations from sham (SHAM), ovariectomized (OVX), diabetic (DM), and diabetic-ovariectomized (DM-OVX) rats. *B*: Comparisons of the maximum Ca^{2+}-dependent actomyosin MgATPase activity of cardiac myofibrillar preparations from various groups of DM-OVX rats with estrogen (E_2) and/or insulin (INS) supplementations. Data are means ± SE from 7-8 preparations. *P<0.05 and $^{\#}$P<0.05 represent a significant difference from SHAM and DM-OVX groups, respectively, using Student-Newman-Keuls test after ANOVA.

On the other hand, increased myofilament sensitivity to Ca^{2+} (reported as pCa_{50}) was only detected in OVX (pCa_{50} = 6.06 ± 0.02) but not in DM (pCa_{50} = 5.96 ± 0.02) hearts when compared to sham controls (pCa_{50} = 5.93 ± 0.02) (Figure 13A). The myofilament Ca^{2+} hypersensitivity detected in sex hormone-deficient hearts was also observed in DM-OVX hearts (pCa_{50} = 6.01 ± 0.02), and reversed upon E_2 or E_2 + INS supplementation (Figure 13B). In all cases there were no significant differences in the Hill coefficient of pCa-myofilament ATPase relationship among all groups of animal (data not shown). These results indicated that estrogen and insulin affect the cardiac contractile activation partly through a common final pathway whilst deficiency of E_2 dominantly induces an adaptive response of the myofilament to be more sensitive to Ca^{2+} even under diabetes complication.

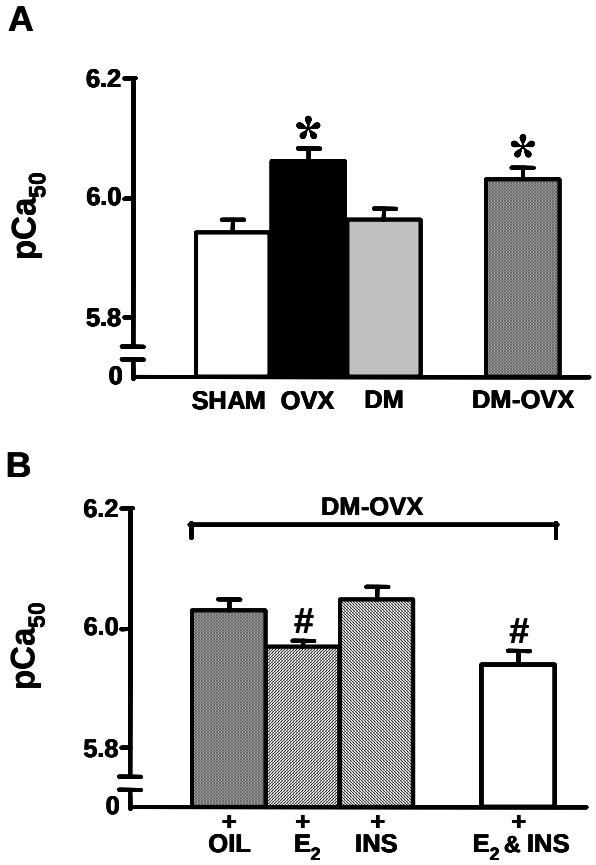

Figure 13. *A*: Comparisons of the myofilament Ca^{2+} sensitivity (pCa_{50}) of cardiac myofibrillar preparations from SHAM, OVX, DM, and DM-OVX rats. *B*: Comparisons of pCa_{50} of cardiac myofibrillar preparations from various groups of DM-OVX rats with E_2 and/or INS supplementations. Data are means $\pm$ SE from 7-8 preparations. *P<0.05 and $^{\#}$P<0.05 represent a significant difference from SHAM and DM-OVX groups, respectively, using Student-Newman-Keuls test after ANOVA.

To determine whether alterations in the β_1-adrenergic receptor in these hearts were associated with changes in myofilament response to Ca^{2+}, we measured the density, binding affinity, and protein content of β_1-adrenergic receptor in these hearts using sarcolemmal preparation and left ventricular homogenate. Similar to the increased Ca^{2+} sensitivity of the myofilament, a significant increase in the β_1-adrenoceptor density was observed only in OVX (~23%) but not in DM hearts compared to shams (Figure 14A). Moreover, a similar magnitude of enhancement in β_1-adrenoceptor density was also observed in DM-OVX hearts (~17%), which was completely restored upon E_2 supplementation (Figure 14B). In agreement with data of receptor density, results from Western blot analysis using a specific anti-β_1-adrenergic receptor antibody demonstrated a significant increase in β_1-adrenergic receptor content in OVX (~48%) and DM-OVX (~49%) hearts, and the receptor upregulation in DM-OVX hearts could be completely reversed by E_2 supplementation (Figure 15). In all groups there were no differences in the binding affinity of β_1-adrenergic receptor among the groups, as summarized in Figure16. Thus alterations in β_1-adrenoceptor expression underlie the protective role of estrogen in cardiac contractile response to Ca^{2+}.

