รายงานวิจัยฉบับสมบูรณ์

โครงการ: บทบาทของฮอร์โมนเพศหญิงต่อความฝืดแข็งของกล้ามเนื้อหัวใจหนูตัดรังไข่ และเมื่อมีเบาหวานแทรกซ้อน

โดย นายเทพมนัส บุปผาอินทร์

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นายเทพมนัส บุปผาอินทร์
ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกอ. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

การลดลงของอัตราเร็วของเลือดที่ไหลกลับเข้าหัวใจในผู้หญิงหลังหมดประจำเดือนบ่งชี้ถึงความผิดปกติในการคลายตัวของหัวใจ งานวิจัยชิ้นนี้จึงได้ศึกษาบทบาทของฮอร์โมนเพศหญิงต่อความสามารถในการยืดขยายของกล้ามเนื้อหัวใจโดยเปรียบเทียบแรงตึงของเส้นใยกล้ามเนื้อหัวใจที่ความยาวกล้ามเนื้อต่างๆกัน โดยการศึกษานี้ใช้หนูขาวตัดรังไข่เพื่อเลียนแบบภาวะหมดประจำเดือน และเหนี่ยวนำให้หนูเป็นเบาหวานร่วมด้วยเพื่อดูผลในการป้องกันโรคของฮอร์โมนเพศหญิง ผลการทดลองพบว่าการขาดฮฮร์โมนเพศอย่างเดียวไม่มีอิทธิพลของต่อความฝืดแข็งของกล้ามเนื้อหัวใจ อัตราส่วนของไททิน และปริมาณคอลลาเจน ส่วนในหนูเบาหวานนั้นพบว่าการมีฮอร์โมนเพศกลับทำให้ความฝืดแข็งของกล้ามเนื้อหัวใจเพิ่มขึ้น โดยเป็นผลจากการเพิ่มขึ้นของปริมาณคอลลาเจน ซึ่งเกิดจากการเพิ่มขึ้นของปริมาณโปรคอลลาเจนและสแมสทูโปรตีน ที่น่าแปลกคือไม่พบการเปลี่ยนแปลงค่าดังกล่าวในหนูตัดรังไข่ที่เป็นเบาหวาน ภาวะเบาหวานนั้นเหนี่ยวนำให้มีระดับของไททีนชนิดเอนทูบีเอมากขึ้นและยิ่งมากขึ้นอีกเมื่อขาดฮอร์โมนเพศ เมื่อศึกษาความสามารถในการหดตัวของกล้ามเนื้อหัวใจที่ระดับความเข้นข้นของแคลเซียมต่างๆพบว่าฮอร์โมนเพศหญิงสามารถป้องกันผลของเบาหวานที่จะมาลดความสามารถในการตอบสนองต่อการกระตุ้นของแคลเซียมของเส้นใยกล้ามเนื้อหัวใจในช่วงความยาวกล้ามเนื้อต่างๆได้ ผลการทดลองสามารถสรุปได้ว่าการมีฮอร์โมนเพศหญิงช่วยให้เนื้อเยื่อหัวใจมีความฝืดแข็งมากขึ้นในสภาวะที่มีโรคเบาหวานแทรกซ้อน ซึ่งการเพิ่มความฝืดแข็งน่าจะเป็นการปรับตัวของกล้ามเนื้อหัวใจเพื่อที่จะรักษาการทำงานของเส้นใยกล้ามเนื้อในช่วงที่มีการหดตัว

ABSTRACT

A decrease in peak early diastolic filling velocity in postmenopausal women implies a sex hormone-related diastolic dysfunction. The regulatory effect of female sex hormones on cardiac distensibility therefore was evaluated in ovariectomized rat by determining the sarcomere length-passive tension relationship of ventricular skinned fiber preparations. Diabetes also was induced in the rat to assess the protective significance of female sex hormones on diastolic function. While ovariectomy had no effect on myocardial stiffness, collagen content or titin ratio, a significant increase in myocardial stiffness was observed in diabetic rat only when female sex hormones were intact. The increased stiffness in diabetic rat was accompanied by an elevated collagen content due to increases in the levels of procollagen and Smad2. Surprisingly, the increased myocardial stiffness in diabetic rat was accompanied by a shift towards a more compliant N2BA of cardiac titin isoforms, even though a higher extent of shift was detected in diabetic-ovariectomized rat in which an increase of cardiac dilatation was observed. The pCa-active tension relationship was analyzed at fixed sarcomere lengths of 2.0 and 2.3 μm to determine the effects of titin isoforms on the magnitude of changes in myofilament Ca^{2+} sensitivity between the two sarcomere lengths. Interestingly, high expression of N2BA titin was associated with a suppressed magnitude of changes in myofilament Ca^{2+} sensitivity only in diabetic-ovariectomized condition. These results indicate a restrictive adaptation of myocardium governed by female sex hormones to maintain myofilament activity in compensation to the pathophysiological induction of cardiac dilatation by the diabetic condition.

หน้าสรุปโครงการ (Executive Summary)

1. ชื่อโครงการ (ภาษาไทย) บทบาทของฮอร์โมนเพศหญิงต่อความฝืดแข็งของกล้ามเนื้อหัวใจหนูตัดรังไข่ และเมื่อมีเบาหวานแทรกซ้อน

   (ภาษาอังกฤษ) Role of female sex hormones in myocardial stiffness of ovariectomized rats with and without diabetes complication

2. ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail

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3. สาขาวิชาที่ทำการวิจัย สรีรวิทยาของกล้ามเนื้อหัวใจ (cardiac muscle physiology)

5. ระยะเวลาดำเนินงาน 2 ปี

6. เนื้อหาวิจัยโดยย่อ

Female sex hormones have been demonstrated to exert many modulating effects on both systolic and diastolic functions of the heart. Clinically, in menopausal women cardiac systolic dysfunctions including a decrease in left ventricular ejection fraction with an increase in heart size were clearly demonstrated. In addition to systolic dysfunctions, cardiac diastolic dysfunction represented by a decrease in ventricular blood filling was also observed. These changes could be improved by hormone replacement therapy. Experimentally, we have reported that ovariectomy induced suppressions of both the chemical and mechanical properties of the cardiac contractile activity in which estrogen supplementation could restore the suppression. However, role of female sex hormones on the diastolic function have never been clarified. In order to specify the protective effect of female sex hormones on diastolic function, diabetic complication which known for causing diastolic dysfunction was introduced.

In this study, the protective effect of female sex hormones on the diastolic function concerning myocardial expansion ability or stiffness was investigated in ovariectomized rats with and without diabetic complication. The passive tension at various sarcomere lengths of cardiac muscle was measured to represent the mechanical stiffness. The content of collagen, the expression of titin isoforms, and the activity of matrix metalloproteinase were quantified to search for the potential mechanism underlying changes in the myocardial stiffness. Length-dependent myofilament Ca^{2+} sensitivity of the heart was analyzed to find out any effect of altered myocardial stiffness on myocardial contractility.

Results demonstrated that, deprivation of ovarian sex hormones in non-diabetic rats exerted no effects on myocardial stiffness, titin isoform ratio or collagen content. But stiffness of the ventricular bundle is significantly increased in female sex hormone-intact diabetic but not in diabetic-ovariectomized rats. Increased stiffness of the heart in diabetic rat is due mainly to elevated collagen content, supported by increase in procollagen and Smad2 contents. On the other hand, N2BA/N2B titin ratio in diabetic rat heart is significantly shifted towards the more compliant N2BA isoform, with a higher shift in diabetic-ovariectomized than in diabetic rats. Moreover, the measurement of pCa-active tension relation demonstrated that the presence of female sex hormones prevented diabetic-induced suppressed myofilament Ca^{2+} sensitivity and decreased length-dependent myofilament Ca^{2+} sensitivity.

In conclusion, our findings suggested that under diabetes complication the presence of female sex hormones induces a restrictive adaptation of myocardium in compensation to the pathophysiology of diabetes-induced dilatation of the heart. This compensation might be an underlying mechanism maintaining normal cardiac myofilament activation during systole.

เนื้อหางานวิจัย

Hypothesis:

1. Female sex hormones play significant role in the myocardial stiffness property.
2. Female sex hormones can prevent changes in the myocardial stiffness in diabetic-complicated rats.

