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EFFECTS OF TESTOSTERONE ON OBESITY-RELATED CARDIAC HYPERTROPHY AND FIBROSIS

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Both testosterone and obesity are known to increase renin-angiotensin system activity, leading to cardiovascular dysfunction. This study determined the interactive effects of obesity and testosterone on left ventricular hypertrophy and cardiac fibrotic factors. Male New Zealand White rabbits were fed a lean or 10% added fat diet. After 12 weeks, fat-fed rabbits exhibited increased left ventricular weight (6.05 ± 0.16 vs. 4.75 ± 0.10 g, respectively, $p \leq 0.05$) and cardiomyocyte cross-sectional area compared to lean rabbits (372.3 ± 19.0 vs. 305.0 ± 13.4 μm^2, respectively; $p \leq 0.01$). These effects were attenuated by both castration and treatment with the angiotensin type 1 receptor blocker, losartan. Obese rabbits did not exhibit increased myocardial collagen as expected. However, castration and losartan treatment increased matrix metalloproteinase-2 (MMP-2) activity in obese rabbits. Despite the effects of castration on hypertrophy and MMP-2 activity, castration did not attenuate plasma renin activity or aldosterone. These data suggest that testosterone contributes to obesity-related left ventricular hypertrophy and decreases collagen degradation, independent of renin activity.

EFFECTS OF TESTOSTERONE ON OBESITY-RELATED CARDIAC FIBROSIS

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CHAPTER I

INTRODUCTION

Scope of the Problem

Cardiovascular disease is the leading cause of death in the United States and internationally.[1] Although a great deal of research is devoted to this topic, many factors affecting its incidence remain to be fully understood. One such factor is obesity. Obesity is known to initiate a pro-inflammatory immune state, thus altering the body's ability to maintain homeostasis.[2] Obesity-related alterations in immune function, as well as in endocrine function, may contribute to negative cardiovascular sequelae, such as hypertension. Chronic hypertension in turn may play a role in the development of left ventricular hypertrophy (LVH) and remodeling, which then contribute to diastolic and/or systolic dysfunction.[3]

Gender also significantly impacts cardiovascular disease incidence. Men have a greater early risk of hypertension and mortality from heart disease than do women, and this effect has been attributed to testosterone.[4-6] The reasons for this are unclear. However activation of the renin-angiotensin system (RAS) is believed to play an important role.[7-9]

Given the high incidence of both obesity and cardiovascular disease in men, the effects of obesity and testosterone on cardiovascular structure and function are of interest.

For this reason, this study will examine the effects of obesity and testosterone on LVH and myocardial fibrosis.

Role of Fibrosis in Hypertensive Heart Failure

Reactive fibrosis is defined as an abnormal accumulation of collagen in a tissue.[10] Usually, this occurs in response to increased pressure or volume load in the cardiovascular system. Cardiac fibroblasts routinely synthesize and degrade collagen, but a change in their activity can change the rate of collagen turnover, resulting in either an accumulation or a deficit of collagen in the extracellular matrix (ECM).[11] These effects are mediated by changes in expression and proteolytic activity of matrix metalloproteinases (e.g., MMP-2 and MMP-9), which degrade collagen, and tissue inhibitors of matrix metalloproteinases (e.g., TIMP-1).[12]

Cardiac function depends upon the ECM as scaffolding upon which myocytes are organized.[13] This organization allows the myocardium to contract with the greatest efficiency. In pathological states, turnover of the ECM can become unbalanced, leading to an ill-formed scaffolding. This can cause myocyte slippage and overall remodeling of the left ventricle (LV), resulting in a dilated ventricle with reduced compliance.[14] In this state, the LV is less able to expand in response to an inflow of blood and subsequent increase in pressure, resulting in diastolic dysfunction.[15, 16] If the interstitial framework changes in such a way that myocytes are no longer organized and are unable to contract most efficiently, systolic dysfunction can also result.[14]

Chronic hypertension leads to a change in activity of cardiac fibroblasts in the heart. Hypertensive heart disease often begins with LVH and diastolic heart failure.[17] Lopez et al. and others have proposed that the initial response to chronic hypertension is increased myocyte size and collagen deposition.[18, 19] These changes most often result in diastolic dysfunction. However, as hypertension persists and cardiac function worsens an eventual decrease in interstitial collagen occurs such that the ventricle becomes dilated, and systolic heart failure with impaired ejection fraction may result.[18, 19] In this case, the heart suffers from both diastolic and systolic dysfunction.