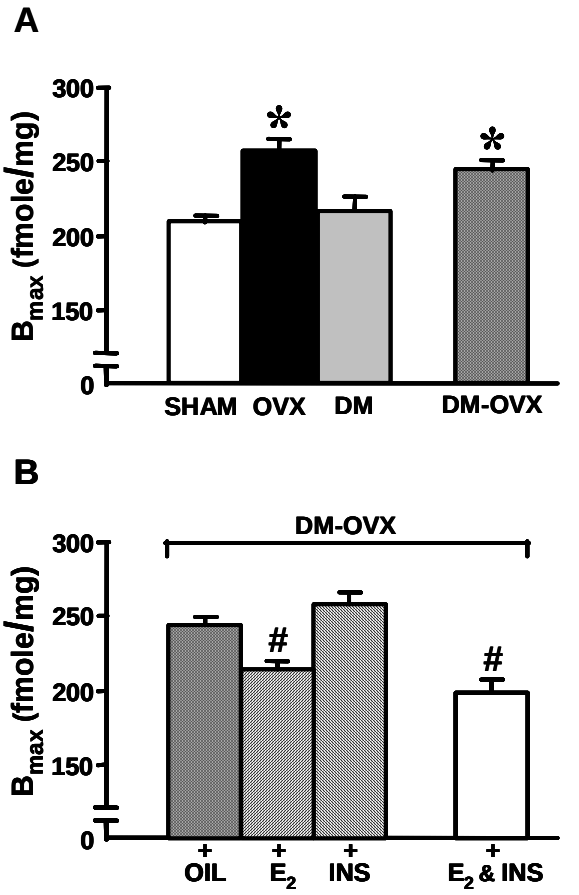

Figure 14.

A: Comparisons of the density (B_{max}) of β_1-adrenergic receptors in left ventricular membrane preparations from SHAM, OVX, DM, and DM-OVX rats.

B: Comparisons of B_{max} of β_1-adrenergic receptors in left ventricular membrane preparations from DM-OVX hearts with E_2 and/or INS supplementations.

Data are means ± SE of 8-10 hearts. *P<0.05 and #P<0.05 represent a significant difference from SHAM and DM-OVX groups, respectively, using Student-Newman-Keuls test after ANOVA.

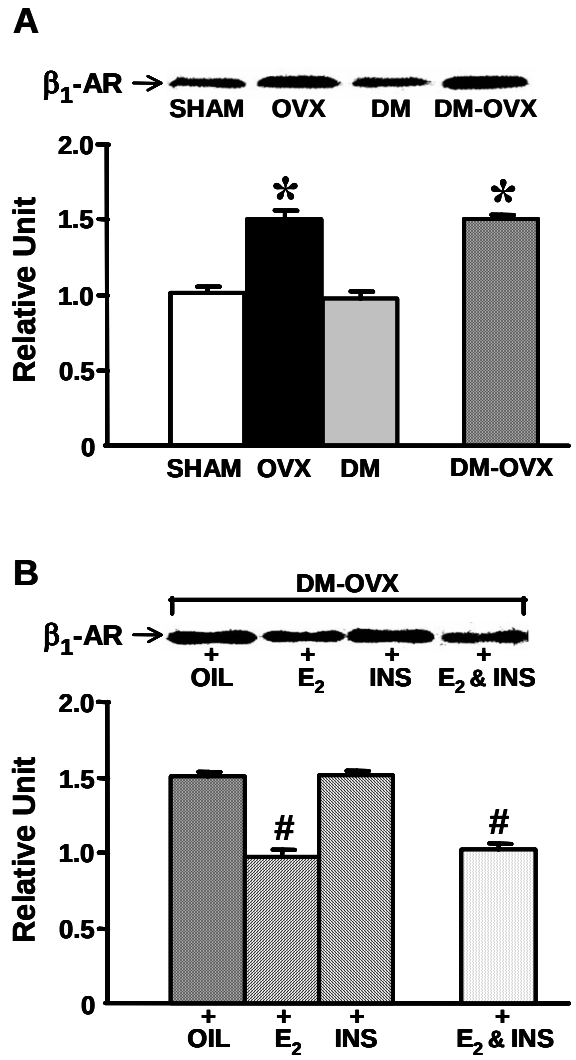

Figure 15.

A: Immunoblot analyses of β_1-adrenergic receptor (β_1-AR) proteins and comparisons of the relative band intensity of left ventricular homogenates from SHAM, OVX, DM, and DM-OVX rats.

B: Immunoblot analyses of β_1-AR proteins and comparisons of the relative band intensity of left ventricular homogenates from DM-OVX rats with E_2 and/or INS supplementations.

Data are means ± SE of 8 hearts. *P<0.05 and #P<0.05 represent a significant difference from SHAM and DM-OVX groups, respectively, using Student-Newman-Keuls test after ANOVA