Specific aims of the project:

1. To study the mechanical stiffness of ventricular muscle isolated from ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations
2. To study the deposition of collagen in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations
3. To study the expression of titin isoforms in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations
4. To study the expression of procollagen and smad2 in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations
5. To study the expression of protein matrix metalloproteinase in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations
6. To study the length-dependent myofilament Ca^{2+} sensitivity of the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations

ระเบียบวิธีวิจัย

Mechanical, histological, and biochemical studies representing the myocardial stiffness will be evaluated in sham-operated, ovariectomized, diabetic, and diabetic-ovariectomized rats with and without hormone supplementation.

ANIMAL GROUPS & PREPARATIONS

Eight-weeks female Sprague-Dawley rats weighing between 180-200 g (8-9 weeks old) were randomly divided into six experimental groups as follow:

1. Sham-operated group (Sham)
2. Ovariectomized group (OVX)
3. Diabetic group (DM)
4. Diabetic-ovariectomized group (DM-OVX)
5. DM-OVX with oil injection (DM-OVX + O)
6. DM-OVX with estrogen supplementation (DM-OVX + E)

EXPERIMENTAL METHODS:

Approach to objective # 1:

To study the mechanical stiffness of ventricular muscle isolated from ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations

The role of female sex hormones in the capability of ventricular expansion during diastole was determined by the stiffness of myocardium. We expect that female sex hormones play a crucial role in the myocardial stiffness both in normal condition and under diabetic complication. The passive stiffness of the muscle bundle representing the total muscle stiffness was determined from the relationship between passive tension developments at various sarcomere lengths. The collagen-based as well as the titin-based stiffness of the muscle bundle were further quantified.

Approach to objective # 2:

To study the deposition of collagen in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations

To understand the mechanism underlying changes in the mechanical stiffness of the hearts, which is determined mainly by alterations in the content of collagen deposition, the total content and the isoforms of collagen were analyzed. Histochemistry and immunofluorescent were performed to quantify the amount of collagen content per tissue area.

Approach to objective # 3:

To study the expression of titin isoforms in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations

Titin is an important sarcomeric protein affecting the myocardial stiffness. The molecular structure of titin is a spring coil connecting the myofibillar contractile portion to the Z-disc of the sarcomere. The expression of titin isoforms in the heart was separated using Agarose gel electrophoresis.

Approach to objective #4:

To study the procollagen and Smad2 in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations

Collagen is a major cardiac component disturbing the stiffness of the heart. Either changed collagen synthesis or altered degradation can affect collagen deposition. In this study, therefore, the measurement of Smad2, a major collagen transcription factor, and procollagen using general immunoblot analysis could reveal the collagen synthesis activity.

Approach to objective # 5:

To study the expression of protein matrix metalloproteinase in the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations

The extracellular matrix is another cardiac component disturbing the stiffness of the heart. Degradation of the extracellular matrix results in changes in the cardiac structure and therefore increases in collagen deposition. Reconstruction of the extracellular matrix is

mainly under the control of matrix metalloproteinase enzymes (MMPs). In this study, therefore, measured the expression of MMPs using general immunoblot analysis.

Approach to objective # 6:

To study the length-dependent myofilament Ca2+ sensitivity of the left ventricle of ovariectomized and diabetic-ovariectomized rats with and without hormone supplementations

Change in diastolic function directly affects the systolic function based on Frank-Starling mechanism. With the sarcomere length-associated myofilament Ca^{2+} sensitivity, this study, therefore, measured the Ca^{2+} concentration-force contraction relationship at two different sarcomere lengths using skinned fiber preparation. A Ca^{2+} concentration that gives the half maximal force contraction indicates the myofilament Ca^{2+} sensitivity of the heart.

RESULTS

Myocardial stiffness. Sarcomere passive length-tension relationships were determined to evaluate the effect of female sex hormone deprivation on myocardial stiffness in non-DM and DM conditions. In non- DM condition, there was no difference in the passive tension of isolated trabecular bundle from heart of SHAM and OVX rats at every sarcomere lengths (Figure 1). However, the passive force of both right ventricular trabeculae and stripped left ventricular papillary muscles from DM heart was significantly elevated, starting from a sarcomere length of 2.3 μm. Interestingly, ovariectomy resulted in the disappearance of enhanced cardiac stiffness in DM group. Increased myocardial stiffness in DM rats was confirmed by an increase in the exponential constant (K) of sarcomere length-passive tension relationship (Table 1). These results indicate that the development of ventricular stiffness in the heart under diabetic condition is induced only when female sex hormones are present.

Collagen deposition. As the major molecular structure contributing to cardiac stiffness is extracellular collagen deposition, the collagen content in ventricular tissues from SHAM, OVX, DM, and DM-OVX rats were compared. Collagen content (red color) per tissue area was higher in heart of DM than of SHAM group (Figure 2). Analysis using color histogram demonstrated no change in collagen content of OVX compared to SHAM rat heart (3.10 ± 0.23 % of tissue section), but there is a significant increase in DM rat heart

(5.06 ± 0.27 %). Similar to the results of mechanical measurements, collagen content in the ventricle of DM-OVX rat (3.95 ± 0.23 % of tissue section) is not significantly different from that of SHAM. These results indicate that one mechanism for diabetes-induced myocardial stiffness is through an increase in collagen deposition.

Titin isoform expression. The effect of female sex hormones on intracellular molecules associated with stiffness of the heart was also determined by analyzing titin isoform levels in ventricles of SHAM, OVX, DM, and DM-OVX rats. Neonatal heart was used as control for titin isoforms. There was no change in N2BA/N2B titin ratio in the heart of OVX rat heart (7.7 ± 1.8) compared to SHAM (5.2 ± 0.2) (Figure 3). On the other hand, a significant increase in the titin isoform ratio was observed in both DM (17.6 ± 1.2) and DM-OVX (34.1 ± 4.3) rats. There was a decrease also in serum triiodothyronine level in DM and DM-OVX rats concomitant with the reduction in titin isoform ratio (data not shown). These results indicate that female sex hormones are able to attenuate the shift towards titin compliant isoform in the heart under diabetic condition.

Collagen remodeling proteins. As collagen level in tissue depends on the balance between collagen synthesis and degradation, we measured levels of Smad2, a major collagen gene transcription factor, procollagen subtype I, and MMP-2 and MMP-9, two major extracellular matrix degrading enzymes. Similar to the results for collagen content, significant increases in Smad2 (~45%) and procollagen (~50%) levels were observed only in DM compared to SHAM group (Figure 4 & 5). The increase in Smad2 and procollagen levels in DM but not DM-OVX rats indicate a significant regulatory effect of female sex hormones on collagen synthesis in heart under diabetic condition.

Immunoblot analysis of latent and active forms of MMP-2 in cardiac tissues from the four groups of rat showed that total MMP-2 content is significantly increased in both DM and DM-OVX rats to a similar degree (53 ± 15 % and 66 ± 19 % respectively) compared to SHAM (Figure 6). However, there was no change in the ratio of active to total forms of MMP-2 among the four animal groups. There were no changes in MMP-9 expression observed among all experimental groups (data not shown). These results indicate that female sex hormones have no impact on expression of the major extracellular matrix degrading enzymes in the heart, but diabetes is able to increase MMP-2 content.

Length-dependent myofilament Ca^{2+} sensitivity. Based on the Frank-Starling effect, impact of diastolic distensibility on myofilament activation was analyzed using skinned fiber

preparations by measuring Ca^{2+} sensitivity of myofilaments at two fixed sarcomere lengths. As expected, the increase in cardiac myofilament Ca^{2+} sensitivity was observed at both sarcomere lengths in OVX but only at long sarcomere length in DM rats compared to sham controls (Figure 7A and Table 1). However, the magnitude of the shifts in myofilament Ca^{2+} sensitivity was not different among the groups (Figure 7B). Interestingly, the magnitude of shift in myofilament Ca^{2+} sensitivity between the two sarcomere lengths (ΔpCa_{50}) is significantly reduced in the heart of DM-OVX rat. These results indicate a significant role of female sex hormones in maintaining the Starling mechanism of the heart even under diabetic condition.