Factors regulating the synthesis and degradation of collagen in the heart

Increased fibrosis can occur due to either an increase in collagen synthesis or a decrease in degradation. Figure 1 illustrates the relationship among the chief factors in these processes. An increase in collagen synthesis most often occurs when myocardial fibroblasts or myocytes are stimulated by angiotensin II (Ang II) or aldosterone (Aldo).[10, 11] Weber et al. demonstrated that these hormones, produced either locally or systemically, increased the activity of fibroblasts independent of their blood pressure effects.[10] Upon binding to its receptor on the cell, Ang II induces transforming growth factor beta-1 (TGF-β1).[20] TGF-β1 then activates its heteromeric type I and type II receptor kinases which in turn phosphorylate Smad 2 and 3. These proteins translocate to the nucleus where they activate the transcription of the genes for collagens type I and type III.[21] TGF-β1 may also foster fibrosis by inhibiting MMPs.[22] Decorin, a small

proteoglycan, inhibits these effects by binding to TGF- β1.[23] This prevents activation of the receptor kinases and halts the downstream signaling processes.

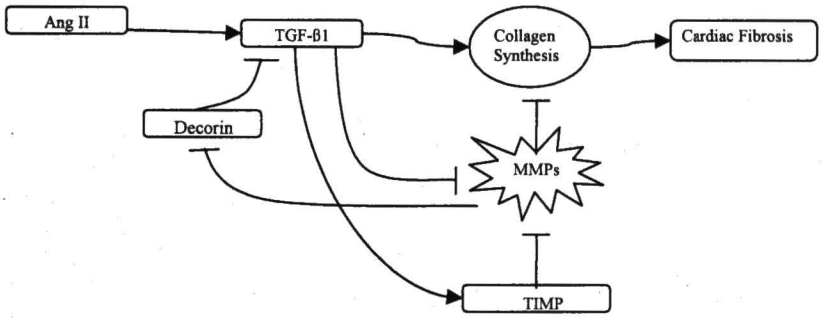

Figure 1: ***Regulation of cardiac fibrosis via Ang II.*** Ang II regulates cardiac fibrosis via TGF- β1 to directly stimulate collagen synthesis and indirectly inhibit its degradation. Ang II = Angiotensin II; TGF-β1 = Transforming growth factor β1; MMP = Matrix metalloproteinases; TIMP = Tissue inhibitor of matrix metalloproteinases.

Collagen accumulation is inhibited by MMP activity or by a reduction in TIMP activity. Conversely, fibrosis may be fostered by reduced synthesis or activity of MMPs, or increased synthesis or activity of TIMPs.[24] These are two of the most important factors in the overall regulation of ECM turnover and the development of cardiac fibrosis.

Two major classes of MMPs are collagenases and gelatinases. MMP-1 is a collagenase and hydrolyzes collagens I, II, and III, by denaturing their characteristic helical structure. The subsequent fragments form gelatin which is degraded by the gelatinases, MMP-2 and MMP-9. Gelatinases also hydrolyze elastin, and collagens IV, V, VII, and X, suggesting they have a critical role in both the degradation of basement

membrane and the collagen framework.[12] MMPs can degrade decorin as well, causing it to dissociate from TGF-β1.[25] This allows TGF-β1 to bind to its receptor kinases, as previously described, and to ultimately induce the expression of collagen. Thus, MMPs directly degrade collagen and also indirectly stimulate its synthesis. Under physiological conditions, MMPs play a vital role in regulating collagen turnover. TIMPs inhibit this process by binding directly to MMPs, inhibiting proteolytic activity.[26] Under pathological conditions, expression of MMPs and TIMPs can be altered to change the rate of ECM turnover and remodel the myocardial framework.

MMPs and TIMPs are regulated by many different mechanisms, although their regulation remains incompletely understood. Ang II is one of the many hormones regulating their expression.[22, 27] In a study by Stacy et al., Ang II decreased the activities of MMP-2 and -9 in mouse cardiac fibroblast cultures. Upon the addition of losartan (LOS), an Ang II type 1 receptor antagonist, to the media, gelatinase activity was restored in a dose-dependent manner. Treatment with an Ang II type 2 receptor antagonist however, gelatinase activity was inhibited and was only restored when the concentration of the Ang II type 2 receptor antagonist was decreased logarithmically.[28] Brilla et al. also observed a decrease in collagenase activity of cultured rat cardiac fibroblasts upon incubation with Ang II, but only type 2 receptor blockade successfully attenuated it.[29] Also, in an experiment by Varo et al., Ang II type 1 receptor blockade by LOS reduced TIMP-1 expression, which led to an increase in degradation of collagen type I.[27] These effects were independent of the anti-hypertensive effects of LOS. Other research has shown that TGF-β1 can inhibit MMP activity and stimulate TIMP activity.[22] Taken

together these studies suggest that Ang II is an important mediator of collagen synthesis via regulation of TGF-β1, MMPs, and TIMPs (Figure 1).