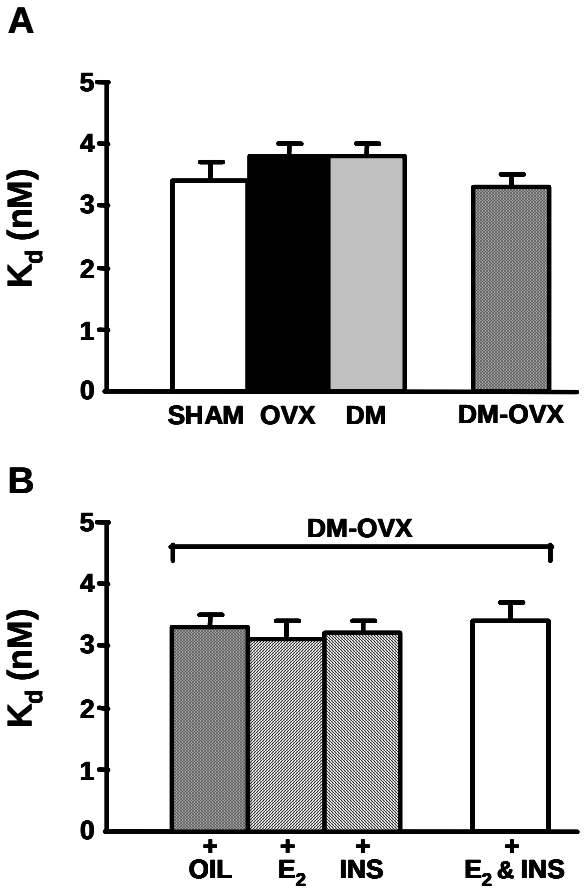

Figure 16. *A*. Comparisons of the dissociation constant (K_d) of β_1-adrenergic receptors of left ventricular membrane preparations from SHAM, OVX, DM, and DM-OVX rats. *B*: Comparisons of the K_d of β_1-adrenergic receptors of left ventricular membrane preparations from DM-OVX rats with E_2 and/or INS supplementations. Data are means $\pm$ SE of 8-10 hearts.

To further investigate whether changes in myofilament response to Ca^{2+} were associated with loss in the cardioprotective effect through expression of HSP72, we determined the amount of this factor using immunoblot analysis. As shown in Figure 17A, the same magnitude of decrease in HSP72 content was demonstrated in OVX (~27%) and DM (~25%) hearts compared to shams. Similarly, the expression of HSP72 in DM-OVX hearts was suppressed to a comparable degree (~22%) compared to shams, and increased upon supplementation with both E_2 and INS (Figure 17B). These results demonstrated that loss in cardioprotective effect through HSP72 expression in sex hormone- or insulin-deficit heart parallels the suppression of maximum myofibrillar ATPase activity but not the hormone-associated hypersensitivity of myofilament to Ca^{2+}.

It is likely that while the homeostatic balance of β_1-adrenergic receptor and HSP72 protective factors is physiologically regulated by E_2, only the protective factors are contributed by insulin. Our data confirm the physiologically cardioprotective function of E_2 even under diabetes complication on the contractile response to Ca^{2+}. The data also provide further support for the beneficial use of E_2 and β_1-blocker in postmenopausal women in preventing maladaptation of estrogen-deficient heart and thereby lowering the incidence of heart failure.

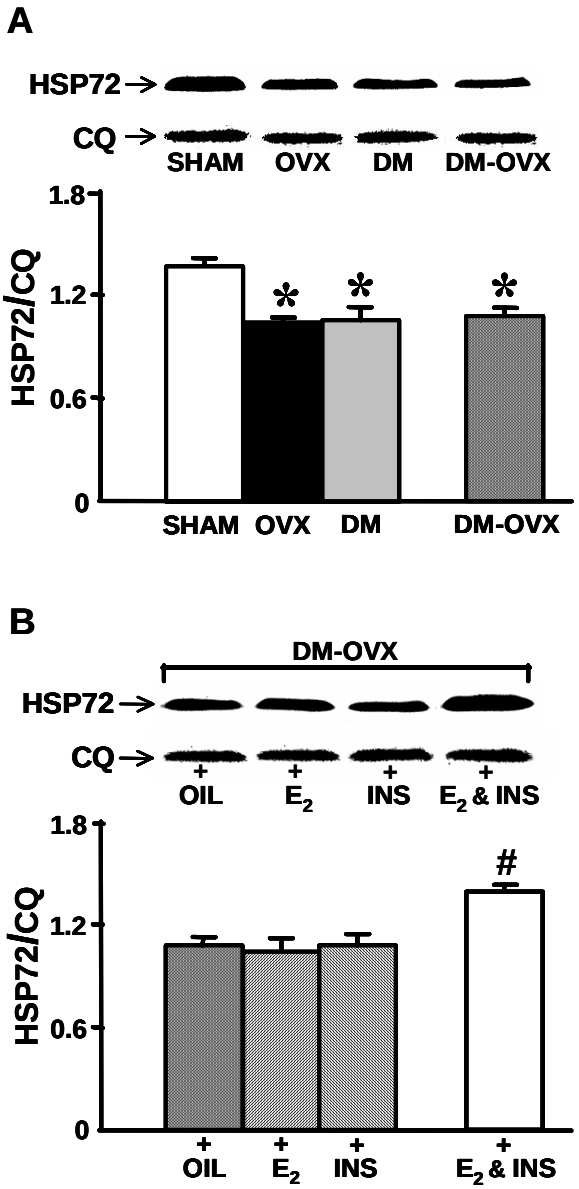

Figure 17. *A*: Immunoblot analyses of heat shock protein (HSP) 72 and calsequestrin (CQ) and comparisons of the band intensity expressed as a ratio of SERCA:CQ of left ventricular homogenates from SHAM, OVX, DM, and DM-OVX rats. *B*. Immunoblot analyses of HSP72 and CQ and comparisons of the band intensity expressed as a ratio of SERCA:CQ of left ventricular homogenates from DM-OVX rats with E_2 and/or Ins supplementations. Data are means ± SE of 8 hearts. *P<0.05 and $^{\#}$P<0.05 represent a significant difference from SHAM and DM-OVX groups, respectively, using Student-Newman-Keuls test after ANOVA.