Table 1 Passive stiffness and Ca^{2+} sensitivity of tension development of skinned fiber preparations from right ventricular trabeculae and/or stripped left ventricular papillary muscle of sham-operated (SHAM), ovariectomized (OVX), diabetic (DM), and diabetic-ovariectomized (DM-OVX) rats.

	PASSIVE STIFFNESS		pCa_{50}	
	RV Trabeculae	LV Papillary M.	SL = 2.0 μm	SL = 2.3 μm
SHAM	10.10 ± 0.99 (8)	10.66 ± 0.64 (8)	5.56 ± 0.02 (12)	5.68 ± 0.02
OVX	10.26 ± 0.87 (10)	9.94 ± 0.69 (9)	5.63 ± 0.02* (10)	5.75 ± 0.02*
DM	13.31 ± 1.18 (12)	12.76 ± 1.20 (8)	5.60 ± 0.02 (9)	5.73 ± 0.01*
DM-OVX	9.67 ± 0.75† (10)	9.10 ± 1.03† (11)	5.52 ± 0.01† (11)	5.61 ± 0.01*†

Data are mean ± SE from number of fiber preparations in parenthesis. *, $^{\dagger}P < 0.05$, significantly different from SHAM and DM, respectively, using Student-Newman-Keuls test after ANOVA.

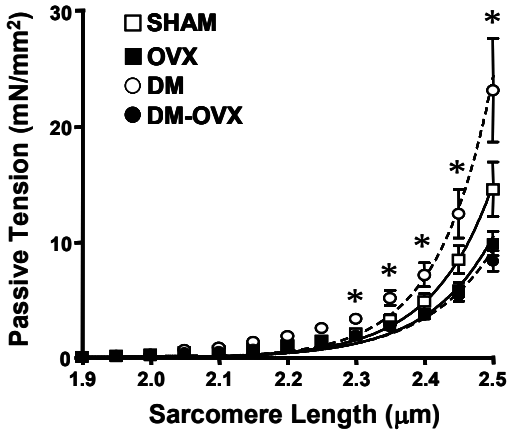

Figure 1 Effect of ovariectomy and diabetes on myocardial stiffness of rat heart. Passive tension of right ventricular trabeculae and left ventricular papillary fibers were measured at various sarcomere lengths. Data are mean ± SE from 16-20 fibers of each group. *Significantly different (P < 0.05) from SHAM at the same sarcomere length using Student-Newman-Keuls test after ANOVA. SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

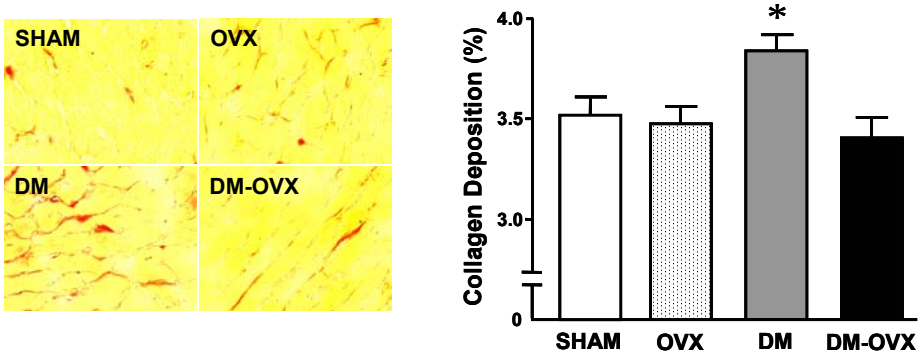

Figure 2 Effect of ovariectomy and diabetes on collagen deposition in rat heart. Left, cardiac section stained with Prico-Sirius red and viewed under light microscope (400x magnification); right, percent collagen content per tissue area. Data are mean ± SE from 400 pictures of four hearts in each group. *Significantly different (P < 0.05) from SHAM using Student-Newman-Keuls test after ANOVA.

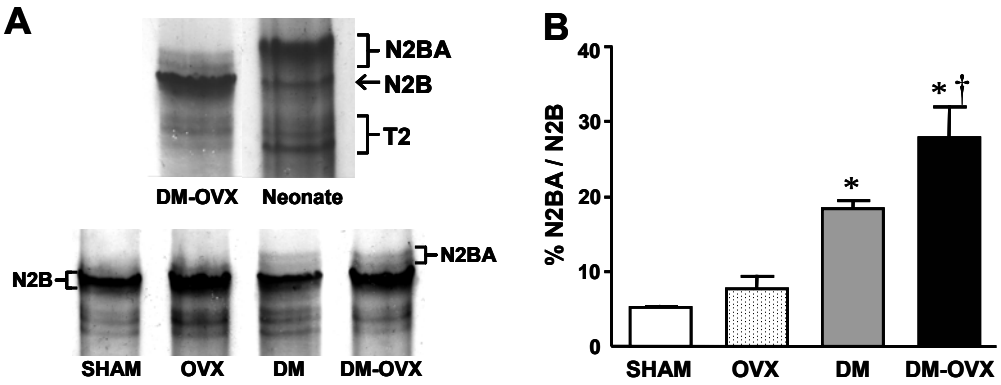

Figure 3 Effect of ovariectomy and diabetes on titin isoforms in rat heart. *A*, silver stained bands of cardiac titin isoforms following 2% SDS-polyacrylamide gel-electrophoresis; *B*, ratio of N2BA to N2B titin isoform. Data are mean ± SE from four hearts in each group. *, † Significantly different ($P < 0.05$) from SHAM and DM group respectively, using Student-Newman-Keuls test after ANOVA. Neonate, cardiac sample from 2-day neonatal rat.

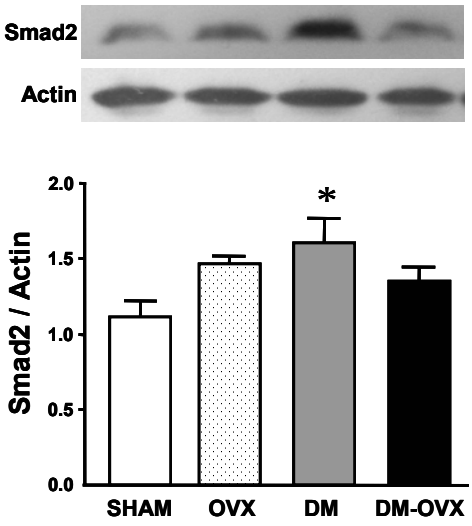

Figure 4 Effect of ovariectomy and diabetes on Samd2 protein level in rat heart. Top, immunoblot analysis of Smad2 (MW~60 kDa) and actin (MW~ 45 kDa) from left ventricular homogenate; bottom, ratio of Smad2 to actin. Data are mean ± SE from four hearts in each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA.

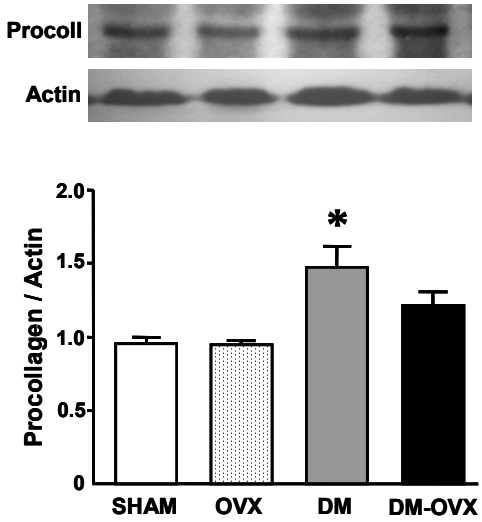

Figure 5 Effect of ovariectomy and diabetes on procollagen expression in rat heart. Top, immunoblot analysis of procollagen (procoll; MW ~60 kDa) and actin from left ventricular homogenate; bottom, ratio of procollagen to actin. Data are mean ± SE from 4-5 hearts in each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA.