As mentioned previously, changes in protein expression and enzyme activity are different in the early and late stages of heart failure. However, many studies have reported different findings. One study demonstrated increased MMP-2 in the early phases of heart failure when the animals only exhibited compensated LVH. As the animals progressed into heart failure, MMP-2 activity returned to normal as it was counteracted by an increase in TIMP-2. Increases in collagen, however, were observed in both stages, possibly because Ang II- or Aldo-stimulated synthesis of collagen by TGF-β1.[30] On the other hand, another study demonstrated an activation of MMP-2 and increased transcription and expression of TIMP-2 and -4 only after the transition from LVH to heart failure in salt-sensitive rats.[19]

Yet another study examined the differences in MMPs and TIMPs in diastolic and systolic heart failure. Patients with diastolic heart failure exhibited proportionately greater increases in TIMP-1, while patients with systolic heart failure had proportionately greater MMP-1 activity. This resulted in an increase in interstitial collagen in the diastolic heart failure group while the systolic heart failure group had decreased interstitial collagen and increased perivascular collagen and scarring.[18] Early in hypertensive heart disease collagen deposition appeared to be increased by the activity of TIMPs causing a stiffening of the myocardium and diastolic heart failure. However as the disease continues, MMPs are activated in response to the increased interstitial collagen, leading to LV dilation, scarring, and systolic heart failure.[18] These and other

studies are summarized in Table 1. Differences between studies may be due to differences in species, disease processes, underlying genetic mutations, or duration of the disease. The regulation of fibrotic regulatory mechanisms during developing heart failure is extremely complex, and conflicting studies warrant further examination.

Table 1. ***Activity/expression of MMPs and TIMPs in the progression of heart failure.***

Citation	Species	Condition	MMP activity	TIMP expression
Iwanaga et al., 2002[19]	Dahl salt-sensitive rat	LVH	↔ MMP-2	↔ TIMP-2, 4
		CHF	↑ MMP-2	↑ TIMP-2, 4
Sakata et al., 2004[31]	Dahl salt-sensitive rat	LVH	↑ MMP-2	↑ TIMP-1, 2
		CHF	↑↑ MMP-2, 9	↑ TIMP-1, 2
Lopez et al., 2006[18]	Human	DHF	↑ MMP-1	↑↑ TIMP-1
		SHF	↑↑ MMP-1	↑ TIMP-1
Zile et al., 2005[15]	Human	LVH	↓ MMP-2, ↑ MMP-9	↔ TIMP-1, 2
		CHF	↔ MMP-2, ↑ MMP-9	↑ TIMP-1, ↔ TIMP-2
Tozzi et al., 2007[30]	Guinea pig	LVH	↑ MMP-2	↔ TIMP-2
		CHF	↔ MMP-2	↑ TIMP-2

LVH = left ventricular hypertrophy, CHF = chronic heart failure, DHF = diastolic heart failure, SHF = systolic heart failure, MMP = matrix metalloproteinases, and TIMP = tissue inhibitor of metalloproteinases.

Effects of obesity on cardiac fibrosis

Obesity is a major risk factor for heart failure due to its complex hemodynamic, immune, and endocrine effects. Obesity is associated with hypertension, insulin resistance, a pro-inflammatory immune state, an increase in RAS activity, and activation of the sympathetic nervous system.[32,33,34,35]

The increase in adiposity that characterizes obesity has a significant hemodynamic effect. Adipose tissue is highly metabolic and therefore requires increased vascularity and intravascular volume.[36,37] The heart adapts to the increased preload by increasing stroke volume and systolic pressure. When hypertension is present, afterload and wall stress are also increased. In an attempt to compensate for these changes, the left ventricle is remodeled, increasing wall thickness and/or chamber diameter (ventricular dilatation).[38, 39] Myocardial fibrosis is also associated with obesity.[2, 40] Increased collagen in the myocardium causes it to become stiff which creates a greater resistance to diastolic filling resulting in diastolic dysfunction. If the LV continues to dilate, circumferential fractional shortening declines, decreasing systolic function.[32] This results in systolic heart failure. When comparing lean and obese heart failure patients, obese patients demonstrate myocyte hypertrophy and increased heart weight, but LV weight proportional to body weight.

Adiposity has also been linked to LV dysfunction independent of hemodynamic effects. This is in large part due to changes in endocrine function and metabolism.[41, 32] Insulin resistance is strongly associated with obesity, and can contribute to cardiovascular pathology.[33, 42, 43] Increased visceral body fat produces a chronic inflammatory state.[2] Adipocytes release pro-inflammatory cytokines (e.g. TNF-α) which decrease responsiveness to insulin.[44, 45] Other adipose-derived cytokines, or adipokines, are resistin, leptin and adiponectin.[46] Resistin is increased in obesity and has been shown to impair insulin sensitivity and glucose tolerance in mice.[47] In contrast, leptin and adiponectin are known to increase insulin sensitivity.[46,48] However, adiponectin