Project V: Complication of diabetes on the relationship between shifts in myosin heavy chain isoforms and changes in the cardiac myofilament ATPase activity in ovariectomized rats

The interactive effect of female sex hormones and insulin on the maximum myofibrillar ATPase activity reported from our laboratory indicates a final common pathway of the hormones in regulating the cardiac contractile activity. A shift in the isoform population of MHC is a well recognizable mechanism underlying 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 MHC isoform shift in responsible for the regulatory effects of estrogen on the cardiac contractile activation.

To evaluate the potential regulatory effects of estrogen on the cross-bridge interactions, we therefore compared the relationships of % α-MHC and maximum myofibrillar ATPase activities from various experimental groups. The body, heart, and uterine weights of nine experimental groups including sham, ovariectomized (OVX) rats with and without estrogen supplementation, diabetes (DM) rats with and without insulin supplementation, and DM-OVX with and without supplementation with estrogen (E_2), insulin (INS) or E_2+INS were summarized in Table 3. As expected, uterine weights were markedly decreased in OVX and DM-OVX groups compared to sham and increased upon estrogen supplementation. Uterine weights were also reduced in DM rats in a smaller magnitude compared with OVX groups. Both heart and body weights were increased in OVX rats, but decreased in DM rats. However, a decrease in body weight was observed in DM-OVX rats without insulin supplementation. As a result, hypertrophy of the heart represented by an increased heart weight-to-body weight ratio was shown in DM and DM-OVX groups without insulin supplementation. In contrast, hypothyroid was detected in DM and DM-OVX rats without insulin supplementation.

Table 3. Body weight (BW), heart weight (HW), uterine weight (UW), % heart weight/ body weight (% HW/BW), and plasma T_3 level

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

Values are means ± SE from 8-12 rats. OVX: ovariectomized; DM: diabetes; DM-OVX: diabetic-ovariectomized; E_2: estrogen; INS: insulin. $P<0.05$, significant difference from SHAM (*) and all (#) groups.

Maximum myofibrillar ATPase activity and % α-MHC of the nine experimental groups were shown in Figure 18 and 19, respectively. As shown in Figure 18, the same degree of suppression in maximum ATPase activity demonstrated in the hearts of OVX and DM rats was also observed in DM-OVX rats compared to sham. The suppression of maximum ATPase activity was prevented by estrogen and insulin supplementation in OVX and DM rats, respectively. Only the combined administration of estrogen and insulin could prevent the suppressed maximum ATPase activity in DM-OVX rats. On the other hand, MHC analysis demonstrated the different levels of relative α-MHC expression in the hearts of OVX (~51%) and DM (~34%) rats compared to sham (~70%) control (Fig. 19A). The pronounced reduction in α-MHC expression detected in DM rats was also observed in the hearts of DM-OVX (~36%) rats (Fig. 19B). Similar to the myofibrillar ATPase activity, the isoform shift of MHC could be prevented by estrogen and insulin in OVX and DM rats, respectively, whereas combined administration of estrogen and insulin was needed for the complete prevention in DM-OVX rats (Fig. 19B). These results indicate differential effects of estrogen and insulin in regulating maximum myofibrillar ATPase activity.

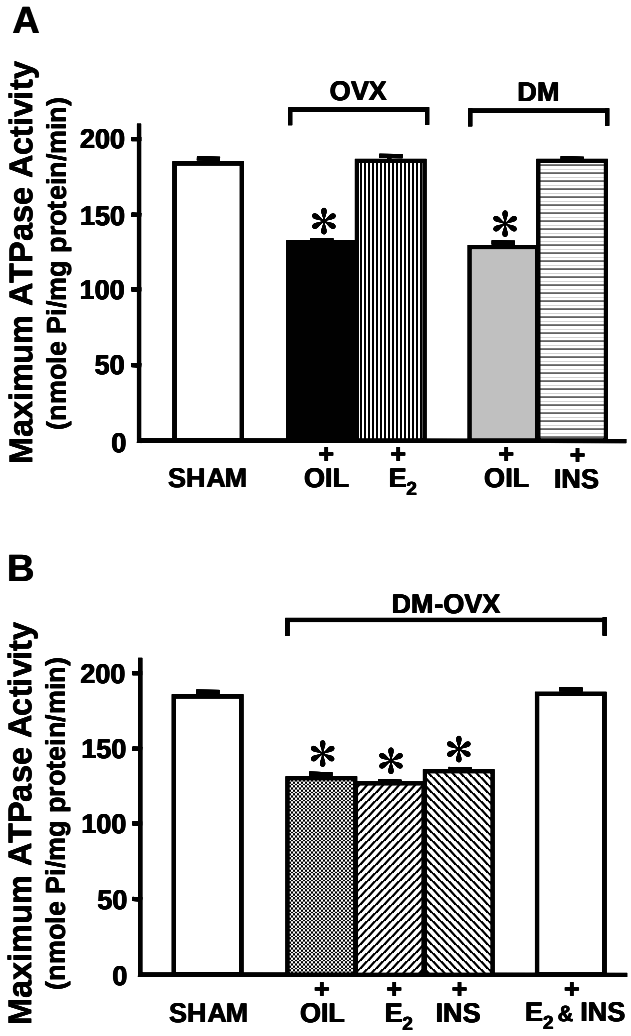

Figure 18. Comparison of the maximum Ca^{2+}-dependent actomyosin Mg^{2+}-ATPase activities in cardiac myofibrillar preparations from sham (SHAM), ovariec-tomized (OVX) with/without estrogen (E_2) supplementation, diabetic (DM) with/without insulin (INS) supplementation *(A),* and from diabetic-ovariectomized (DM-OVX) with/ without E_2 and/or INS supplementation *(B)*. Data are means±SE from 10-13 preparations. *P<0.05, significant difference from SHAM group using Student-Newman-Keuls test after ANOVA.