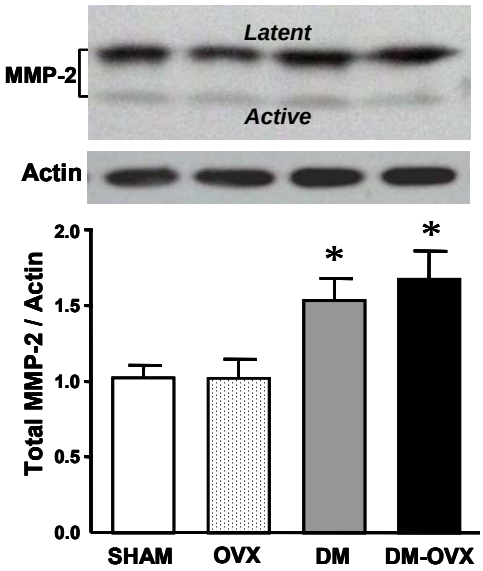

Figure 6 Effect of ovariectomy and diabetes on MMP-2 protein expression in rat heart. Top, immunoblot analysis of latent (70 kDa) and active (65 kDa) components of MMP-2 from left ventricular homogenate; bottom, ratio of total MMP-2 to actin. Data are mean ± SE from six hearts in each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA.

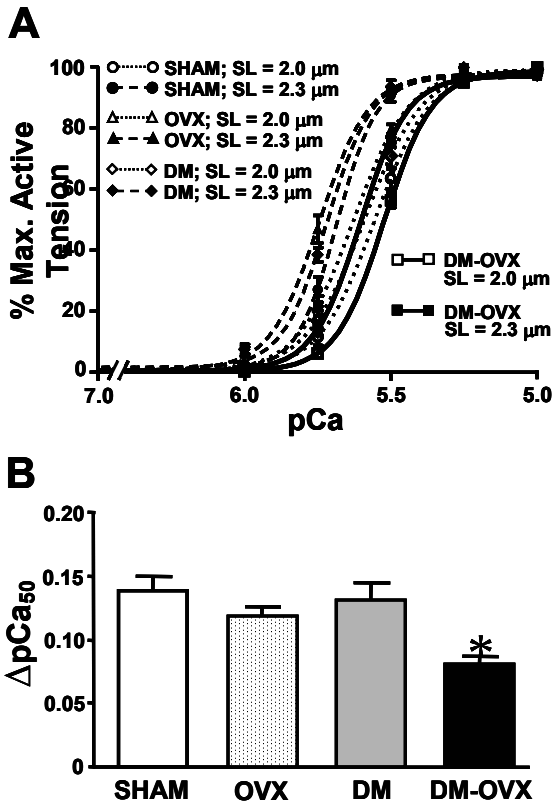

Figure 7 Effect of ovariectomy and diabetes on length-dependent myofilament Ca^{2+} sensitivity in rat heart. pCa-tension relations of skinned papillary muscle from SHAM, OVX, DM, and DM-OVX (*A*) at two sarcomere lengths of 2.0 and 2.3 μm and data summarizing magnitude of shifts in pCa_{50} (ΔpCa_{50}) between the two sarcomere lengths *(B)* from each group. Data are means ± SE from 9-12 preparations of 3-4 hearts in each group. *Significantly different from SHAM ($P < 0.05$) using Student-Newman-Keuls test after ANOVA.

Output ที่ได้จากโครงการ

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ภาคผนวก

INCREASED MYOCARDIAL STIFFNESS WITH MAINTENANCE OF LENGTH-DEPENDENT CALCIUM ACTIVATION BY FEMALE SEX HORMONES IN DIABETIC RAT

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Running head: MYOCARDIAL STIFFNESS IN DIABETIC-OVARIECTOMIZED RAT

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ABSTRACT

A decrease in peak early diastolic filling velocity in postmenopausal women implies a sex hormone-related diastolic dysfunction. The regulatory effect of female sex hormones on cardiac distensibility therefore was evaluated in ovariectomized rat by determining the sarcomere length-passive tension relationship of ventricular skinned fiber preparations. Diabetes also was induced in the rat to assess the protective significance of female sex hormones on diastolic function. While ovariectomy had no effect on myocardial stiffness, collagen content or titin ratio, a significant increase in myocardial stiffness was observed in diabetic rat only when female sex hormones were intact. The increased stiffness in diabetic rat was accompanied by an elevated collagen content due to increases in the levels of procollagen and Smad2. Surprisingly, the increased myocardial stiffness in diabetic rat was accompanied by a shift towards a more compliant N2BA of cardiac titin isoforms, even though a higher extent of shift was detected in diabetic-ovariectomized rat in which an increase of cardiac dilatation was observed. The pCa-active tension relationship was analyzed at fixed sarcomere lengths of 2.0 and 2.3 μm to determine the effects of titin isoforms on the magnitude of changes in myofilament Ca^{2+} sensitivity between the two sarcomere lengths. Interestingly, high expression of N2BA titin was associated with a suppressed magnitude of changes in myofilament Ca^{2+} sensitivity only in diabetic-ovariectomized condition. These results indicate a restrictive adaptation of myocardium governed by female sex hormones to maintain myofilament activity in compensation to the pathophysiological induction of cardiac dilatation by the diabetic condition.

Keywords: female sex hormones, diabetes, diastolic distension, titin, collagen

INTRODUCTION

The regulatory role of female sex hormones on cardiac diastolic function has been indicated from reports showing a significant decrease in peak early diastolic velocity (E-wave) of the heart in postmenopausal women (1, 18). Of the two major factors affecting diastolic filling velocity, namely, relaxation and distension of myocardium, our previous studies in rat have demonstrated prolonged ventricular relaxation induced after chronic deprivation of ovarian sex hormones (8). A decrease in sarcoplasmic reticulum (SR) Ca^{2+} uptake activity ultimately leads to a delayed decay of intracellular Ca^{2+} level in cardiomyocytes of ovariectomized (OVX) rat, and Ca^{2+} hypersensitivity of cardiac myofilament detected in such animal also impedes myocardial relaxation (7, 8, 31). A preventive effect of estrogen supplementation on all these changes further supports the regulatory role of female sex hormones on ventricular relaxation (7, 8). It is therefore possible that the decreased early diastolic velocity in menopause is due partly to a slower relaxation of myocardium. However, it is not clear whether female sex hormones play any regulatory role on the distension of myocardial chamber.

Induction of cardiac fibrosis following various cardiovascular insults from alterations between the presence and absence of female sex hormones indicates a potential impact of such hormones on myocardial distension (2, 3, 12, 14, 20, 21, 24, 27). The presence of female sex hormones significantly reduces an increase in collagen deposition in myocardium in animal models of aging, hypertension and chronic angiotensin II infusion (2, 3, 20, 32). The effect of estrogen on vasodilatation has been claimed to be responsible for its protective activity (3). Addition of estrogen to cultured cardiac fibroblast disrupts angiotensin II-stimulated fibroblast activities (27). In the ovarian sex hormones deprivation condition, chronic volume overload induces severe ventricular dilation of the heart, in which estrogen supplementation significantly

increases myocardial stiffness (12). Despite knowledge of the beneficial effects of female sex hormones under physiological stress, their failure in preventing fibrous formation in both acute and chronic myocardial infarction has been demonstrated (14, 21, 24). These contradictory data concerning the effect of female sex hormones on myocardial distension indicate that further studies are required to better understand their regulatory influences especially under pathological conditions.

The present study evaluated the effect of female sex hormones on diastolic distension of the heart under both physiological and pathological conditions, using OVX rats with and without diabetes, as it is well-known that diabetes induces vascular-independent cardiomyopathy with increase in collagen deposition (30, 33). We compared sarcomere length passive tension relationship, collagen deposition and titin isoform content among the experimental groups, consisting of sham-operated, and OVX with and without streptozocin-induced diabetes. Active tension of skinned papillary fibers at various Ca^{2+} concentrations also was measured at two sarcomere lengths, 2.0 and 2.3 μm, in order to evaluate the possible influence of female sex hormones on length-dependent myofilament Ca^{2+} sensitivity. The results showed that, while female sex hormones had no effect on diastolic activity under physiological condition, the hormones helped induce a greater myocardial stiffness in protecting myocardial distension under diabetic condition. These observations imply a feasible compensatory mechanism governed by female sex hormones to restrict increased cardiac compliance induced by pathological stress.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma Chemical (St. Louis, MO) and USB Corporation (Cleveland, OH). Electrophoresis reagents were from Bio-Rad (Hercules, CA) or Amersham Pharmacia Biotech (Buckinghamshire, UK), and SeaKem Gold Agarose was from Lonza (Rockland, ME). Peroxidase-conjugated affinipure donkey anti-mouse IgG (H+L) was purchased from Research Diagnostics (Flanders, NJ) and horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (ZyMax grade) from Zymed (San Francisco, CA).