decreases with increasing visceral fat mass, therefore reducing insulin sensitivity in obesity.[48] Leptin increases with increasing adiposity, but obesity is associated with a central and/or peripheral insensitivity to leptin.[49] Steinberg et al. observed that skeletal muscle strips from obese humans were less responsive to leptin administration than those from lean humans, exhibiting decreased stimulation of fatty acid oxidation.[46] When lipid metabolism is impaired in obesity, triglycerides accumulate in skeletal muscle and reduce muscle sensitivity to insulin.[50] When the body becomes less sensitive to insulin, glucose usage declines, and the myocardium becomes more dependent upon fatty acid oxidation. This causes an increase in myocardial oxygen consumption, a decrease in cardiac efficiency, and accumulation of toxic intermediates of fatty acid oxidation in the myocytes, resulting in myocyte contractile dysfunction.[51,52] Hyperinsulinemia also causes activation of the sympathetic nervous system and increases angiotensinogen production in the liver, leading to increased RAS activity and sensitivity.[53] This could exacerbate the effects of AngII on cardiac fibrosis in obesity.

Changes in circulating adipokines associated with obesity may also result in direct end-organ damage. In rat glomerular mesangial cells and neonatal rat cardiac myofibroblasts, leptin increased MMP-2 activity. However, leptin did not change in myocardial collagen volume.[54, 55] Fujita *et al.* utilized adiponectin knock-out mice to test the role of adiponectin in AngII-induced cardiac fibrosis. The knock-out exhibited significant increases in cardiac fibrosis when compared to wild type mice. This was reversed by adenovirus-mediated adiponectin treatment.[56] As obesity is associated with decreased adiponectin concentrations, this study suggests one possible mechanism by

which obesity can cause cardiac fibrosis. More research is needed to determine other direct effects of adipokines on cardiac fibrosis.

Of all of the endocrine abnormalities associated with obesity, increased RAS activity is considered the most important factor regulating cardiac fibrosis. Gene expression of all components of the RAS is upregulated in adipose tissue.[34] Obesity is also associated with an increase in sympathetic nervous system activity[35] which could further increase systemic RAS activity. Increased plasma Ang II concentration has deleterious effects on the myocardium independent of the effects on blood pressure. As stated before, Ang II induces TGF-β1, leading to increased collagen synthesis and subsequent fibrosis.[20] Ang II also exerts hypertrophic effects on cardiac myocytes independent of blood pressure. This was observed in a study by Mazzolai *et al.* in which transgenic mice overexpressing angiotensinogen in cardiomyocytes exhibited significant increases in heart weight and cardiomyocyte size while maintaining normal blood pressure. Blockade of the angiotensin type I receptor reversed these effects.[57] Interestingly, these mice did not exhibit increases in interstitial fibrosis.

In previous studies utilizing the obese (female) rabbit animal model, obesity was associated with hypertension and tachycardia, as well as cardiac eccentric and concentric hypertrophy.[58] When neurohumoral measurements were taken, plasma renin activity (PRA) was found to be 70% higher in obese animals.[59] Obese rabbits also demonstrated reduced diastolic compliance and filling compared to lean controls in isolated heart studies.[58, 60] This spurred another study examining the molecular aspects of cardiac fibrosis. Female obese rabbits exhibited significantly increased expression of collagen I

and III, as well as total interstitial and perivascular collagen. Compared to lean rabbits they also had increased TGF-β1 and reduced decorin.[61] In preliminary studies in male rabbits, obesity increased PRA 9-fold compared to lean males and 2-fold compared to obese females, suggesting that there may be gender differences in obesity-related heart disease (unpublished).

Effects of testosterone on cardiac fibrosis

Epidemiologic studies have shown that men have a greater risk for developing hypertension early in life and also developing other cardiovascular diseases such as coronary artery disease. Gender differences in blood pressure begin in puberty and persist throughout adulthood.[4] The increased risk or mortality from heart disease seen in men is attenuated by orchiectomy.[62] Also, anabolic steroids are associated with hypertension and ventricular hypertrophy.[63]

Many animal models have exhibited gender differences in disease as well. In a hypertensive rat model, male rats had greater blood pressure than females. Upon castration blood pressure was reduced. These effects were reversed by administration of testosterone.[9] Also, it is important to note that these effects were not dependent upon conversion of testosterone to dihydrotestosterone.[6] Male rats also excreted significantly less sodium and water than females under comparable renal perfusion pressures, causing a rightward shift of the pressure-natriuresis relationship.[64] This effect has been seen in humans as well.[65]

The chief factor contributing to these gender differences appears to be the RAS. Males have been shown to have higher PRA values than females in animal models and in humans.[7, 66] This difference was abolished by castration and restored by administration of exogenous testosterone. Increased PRA has also been shown to correlate positively with the dose of testosterone.[67] Male rats have increased angiotensinogen and renin mRNA, which is regulated by testosterone.[8, 9]

Testosterone is believed to directly stimulate RAS activity in the kidney.[68] This effect may be achieved in part by increasing sodium/hydrogen exchangers on the apical membrane of the proximal tubule.[68] This could lead do decreased sodium delivery to the macula densa, which would cause stimulation of the RAS. The significance of testosterone's stimulation of RAS was analyzed in spontaneously hypertensive rats (SHR). Ovariectomized female SHR supplemented with testosterone exhibited increased blood pressure, and this effect was blocked by administration of angiotensin converting enzyme inhibitors.[7] These results indicate that hypertension associated with testosterone is caused by increased RAS activity.