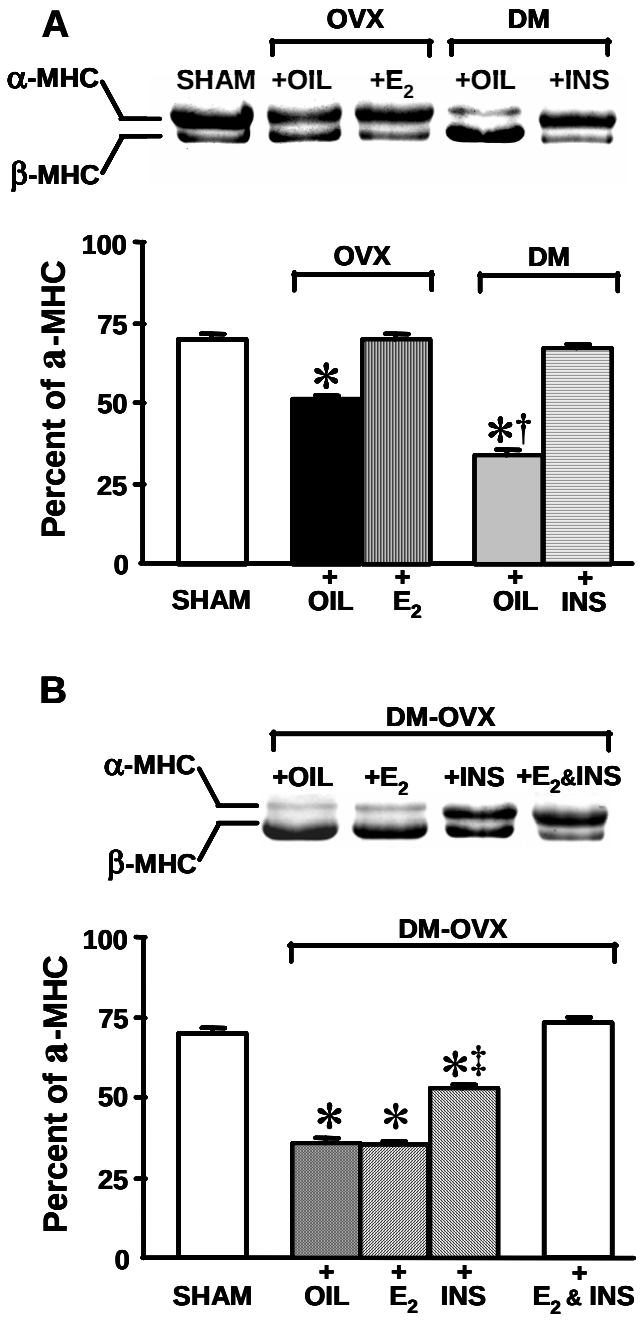

Figure 19.

Myosin heavy chain (MHC) region of SDS gels and comparison of the relative amount of α-MHC (as percenttage of total MHC) of left ventricular trabeculae from SHAM, OVX with/without E_2 supplementation, DM with/without INS supplementation *(A),* and from DM-OVX with/without E_2 and/or INS supplementation *(B)*. Data are means ± SE from 9-11 hearts. P<0.05, significant difference from SHAM (*), OVX (†), and DM-OVX (‡) using Student-Newman-Keuls test after ANOVA.

Relationship of maximum ATPase activity and % α-MHC in these experimental groups was plotted and the linear regression analysis of data from individual experimental group to that of sham control was then fitted to calculate the amount of changes in maximum ATPase activity per unit change in α-MHC which represents changes in the cross-bridge velocity. As shown in Figure 20A, a greater decrease in myofibrillar ATPase activity per unit α-MHC was demonstrated in the hearts of OVX rats compared to DM rats. Regression analysis fitting data of OVX and OVX+E_2 groups gave rise to the same slope as that of OVX and sham rats (Fig. 20B). Similarly, regression analysis of data from DM and DM+INS groups yielded the same level of slope as that of DM and sham rats. Interestingly, the less reduction in myofibrillar ATPase activity per unit α-MHC observed in DM group was also demonstrated in DM-OVX rats (Fig. 20C). As expected, the regression analysis of data from DM-OVX and DM-OVX+E_2+INS groups resulted in the similar magnitude of slope as that of DM-OVX and sham rats (Fig. 20D). These results imply that estrogen, in contrast to insulin, exerts a regulatory effect on the cross-bridge kinetics in addition to its effect on the number of cross-bridge.