Animal preparation. Female Sprague-Dawley rats were sham operated (SHAM) or ovariectomized (OVX) at 8 weeks of age as previously described (29). Individual rats were housed in standard cages and provided with rat chow and water *ad libitum*. Two weeks after surgery, both SHAM and OVX rats were randomly injected intraperitoneally with 60 mg/kg body weight of streptozotocin in order to induce type I diabetes. Non-diabetic animals were injected with citrate buffer. Diabetic status was verified by determining urinary glucose using glucose strip (Roche, Indianapolis, IN) 1 day after induction and on the day animals were sacrificed. Streptozotocin-injected rats with urine glucose level of less than 500 mg/dL were discarded from the study. Animal protocols were approved by Experimental Animal Committee, Faculty of Science, Mahidol University, in accordance with guidelines of National Laboratory Animal Centre, Thailand.

Sarcomere length-passive tension and pCa-active tension measurements. Ten weeks after surgery, rat was anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) following heparinization (1000 units/kg body weight) and heart was removed rapidly.

The excised heart was perfused with low-calcium Krebs-Henseleit solution containing 2,3-butanedione monoxime in a modified Langendorff perfusion system as previously described (6). Both non-branched trabeculae from the free wall of right ventricle and the left ventricular papillary muscle were dissected. The remaining ventricles were rapidly frozen and kept at -80 ^{o}C. Papillary muscle was cut longitudinally into a small fiber bundle (150–250 μm in diameter) in ice-cold high relaxing (HR) buffer (10 mM EGTA, 2 mM free Mg^{2+}, 5 mM $MgATP^{2-}$, 79.2 mM KCl, 12 mM creatine phosphate, 20 mM MOPS, pH 7.0, ionic strength 0.15 M, 2.5 μg/ml pepstatin A, 1 μg/ml leupeptin, and 50 μM PMSF) (8). For measurements of force contraction in various Ca^{2+} concentrations, left ventricular stripped papillary fibers were skinned in HR buffer containing 1% Triton X-100 for one hour at 25^{o}C. Right ventricular trabeculae and the rest of the stripped papillary were skinned overnight at 4^{o}C for passive force measurements. The skinned fiber bundle was attached using aluminum T-clips at one end to a displacement generator and at the other end to a force transducer (KG-7) (16). Active tension was measured at two fixed sarcomere lengths of 2.0 and 2.3 μm at 20^{o}C in a solution containing Ca^{2+} concentrations ranging from pCa 7.0 to 4.5. Passive force was measured at sarcomere lengths ranging from 1.9 to 2.5 μm determined by laser diffraction pattern in HR buffer at 20^{o}C. Cross-sectional area of fiber bundle was calculated based on an elliptical model.

Titin isoform separation. Frozen myocardial tissue (1 mg) was homogenized in 40 μL of urea buffer containing a cocktail of protease inhibitors (Sigma Chemical). The sample was incubated at 60^{o}C for 3 min, immediately placed on ice and centrifuged at 10,000 *g* for 2 min at 4^{o}C. Supernatant (5 μL) was electrophoresed in agarose-strengthened 2% SDS-polyacrylamide gel as previously described (28). Gel was visualized using silver stain-plus kit (Bio-Rad). Titin

bands were analyzed using Image Master Labscan version 3.01 and Image Master Totallab version 1.0 (Amersham Pharmacia Biotech).

Histochemical analysis. Areas of collagen deposition in the left ventricle were determined by Prico-Sirius red staining as follows. The fresh left ventricle was frozen in medium (Sakura Finetek) and cryostat sectioned. Ten μm thick sections were stained with Prico-Sirius red dye (Direct red; Sigma) for 1 hour as previously described (23). Histological sections were examined under light microscope (400x magnification) and recorded using a high resolution digital camera. Red color area in the ventricular tissue represents collagen content. The ratio of collagen content to ventricular myocytes was analyzed based on a color histogram using Image J (NIH).

Immunoblot analysis. Frozen left ventricular tissue was homogenized in RIPA buffer containing a cocktail of protease inhibitors (Sigma Chemical). Protein concentration of the left ventricular homogenate was determined by bicinchoninic acid assay (25). Monoclonal antibodies against Smad2 (1:1,000 dilution) (Cell Signaling Technology) and type II matrix metalloproteinase enzyme, MMP-2 (1:2,000 dilution) (Santa Cruz) were used for analyzing protein contents of Smad2 and MMP-2 respectively, in 300 μg of tissue homogenate. The amount of protein was determined relative to the amount of actin detected using polyclonal antibody against β-actin (1:10,000 dilution) (Aviva) in the same gel. Band density was analyzed using Image Master Labscan version 3.01 and Image Master Totallab version 1.0 (Amersham Pharmacia Biotech).

Data and statistical analysis. Relationship of pCa and percent maximum developed tension was fitted to Hill equation using nonlinear least squares regression analysis (GraphPad Prism version 4.0) to derive pCa_{50} (half-maximal activating Ca^{2+} concentration). Curves relating sarcomere length with passive tension were fitted to an exponential growth equation $Y = Start \cdot Exp^{(K \cdot X)}$ (GraphPad Prism version 4.0) where K is the passive stiffness of the heart. Data are presented as mean ± SE. Significance of difference among groups was determined using one-way ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons. *P* value < 0.05 is considered significantly different.

RESULTS

Animal. Chronic deprivation of ovarian sex hormones induced a significant increase in body weight (358 ± 2 g) compared to sham group (278 ± 2 g). Significant lower body weight was observed in both diabetic (DM) (15%) and diabetic-ovariectomized (DM-OVX) (21%) compared to SHAM and OVX rats respectively, similar to previous study (29). Deficiency of female sex hormones was demonstrated by significant decrease in uterine weight, from 0.56 ± 0.02 g in SHAM to 0.14 ± 0.01 g in OVX group and from 0.53 ± 0.02 g in DM to 0.12 ± 0.01 g in DM-OVX group.

Myocardial stiffness. Sarcomere passive length-tension relationships were determined to evaluate the effect of female sex hormone deprivation on myocardial stiffness in non-DM and DM conditions. In non- DM condition, there was no difference in the passive tension of isolated trabecular bundle from heart of SHAM and OVX rats at every sarcomere lengths (Figure 1). However, the passive force of both right ventricular trabeculae and stripped left ventricular papillary muscles from DM heart was significantly elevated, starting from a sarcomere length of 2.3 μm. Interestingly, ovariectomy resulted in the disappearance of enhanced cardiac stiffness in DM group. Increased myocardial stiffness in DM rats was confirmed by an increase in the exponential constant (K) of sarcomere length-passive tension relationship (Table 1). These results indicate that the development of ventricular stiffness in the heart under diabetic condition is induced only when female sex hormones are present.

Collagen deposition. As the major molecular structure contributing to cardiac stiffness is extracellular collagen deposition (reviewed by Fukuda & Granzier (9)), the collagen content in ventricular tissues from SHAM, OVX, DM, and DM-OVX rats were compared. Collagen

content (red color) per tissue area was higher in heart of DM than of SHAM group (Figure 2). Analysis using color histogram demonstrated no change in collagen content of OVX compared to SHAM rat heart (3.10 ± 0.23 % of tissue section), but there is a significant increase in DM rat heart (5.06 ± 0.27 %). Similar to the results of mechanical measurements, collagen content in the ventricle of DM-OVX rat (3.95 ± 0.23 % of tissue section) is not significantly different from that of SHAM. These results indicate that one mechanism for diabetes-induced myocardial stiffness is through an increase in collagen deposition.