RAS activity contributes to cardiac fibrosis, but studies on the fibrotic effects of testosterone conflict. In a study by Seachrist et al., testosterone increased coronary adventitial collagen and myocardial collagen in SHR, independent of hemodynamic effects. Seachrist proposed that testosterone's effects on cardiac fibrosis were either through a mechanism involving Ang II or through a non-genomic increase in intracellular calcium levels.[69] Ramires et al. also suggested that RAS effector hormones modulated fibrous tissue formation by increasing intracellular calcium.[70] Both testosterone and Ang

II have been shown to increase phospholipase C and intracellular calcium concentration, so it is conceivable that these two hormones could have additive effects contributing to the development of hypertrophy and fibrosis.[71-73] Yet another study observed increases in cardiac hypertrophy and fibrosis in male mice lacking guanylyl cyclase-A, which is a crucial enzyme in natriuretic peptide signaling. This suggests that guanylyl cyclase-A signaling counteracts Ang II effects in the heart. This was confirmed by knocking out the Ang II type 1A receptor, which abolished gender differences in hypertrophy and fibrosis. The investigators suggested that testosterone exerts its effects chiefly by the RAS, acting at the Ang II type 1A receptor, but that these effects are offset by guanylyl cyclase-A signaling in normal mice.[74] Ellmers et al. suggested a mechanism for this effect, in which the natriuretic peptide receptors block Ang II-mediated calcium signaling, and subsequent transcription of pro-hypertrophic and pro-fibrotic genes.[73, 75]

In contrast to these results, Ikeda et al. observed protective effects of testosterone on the heart. This study investigated the relationship between androgens and Ang II-induced cardiac fibrosis by comparing androgen receptor knockout mice (ARKO) to wild type mice. When stimulated with Ang II, ARKO mice had higher collagen types I and III, and TGF-β1 mRNA expression than wild type mice, as well as significant impairment of left ventricular systolic function. This suggested that androgens and their receptors had a protective effect against Ang II-induced cardiac remodeling via down-regulation of TGF-β1 expression.[76] These data suggest that testosterone may attenuate obesity induced cardiac fibrosis.

In vitro studies have further examined the androgen receptor-mediated effects of testosterone on fibrosis. Cardiac myocytes express androgen receptors, and these receptors are known to mediate hypertrophic effects.[77] In one study, testosterone had no effect on MMP-2 gene and protein expression in aortic smooth muscle cells, but it decreased collagen content.[78] On the contrary, a different study showed increased collagen in cultured adventitial fibroblasts treated with testosterone.[79] Taken together, these studies suggest that testosterone has pro- and anti-fibrotic effects, mediated through different processes in different cell types. However, the majority of the currently available data point towards testosterone as a pro-fibrotic factor.

Rationale

As stated earlier, preliminary data showed a greater increase in RAS activity in obese male rabbits than in obese female rabbits versus their respective lean controls. Further, obese castrated male rabbits had significantly reduced PRA compared to obese intact males, but a significantly higher PRA than lean intact males (unpublished data). These data suggested that testosterone may play an important role in obesity-related cardiovascular disease.

Many studies have described the separate effects of obesity and testosterone in up-regulating the RAS.[7-9, 34, 59, 66] Ang II causes hypertrophy and fibrosis in cardiac myocytes[27, 28, 57, 80-82] by regulating the expression of pro- and anti-fibrotic factors, such as MMPs and TIMPs.[20, 28, 83, 84] How obesity and testosterone interact in the regulation of the RAS and the fibrotic factors it regulates remains unknown. Therefore, the present

study was designed to delineate the effects of testosterone and obesity in an *in vivo*, clinically-relevant model.

Significance

CV disease is the leading cause of death in the US.[1] Risk factors include age, sex, obesity, hypertension, high cholesterol, smoking, and diabetes.[85] The recent increase in obesity in the United States has contributed to the prevalence of heart disease through its influence on hypertension, inactivity, and insulin resistance/diabetes.[32] Men suffer disproportionately from heart disease in the US.[62] The factors that may contribute to the development of heart disease in men in the setting of obesity are important to examine. This project will help to elucidate mechanisms behind the greater prevalence of heart disease among men. Specifically, the effects of testosterone and obesity on hypertrophy and fibrosis of the LV will be examined.

Results from this study will also be significant in the realm of drug therapies. Aging men and women often take testosterone supplements to replace that naturally lost with age and to enhance muscle strength. However, the decline in testosterone experienced with increasing age has been hypothesized to have protective effects on cardiovascular and renal health.[86] Thus, such supplementation may have negative consequences. Illicit use of anabolic steroids to enhance athletic performance is currently the most common use of anabolic steroids.[63] Studies have associated steroid use with hypertension, ventricular remodeling, and myocardial infarction.[63] Thus, it is important

to delineate the mechanism by which testosterone elicits these effects, and factors such as obesity which might interact with them,.