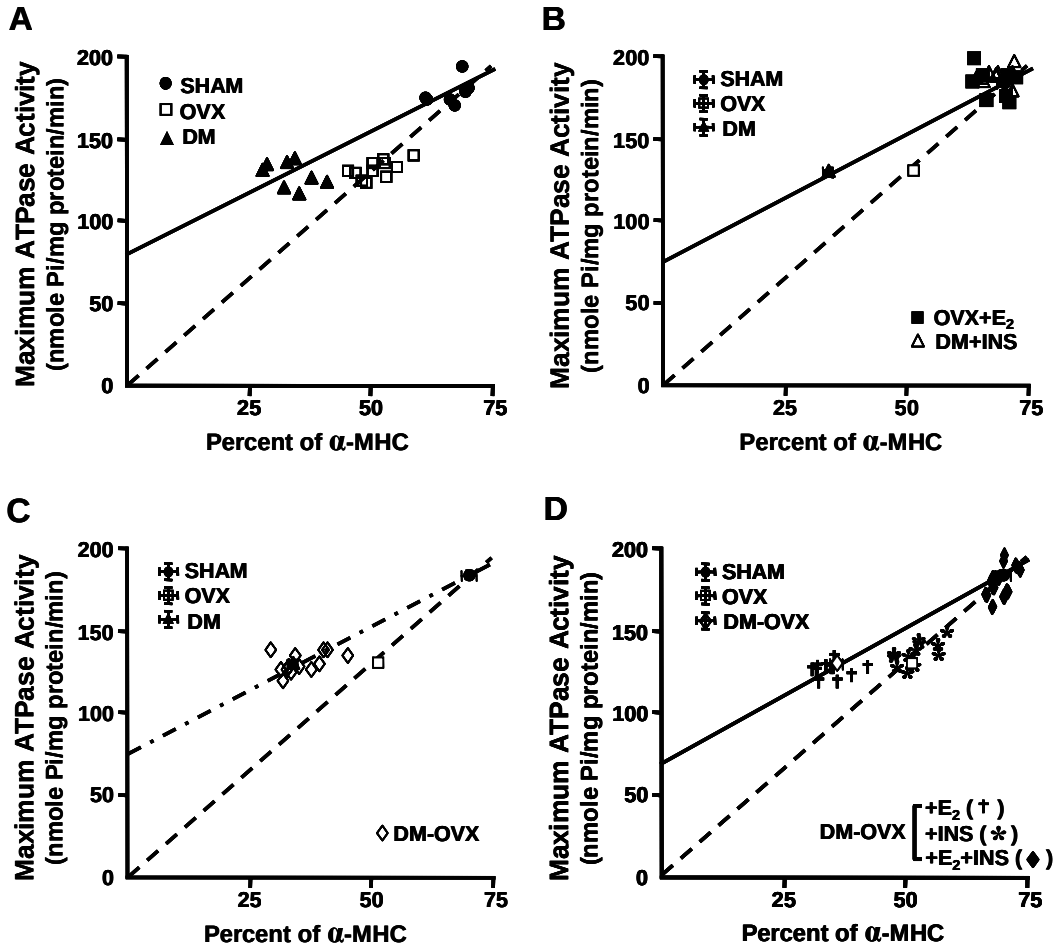

Figure 20. Linear regression analysis of the maximum myofibrillar ATPase activity and % α-myosin heavy chain (MHC) from the combined data of various experimental groups, including A: DM and sham groups (____) and OVX and sham groups (_ _ _), B: DM with insulin supplementation and DM groups (____) and OVX with estrogen supplement and OVX groups (_ _ _ _), C: DM-OVX and sham groups (_ _ _ _), D: DM-OVX supplemented with estrogen and sham groups (____), DM-OVX supplemented with insulin and sham groups (_ _), and DM-OVX supplemented with both estrogen and insulin and DM-OVX groups (____).

To test the significant role of estrogen in the cross-bridge kinetics, the correlation of maximum myofibrillar ATPase activity to various levels of % α-MHC in the hearts of OVX rats was then compared to that of DM rats. Expression of α-MHC in the hearts of OVX and DM rats were varied by manipulating the plasma thyroid status using T_3 injection or PTU administration. The body, heart, and uterine weights, and the plasma thyroid hormone levels of various experimental groups, including sham, hypothyroid-OVX (OVX+PTU), hypothyroid-(DM+PTU) and euthyroid-DM (DM+T_3), and hypothyroid- (DM-OVX+PTU) and euthyroid-DM-OVX (DM-OVX+T_3) rats were summarized in Table 4. As expected, maximum myofibrillar ATPase activity and % α-MHC were further suppressed in every group of hypothyroid induction (Fig. 21). 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 to the same levels as those in sham control. Correlation of maximum ATPase activity to % α-MHC at different thyroid hormone status demonstrated a linear relationship in both DM (Fig. 22A) and DM-OVX (Fig. 22B) groups with the same slope value as those previously detected in DM and DM-OVX data with sham (Fig. 20). In contrast, in OVX rats the correlation of maximum ATPase activity to % α-MHC expression at different thyroid status demonstrated a concave shape relationship fitted by the second degree polynomial equation (Fig. 22C). These results confirm that estrogen, in contrast to insulin, plays a major regulatory

role in the single force of cross-bridge interaction in addition to its effect on the number of cross-bridge. Moreover, the results also indicate that insulin indirectly affects the number of cross-bridge through the effect of thyroid hormone on α-MHC expression.

Table 4. 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 + PTU	292 ± 5	0.85 ± 0.02	0.09 ± 0.01*	0.29 ± 0.01	20.3 ± 1.8 *
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*
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 from 8-12 rats. OVX: ovariectomized; DM: diabetes; DM-OVX: diabetic-ovariectomized; T_3: triiodothyroinin; PTU: propyl-2 thiouracil. *P<0.05 is significant difference from SHAM group.