Titin isoform expression. The effect of female sex hormones on intracellular molecules associated with stiffness of the heart was also determined by analyzing titin isoform levels in ventricles of SHAM, OVX, DM, and DM-OVX rats. Neonatal heart was used as control for titin isoforms. There was no change in N2BA/N2B titin ratio in the heart of OVX rat heart (7.7 ± 1.8) compared to SHAM (5.2 ± 0.2) (Figure 3). On the other hand, a significant increase in the titin isoform ratio was observed in both DM (17.6 ± 1.2) and DM-OVX (34.1 ± 4.3) rats. There was a decrease also in serum triiodothyronine level in DM and DM-OVX rats concomitant with the reduction in titin isoform ratio (data not shown). These results indicate that female sex hormones are able to attenuate the shift towards titin compliant isoform in the heart under diabetic condition.

Collagen remodeling proteins. As collagen level in tissue depends on the balance between collagen synthesis and degradation, we measured levels of Smad2, a major collagen gene transcription factor, procollagen subtype I, and MMP-2 and MMP-9, two major extracellular matrix degrading enzymes. Similar to the results for collagen content, significant increases in Smad2 (~45%) and procollagen (~50%) levels were observed only in DM compared

to SHAM group (Figure 4 & 5). The increase in Smad2 and procollagen levels in DM but not DM-OVX rats indicate a significant regulatory effect of female sex hormones on collagen synthesis in heart under diabetic condition.

Immunoblot analysis of latent and active forms of MMP-2 in cardiac tissues from the four groups of rat showed that total MMP-2 content is significantly increased in both DM and DM-OVX rats to a similar degree (53 ± 15 % and 66 ± 19 % respectively) compared to SHAM (Figure 6). However, there was no change in the ratio of active to total forms of MMP-2 among the four animal groups. There were no changes in MMP-9 expression observed among all experimental groups (data not shown). These results indicate that female sex hormones have no impact on expression of the major extracellular matrix degrading enzymes in the heart, but diabetes is able to increase MMP-2 content.

Length-dependent myofilament Ca^{2+} sensitivity. Based on the Frank-Starling effect, impact of diastolic distensibility on myofilament activation was analyzed using skinned fiber preparations by measuring Ca^{2+} sensitivity of myofilaments at two fixed sarcomere lengths. As expected, the increase in cardiac myofilament Ca^{2+} sensitivity was observed at both sarcomere lengths in OVX but only at long sarcomere length in DM rats compared to sham controls (Figure 7A and Table 1). However, the magnitude of the shifts in myofilament Ca^{2+} sensitivity was not different among the groups (Figure 7B). Interestingly, the magnitude of shift in myofilament Ca^{2+} sensitivity between the two sarcomere lengths (ΔpCa_{50}) is significantly reduced in the heart of DM-OVX rat. These results indicate a significant role of female sex hormones in maintaining the Starling mechanism of the heart even under diabetic condition.

DISCUSSION

The present study is the first to demonstrate a significant role of female sex hormones in modulating ventricular compliance especially under diabetic pathological condition. Female sex hormones partly prevented a shift towards titin compliant isoform and raised collagen content in order to restore heart stiffness induced by diabetes. Female sex hormones also helped to restore the physiological length-dependence on myofilament Ca^{2+} sensitivity altered in the heart under diabetic condition.

It is well accepted that altered myocardial stiffness has a significant functional impact on both diastolic and systolic activities of the heart (9). Theoretically, an altered myocardial distensibility influences ventricular filling velocity and thereby affects the end diastolic volume of the heart with consequent modulation of cardiac muscle contraction force. Based on the well-accepted cellular basis of Frank-Starling law of the heart (22), sarcomere length-dependent variation in myofilament response to Ca^{2+} alteration in myocardial passive stiffness could affect the active tension of contractile activity. This is supported by evidences showing differential regulatory effects of the two titin isoforms on sensing stretch and promoting actomyosin interaction through differences in myofilament Ca^{2+} sensitivity (10, 11). Sarcomere length dependence on Ca^{2+} sensitivity and maximum tension is more pronounced in tissues with high passive tension predominantly expressing N2B titin isoform. The increase in N2BA level in the heart of DM-OVX rat shown in this study indicates a reduction in the effectiveness of length-dependent Ca^{2+} sensitivity and therefore a reduction in active tension development. This implication is further supported by our results showing a reduced shift in myofilament Ca^{2+} sensitivity of tension development in response to change in sarcomere length in DM-OVX rat heart. Moreover, other groups measuring force contraction of skinned fiber preparation from

heart of diabetic male rat have reported decreases in both maximum tension and Ca^{2+} sensitivity (13, 17).

Interestingly, instead of having a low level of passive tension in DM rat heart in which N2BA was high, an increased myocardial stiffness was observed indicating that other factors other than titins are involved in determining myocardial stiffness. Of the two major components that determine compliance/stiffness of the heart, namely, intracellular titin and extracellular collagen contents, our results showed a significant increase in collagen deposition in DM rat heart suggesting that collagen plays a major role in modulating myocardial stiffness. The parallel results of changes in collagen content in diabetic rats, with and without female sex hormones, with that of myocardial passive stiffness demonstrated in the present study also indicate the significant role of female sex hormones in modulating the level of extracellular matrix collagen of the heart. Moreover, concomitant changes in Smad2 and procollagen contents with the increase in collagen deposition in DM rat heart obtained only with intact female sex hormones further suggest that the underlying mechanism of female sex hormones in modulating collagen content is, in part, via regulation of the biosynthesis process. In contrast to previous reports showing a preventive effect of female sex hormones on collagen expression in aging (32), hypertension (2, 3), and chronic angiotensin II infusion (20), differences in cardiac insults may underlie the various compensatory responses of the heart.

Our results suggest that deprivation of female sex hormones after menopause may enhance the pathophysiology of diabetes-induced dilatation of the heart, but it is not clear yet whether sex hormone-induced increased myocardial stiffness observed in the diabetic condition represents a beneficial or detrimental effect on the overall cardiac function. Although a shift of titin isoforms towards the fetal N2BA isoform is similar to those changes found in patients with

dilated cardiomyopathy and heart failure (4, 19), an increased myocardial stiffness in intact female sex hormone-DM rat could well be an adaptive response against the development of ventricular dilatation. The influence of circulating ovarian hormones on the pattern of myocardial remodeling has been reported in a study depicting marked ventricular dilation in OVX rat facing a chronic volume overload (5) in which estrogen supplement could improve reduced stiffness (12). A female-over-male preference in induction of concentric cardiac hypertrophy was also observed in response to hemodynamic overload after myocardial infarction (15). Moreover, adult men demonstrate more severe ventricular dilatation and left ventricular dysfunction than adult women in familial dilated cardiomyopathy arising from cardiac troponin T mutation (26). Thus, female sex hormones are likely to have a more beneficial rather than detrimental effect on adaptive responses of the heart to the pathophysiology of diabetes-induced cardiac dilatation.

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FIGRUE LEGENDS

Figure 1 Effect of ovariectomy and diabetes on myocardial stiffness of rat heart. Passive tension of right ventricular trabeculae and left ventricular papillary fibers were measured at various sarcomere lengths. Data are mean ± SE from 16-20 fibers of each group. *Significantly different ($P < 0.05$) from SHAM at the same sarcomere length using Student-Newman-Keuls test after ANOVA. SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

Figure 2 Effect of ovariectomy and diabetes on collagen deposition in rat heart. Top, cardiac section stained with Prico-Sirius red and viewed under light microscope (400x magnification); bottom, percent collagen content per tissue area. Data are mean ± SE from 400 pictures of four hearts in each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA. SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

Figure 3 Effect of ovariectomy and diabetes on titin isoforms in rat heart. *A*, silver stained bands of cardiac titin isoforms following 2% SDS-polyacrylamide gel-electrophoresis; *B*, ratio of N2BA to N2B titin isoform. Data are mean ± SE from four hearts in each group. *, † Significantly different ($P < 0.05$) from SHAM and DM group respectively, using Student-Newman-Keuls test after ANOVA. Neonate, cardiac sample from 2-day neonatal rat; SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

Figure 4 Effect of ovariectomy and diabetes on Samd2 protein level in rat heart. Top, immunoblot analysis of Smad2 (MW~60 kDa) and actin (MW~ 45 kDa) from left ventricular homogenate; bottom, ratio of Smad2 to actin. Data are mean ± SE from four hearts in each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA. SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