Specific Aim

The overall goal of this study is to delineate the relationship between obesity and testosterone in producing cardiac hypertrophy and fibrosis. In order to achieve this goal, the following specific aim was developed:

Specific Aim I: To test the hypothesis that both obesity and testosterone increase left ventricular hypertrophy and cardiac fibrosis in male rabbits via RAS activation.

Subhypothesis 1: Obese male rabbits will exhibit the greatest cardiac hypertrophy and fibrosis due to the combined influence of obesity- and testosterone-induced RAS activation.

Subhypothesis 2: Obese castrated male rabbits will exhibit a partial reversal of cardiac hypertrophy and fibrosis due to the absence of testosterone and the subsequent partial down-regulation of the RAS.

Subhypothesis 3: Angiotensin II receptor blockade in castrated testosterone-supplemented male rabbits will result in full reversal of cardiac hypertrophy and fibrosis.

Figure 2 illustrates these concepts. Obesity will up-regulate RAS activity, thereby activating myocyte growth, increasing TIMP expression, and decreasing MMP expression (see Figure 1). The result will be increased fibrosis and LVH. Testosterone will also increase left ventricular hypertrophy and cardiac fibrosis by up-regulating RAS activity.

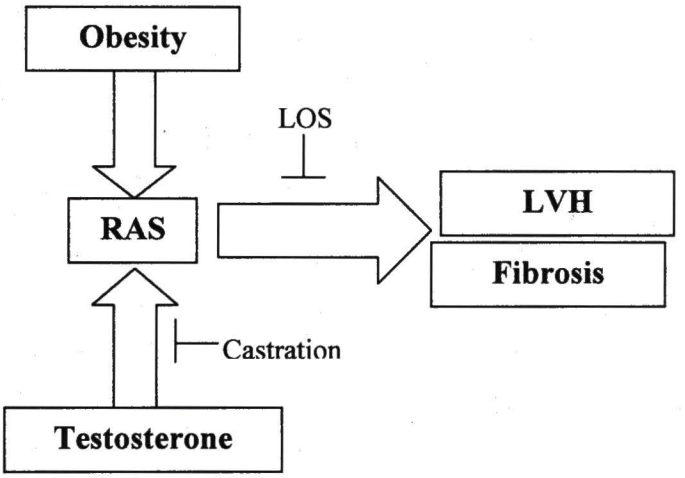

Figure 2: ***Proposed interactions between obesity, testosterone, and the renin angiotensin in the development of left ventricular hypertrophy and fibrosis.*** Obesity and testosterone increases left ventricular hypertrophy *and* cardiac fibrosis by up-regulating the RAS. RAS = Renin angiotensin system; LOS = Losartan; LVH = Left ventricular hypertrophy.

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CHAPTER II

EFFECTS OF TESTOSTERONE ON OBESITY-RELATED CARDIAC HYPERTROPHY AND FIBROSIS

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ABSTRACT

Both testosterone and obesity are known to increase renin-angiotensin system activity, leading to cardiovascular dysfunction. This study determined the interactive effects of obesity and testosterone on left ventricular hypertrophy and cardiac fibrotic factors. Male New Zealand White rabbits were fed either a maintenance diet or 10% added fat diet. After 12 weeks, rabbits fed the high fat diet exhibited resting tachycardia, systolic hypertension, and increased plasma renin activity (PRA). Castration reduced heart rate in these animals but not blood pressure or PRA. Obese rabbits also exhibited increased myocyte cross-sectional area compared to lean rabbits (372.3 ±19.0 vs. 305.0 ± 13.4 μm, respectively; $p \leq 0.01$). Hypertrophic effects were attenuated by both castration and treatment with the angiotensin II type 1 receptor antagonist, losartan. Obese rabbits did not exhibit increased myocardial collagen as expected, nor differences in expression of tissue inhibitors of metalloproteinases or activity of matrix metalloproteinase-2 (MMP-2). However, castration or losartan treatment increased MMP-2 activity only in obese rabbits. These data suggest that testosterone contributes to obesity-related left ventricular hypertrophy and decreases degradation of collagen independent of the renin-angiotensin system.

Keywords: fibrosis, hypertrophy, testosterone, obesity, metalloproteinases

INTRODUCTION

Cardiovascular disease is the leading cause of death in the US.[1] Risk factors include age, sex, hypertension, high cholesterol, smoking, diabetes, and obesity.[2] The current epidemic of obesity in the United States has contributed to the prevalence of heart disease through its influence on hypertension, inactivity, and insulin resistance/diabetes.[3] Men are at greater risk than women for developing hypertension and other cardiovascular disease such as coronary artery disease.[4-6] Therefore, it is important to examine factors such as gender and the attendant hormonal differences that may contribute to the development of heart disease in the setting of obesity.