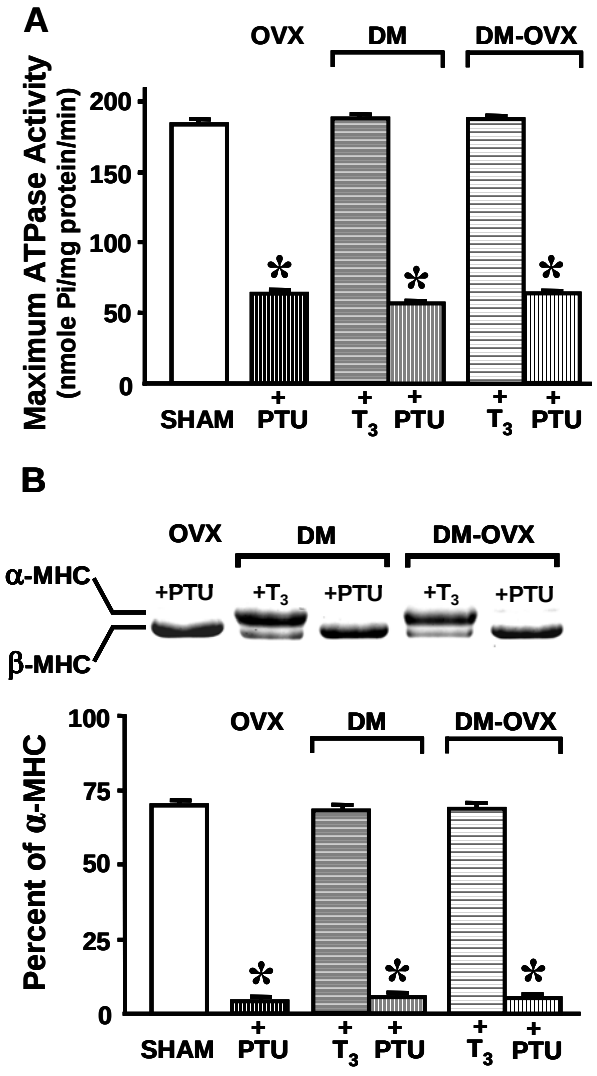

Figure 21.

A: comparison of the maximum Ca^{2+}-dependent actomyosin Mg^{2+}-ATPase activity of cardiac myofibrillar preparations at pH 7.0 from SHAM, OVX rat with 6-n-propyl-2-thiouracil (PTU) treatment, DM rat with either triiodothyronin (T_3) or PTU treatment, and DM-OVX rat with either T_3 or PTU treatment.

B: myosin heavy chain (MHC) region of SDS gels and comparison of the relative amount of α-MHC (as a percenttage of total MHC) of left ventricular trabeculae from SHAM, OVX rats with PTU treatment, DM rat with either T_3 or PTU treatment, and DM-OVX with OVX rats with either T_3 or PTU treatment.

Data are means ± SE from 12 preparations. *P<0.05, significant difference from SHAM using Student-Newman-Keuls test after ANOVA