Figure 5 Effect of ovariectomy and diabetes on procollagen expression in rat heart. Top, immunoblot analysis of procollagen (procoll; MW ~60 kDa) and actin from left ventricular homogenate; bottom, ratio of procollagen to actin. Data are mean ± SE from 4-5 hearts in each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA. SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

Figure 6 Effect of ovariectomy and diabetes on MMP-2 protein expression in rat heart. Top, immunoblot analysis of latent (70 kDa) and active (65 kDa) components of MMP-2 from left ventricular homogenate; bottom, ratio of total MMP-2 to actin. Data are mean ± SE from six hearts in each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA. SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

Figure 7 Effect of ovariectomy and diabetes on length-dependent myofilament Ca^{2+} sensitivity in rat heart. pCa-tension relations of skinned papillary muscle from SHAM, OVX,

DM, and DM-OVX (*A*) at two sarcomere lengths of 2.0 and 2.3 μm and data summarizing magnitude of shifts in pCa_{50} (ΔpCa_{50}) between the two sarcomere lengths *(B)* from each group. Data are means ± SE from 9-12 preparations of 3-4 hearts in each group. *Significantly different from SHAM ($P < 0.05$) using Student-Newman-Keuls test after ANOVA. SHAM, sham operated; OVX, ovariectomized; DM, diabetic; DM-OVX, diabetic ovariectomized.

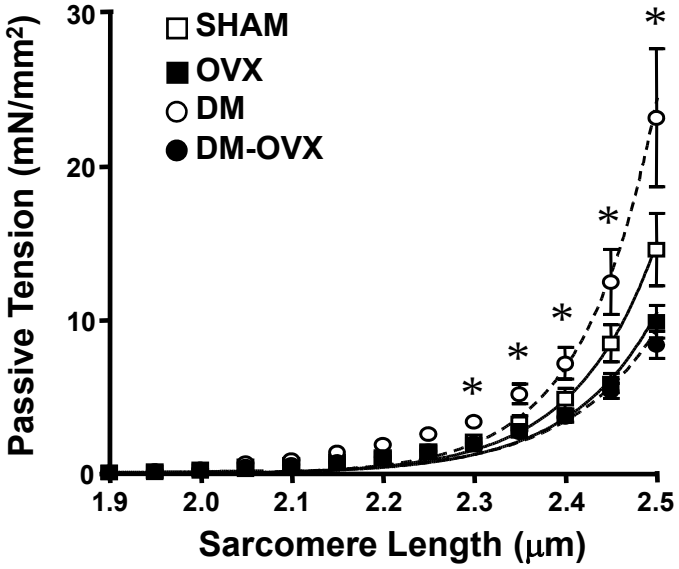
SHAM
OVX
DM
DM-OVX
Passive Tension (mN/mm²)
30
20
10
0
1.9
2.0
2.1
2.2
2.3
2.4
2.5
Sarcomere Length (μm)
*
*
*
*
*

Figure 1.

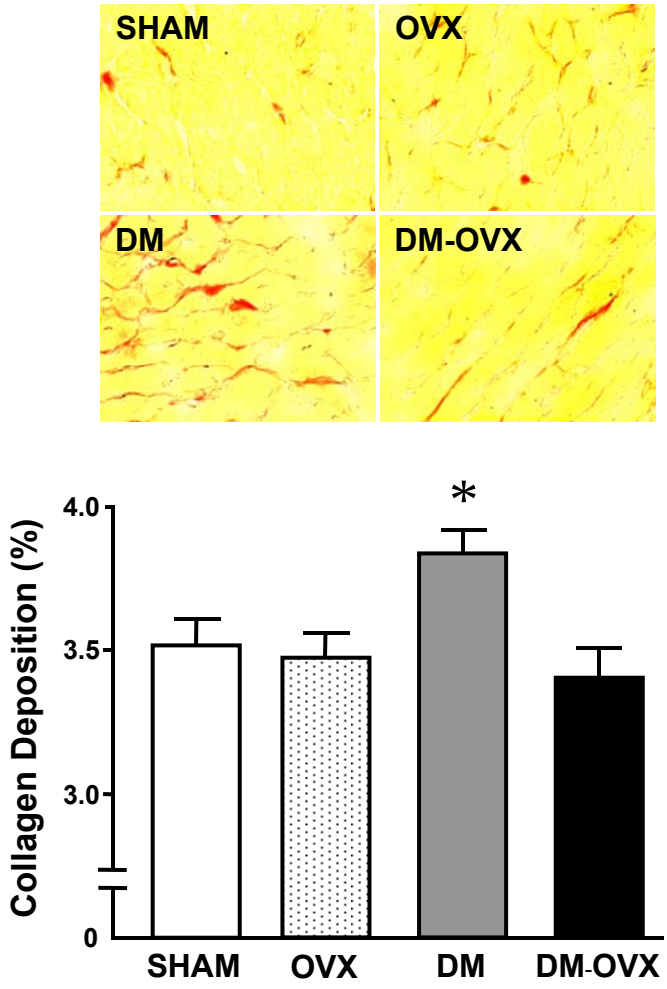
SHAM
OVX
DM
DM-OVX
Collagen Deposition (%)
4.0
3.5
3.0
0
*
SHAM
OVX
DM
DM-OVX

Figure 2.

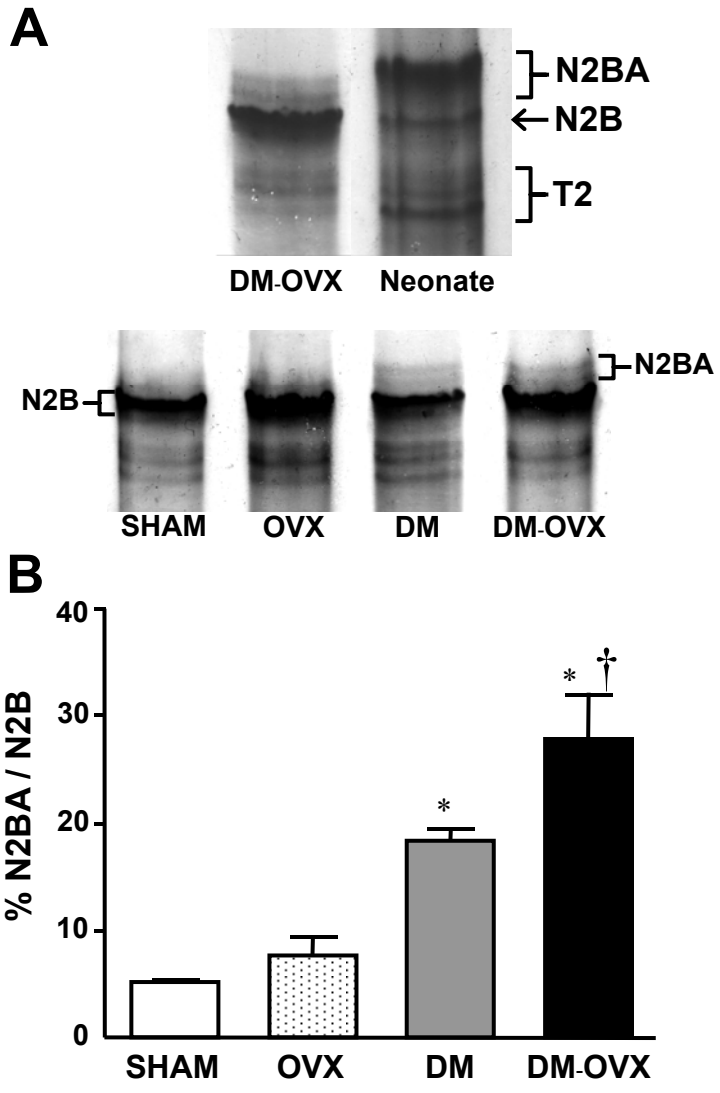
A
N2BA
N2B
T2
DM-OVX
Neonate
N2B
N2BA
SHAM
OVX
DM
DM-OVX
B
% N2BA / N2B
40
30
20
10
0
*
*
†
SHAM
OVX
DM
DM-OVX

Figure 3.