Two characteristics associated with some forms of cardiovascular disease are left ventricular hypertrophy (LVH) and cardiac fibrosis. One of the most important factors contributing to the development of LVH and cardiac fibrosis is activation of the renin-angiotensin system (RAS).[7-12] Angiotensin II (Ang II), a product of the RAS, causes hypertrophy and fibrosis in cardiac myocytes.[9, 12-16] Ang II regulates the cardiac expression of pro- and anti-fibrotic factors, such as transforming growth factor-β1 (TGF-β1), decorin, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).[15, 17-19]

Many studies have shown that obesity is associated with increases in expression and activity of the RAS.[20-24] In obesity, gene expression of all components of the RAS is upregulated in adipose tissue.[23] The overexpression of angiotensinogen in adipose tissue has been associated with increased blood pressure.[25, 26] Obesity-related alterations in both insulin sensitivity and circulating adipokines may also contribute to changes in RAS

activity.[3, 27, 28] Finally, obesity is associated with an increase in sympathetic nervous system activity which could further increase systemic RAS activity.[29]

Testosterone is also thought to contribute to RAS activation. Males have been shown to have higher plasma renin activity (PRA) than females in both animals[30] and humans.[24] Further, animal studies demonstrated reduced PRA after castration which was restored by administration of testosterone. Testosterone has been associated with increased hepatic[21] and adipose tissue[31] angiotensinogen mRNA. Testosterone also increased, while castration reduced, RAS expression in rat epididymis[32] and renal angiotensinogen mRNA.[22] Preliminary data in obese male rabbits showed a greater increase in PRA than in obese females (unpublished data). In addition, castration in obese male rabbits reduced both PRA and left ventricular mass. This suggested that testosterone may play a role in obesity-related RAS activation and cardiovascular sequelae such as LVH.

Despite the greater incidence of cardiovascular diseases in men, little basic research has focused on the effect of gender on the relationship between obesity and cardiac structure or function. Therefore, the purpose of this study was to determine whether testosterone potentiated the effects of obesity on LVH and cardiac fibrosis. Further, because both obesity and testosterone have been shown to increase RAS activity, we hypothesized that Ang II type I receptor blockade would block these effects. In addition, we determined the influence of obesity and testosterone on selected pro- and anti-fibrotic factors responsible for cardiac fibrosis.

METHODS

Animals and surgical procedures:

Animal use was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center, and was carried out according to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 86-23, revised 1985) and provisions of the Animal Welfare Act. Eighty adult male New Zealand white rabbits (7-8 lbs, Myrtle's Rabbitry, Thompson Station, TN) were randomized to eight groups: lean (LM, n = 10), lean castrated (LCM, n = 10), lean castrated with testosterone supplemented (LCM/T, n = 10), lean castrated with testosterone supplemented and treated with losartan (LCM/T/LOS, n = 11), obese (OM, n = 9), obese castrated (OCM, n = 10), obese castrated with testosterone supplemented (OCM/T, n = 10), and obese castrated with testosterone supplemented and treated with losartan (OCM/T/LOS, n = 9).

Prior to starting the feeding protocol, rabbits in designated groups underwent surgical castration. Rabbits were sedated with ketamine/xylazine (8.0 mg/kg and 3.0 mg/kg, respectively) prior to induction of general anesthesia with 4-5% isoflurane with an oxygen flow of 0.6 L/min and administered with a facemask. Castration was performed using standard procedures. Rabbits recovered for 1 wk before starting the diet protocol. Rabbits assigned to receive exogenous testosterone had 6-8 testosterone-filled

silastic pellets implanted subcutaneously at the start of the feeding protocol, and every 3 wks afterward. Rabbits assigned to obese groups were fed an ad-lib high-fat diet (10% added fat; 2:1, corn oil: lard) while rabbits assigned to remain lean were fed 100-120 g/d of normal rabbit chow. Rabbits remained on assigned diets for 12 wks. Losartan (LOS; 25 mg/kg/d) was administered in drinking water to the appropriate groups throughout the 12-wk protocol.

Protocol Summary:

At the end of the 12-wk protocol, rabbits were placed in a Lucite restrainer, and the central ear artery was cannulated with a saline-filled PE-50 catheter. A blood pressure transducer was connected to the fluid filled catheter, and resting blood pressure was recorded for 45 minutes in a quiet room (ADInstruments, Colorado Springs, CO). Heart rate was calculated from pressure wave peaks. After blood pressure measurement, arterial blood was collected for measurements of PRA, aldosterone (Aldo), and testosterone. Samples were placed in appropriate chilled blood collection tubes and centrifuged at 1200 X g for 15 min at 4°C. Plasma was stored at -90°C until assay.