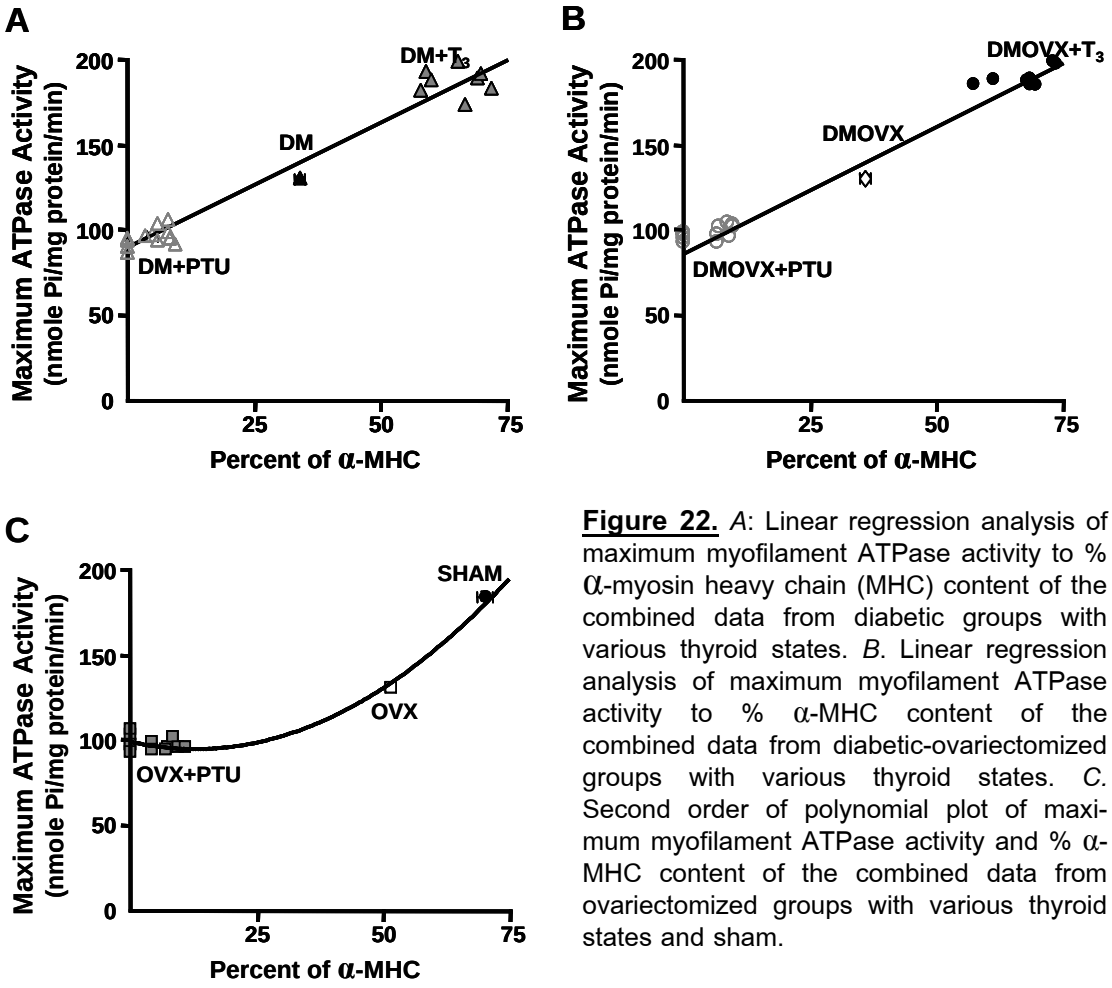

Figure 22. *A*: Linear regression analysis of maximum myofilament ATPase activity to % α-myosin heavy chain (MHC) content of the combined data from diabetic groups with various thyroid states. *B*. Linear regression analysis of maximum myofilament ATPase activity to % α-MHC content of the combined data from diabetic-ovariectomized groups with various thyroid states. *C*. Second order of polynomial plot of maximum myofilament ATPase activity and % α-MHC content of the combined data from ovariectomized groups with various thyroid states and sham.

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RESEARCH OUTPUT

I. INTERNATIONAL PUBLICATIONS:

1. Thawornkaiwong A., J Pantharanontaga, and **J Wattanapermpool.**
   Hypersensitivity of myofilament response to Ca^{2+} in association with maladaptation of estrogen-deficient heart under diabetes complication.
   Am. J. Physiol. Regul. Integr Comp. Physiol. 292: R844-851, 2007.
   Impact Factor = 3.685 (Journal Citation Reports 2006)

2. Bupha-Intr T., **J. Wattanapermpool,** JR Pena, BM Wolska, and RJ Solaro.
   Myofilament response to Ca^{2+} and Na^{+}/H^{+} exchanger activity in sex hormone related protection of cardiac myocytes from deactivation in hypercapnic acidosis.
   Am. J. Physiol. Regul. Integr Comp. Physiol. 292: R837-843, 2007.
   Impact Factor = 3.685 (Journal Citation Reports 2006)

3. Bupha-Intr T. and **J. Wattanapermpool.**
   Reguratory role of ovarian sex hormones in the calcium uptake activity of cardiac sarcoplasmic reticulum.
   Am. J. Physiol. Haert Circ Physiol. 291:1101-1108, 2006.
   Impact Factor = 3.724 (Journal Citation Reports 2006)

II. ABSTRACT & ORAL/POSTER PRESENTATION:

1. Laosiripisan J and Wattanapermpool J. Cardioprotective effects of exercise training on changes in the SR Ca^{2+}-uptake activity in ovariectomized rats. The Scientific Congress of the 1st Asian Indoor Games: The Challenging Role of Sports Science for Better Sports Performance, Asia Hotel, Bangkok, October 17-19, 2005. (Abstract for Oral Presentation by J. Laosiripaisan)

2. Wattanapermpool J, Bupha-Intr T, and Laosiripaisan J. Cardioprotective effects of female sex hormones and exercise training on the Ca^{2+} uptake activity of sarcoplasmic reticulum. Poster # P-S2C-03 ในการประชุม นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. วันที่ 12-14 ตุลาคม 2549 ณ โรงแรมรีเจนท์ ชะอำ จังหวัดเพชรบุรี

3. Laosiripisan J and Wattanapermpool J. Cardioprotective effects of exercise training on the altered SR Ca^{2+}-uptake function in ovariectomized rats. *J Mol Cell Cardiol. 40:910 (Abstract A 119), 2006.* The 28th Annual International Society for Heart Research North American Section Meeting, June 13-16, 2006. Toronto, Canada.

III. AS INVITED SPEAKER

Oral presentation in two meetings and one special seminar

1. Wattanapermpool J. Significance of estrogen on cardiac contractile activity and the benefit of phytoestrogens. Proceedings for the Conference on Research and Product Development from Soybean June 27-28, 2005. Faculty of Pharmaceutical Sciences, Ubonratchathani University. 41-60, 2005.

2. Wattanapermpool J. Beautiful Heart in Healthy Women. การอบรมเชิงปฏิบัติการสรีรวิทยา-พยาธิสรีรวิทยา ครั้งที่ 24 เรื่อง HOW TO LIVE FIT AND WELL ระหว่างวันที่ 27-31 มีนาคม 2549 คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
3. Wattanapermpool J. Female Sex Hormones and Cardiac Muscle Activation. Guest speaker for special seminar at Department of Physiology & Biophysics, College of Medicine, University of Illinois at Chicago, USA. June 22, 2006.

IV. การสร้างนักวิจัยรุ่นใหม่

สร้างนักวิจัยรุ่นใหม่ เป็นนักศึกษาระดับ**ปริญญาโท** 1 คน คือ

นางสาวจิตานันท์ เหล่าศิริไพศาล

สร้างนักวิจัยรุ่นใหม่ เป็นนักศึกษาระดับ**ปริญญาเอก** 1 คน คือ

นางสาวอริยพร ถาวรไกรวงศ์

APPENDIX