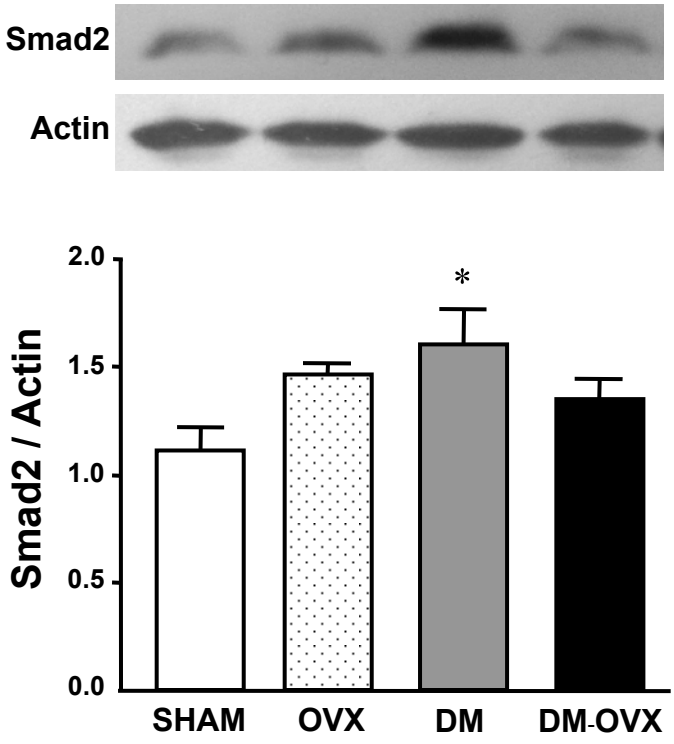

Figure 4.

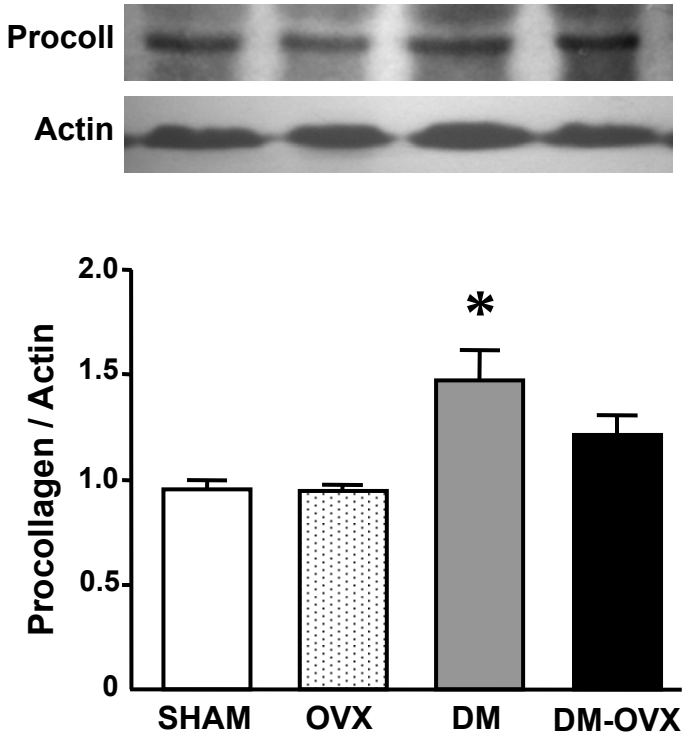
Procoll
Actin
Procollagen / Actin
2.0
1.5
1.0
0.5
0
*
SHAM
OVX
DM
DM-OVX

Figure 5.

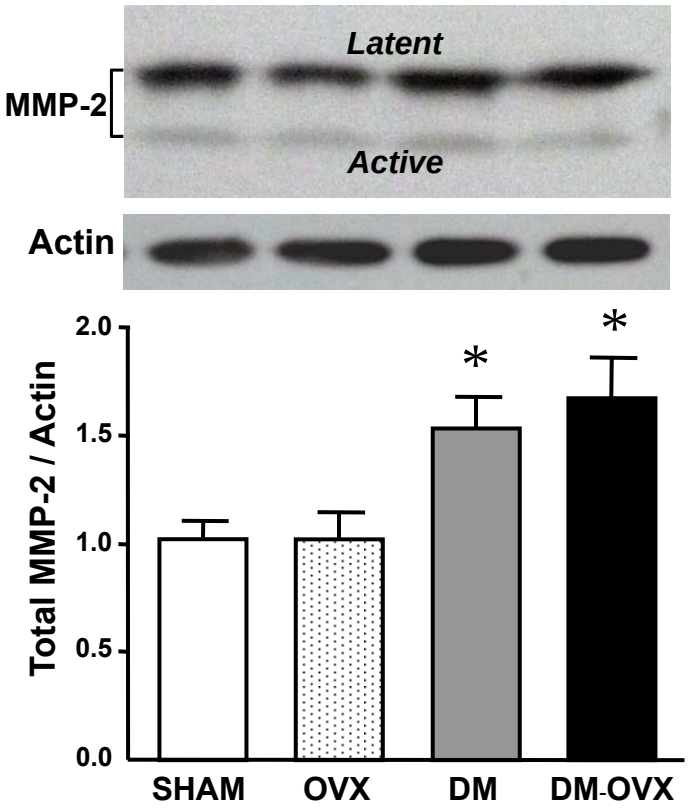
Latent
MMP-2
Active
Actin
Total MMP-2 / Actin
2.0
1.5
1.0
0.5
0.0
*
*
SHAM
OVX
DM
DM-OVX

Figure 6.

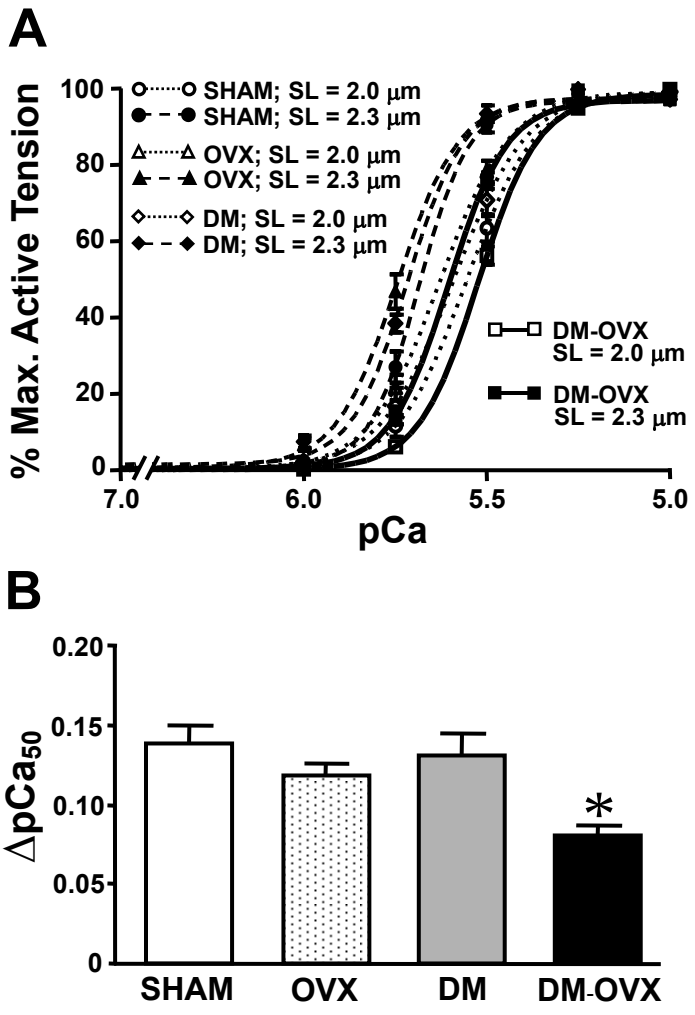
A
% Max. Active Tension
100
80
60
40
20
0
7.0
6.0
5.5
5.0
pCa
SHAM; SL = 2.0 μm
SHAM; SL = 2.3 μm
OVX; SL = 2.0 μm
OVX; SL = 2.3 μm
DM; SL = 2.0 μm
DM; SL = 2.3 μm
DM-OVX
SL = 2.0 μm
DM-OVX
SL = 2.3 μm
B
ΔpCa50
0.20
0.15
0.10
0.05
0
SHAM
OVX
DM
DM-OVX
*

Figure 7.