Rabbits were then anesthetized with 5% isoflurane with 0.6 L/min O_2 flow, administered with facemask, and placed in a right lateral position for echocardiography. Two-dimensional directional M-mode images were obtained using a Phillips HDI 5000 ultrasound instrument and a 5-12 MHz phased array transducer. Measurements were taken in a short axis plane at the level of the papillary muscle. Left ventricular internal chamber diameter (LVID), posterior wall thickness (PW), and septal thickness (IVS)

measurements were taken in systole (s) and diastole (d). Heart rate was calculated from M-mode tracings using identifiable landmarks in consecutive cardiac cycles. All parameters were measured as the average of three consecutive cardiac cycles. From these measurements ejection fraction and fractional shortening were calculated.[33]

After echocardiographic analyses were completed, an endotracheal tube was positioned in the trachea for subsequent mechanical ventilation. The heart was subjected to cardioplegic arrest as previously described,[34] then removed and weighed. Transverse sections were immersed in formalin for morphometric and collagen analysis. Remaining left ventricular tissue was frozen in liquid nitrogen and stored at -80°C.

Body Composition:

After sacrifice the carcass was emptied of blood and gastrointestinal contents and then shaved. The remaining carcass was homogenized (Robot Coupe R18, Jackson, MS) and 2-3 g samples were analyzed for fractional water and fat as described previously.[35] Values from quadruplicate samples were averaged for analysis.

Left Ventricular Morphometry and Collagen:

Left ventricular tissue sections were prepared from all animals as described previously.[36] Sections were stained with hematoxylin and eosin for morphometry and with Gomori's 1-step tri-chrome stain for collagen composition. Myofiber cross-sectional area was determined from hematoxylin-eosin-stained sections under 40X magnification. Approximately 20 - 30 cardiomyocytes per slide were measured.

Interstitial collagen volume fraction was measured under 40X objective as the percentage of green-stained tissue area per total myocardial area in each microscopic field.[36] A minimum of 10 randomly selected areas per slide were measured. In addition, at least 10 vessel and associated perivascular collagen cross-sectional areas per slide were measured as described above. Perivascular collagen area was normalized to vessel lumen area within each microscopic field. Analyses were performed using a Nikon TE2000 inverted microscope interfaced with a computer-analysis program (Image Pro-Plus, Version 5.0) and Adobe Photoshop CS3 with Fovea Pro 4.0 Plug-ins (Reindeer Graphics, Asheville, NC).

Hormone Analyses:

Plasma renin activity and Aldo were analyzed by radioimmunoassay as previously described.[37] After benzene and hexane extraction, testosterone concentrations were analyzed with a commercial radioimmunoassay kit (Siemens, Munich, Germany) according to manufacturer's specifications.

Preparation of tissue extracts for protein expression and activity analyses:

Frozen left ventricular tissue was pulverized in liquid nitrogen, homogenized, and extracted in phosphate buffered saline containing 0.5% Triton X-100, 0.01% sodium azide, and Halt™ protease inhibitor cocktail, EDTA-free (Pierce Biotechnology, Rockford, IL). The initial extraction mixture consisted of 100 mg of tissue per 200 μL of extraction buffer. After gently rocking for 18 hrs at 4°C, the samples were centrifuged at

10,000 x g for 10 minutes at 4°C, and the supernatants were collected and stored at -80°C. Protein concentration for each sample was determined by Lowry protein assay.[38]

Immunoblot analysis of TIMP-1:

Protein expression TIMP-1 was determined by immunoblot analysis, using modifications of a previously described procedure.[39] Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed under reducing conditions on a 12.5% Tris-HCl precast gel (BioRad, Hercules, CA). Proteins were transferred onto a nitrocellulose membrane, washed with a TBST solution (10 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween-20), and blocked for non-specific binding with TBST containing 5% nonfat dry milk and 3% bovine serum albumin overnight at 4°C. Next, the membrane was incubated with anti-TIMP-1 monoclonal mouse antibody for 1 hr (2 μL/mL, Calbiochem, La Jolla, CA, clone # 7-6C1). The membrane was washed three times for 5 min with TBST, and then incubated for 1 hr in goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (1:5000, Calbiochem, cat # DC02L). The membrane was then washed with TBST four times for 10 min, and developed using the enhanced chemiluminescence method as per the manufacturer's instructions (SuperSignal® West Pico Chemiluminescent Substrate, Pierce Biotechnology). Bands were visualized using a Fluorchem Imaging System (Alpha Innotech, San Leandro, CA), and quantitated using Image J software (NIH). For normalization, blots were reprobed for actin (1:5000, Calbiochem, cat # CP01).

Zymography analysis of MMP-2 and MMP-9 activity:

MMP enzyme activity was determined using SDS-PAGE zymography. Electrophoresis of 60 μg of left ventricular protein was performed on 10% gelatin-containing Tris-HCl gels (BioRad, Hercules, CA). After electrophoresis, gels were placed in a renaturing solution (2.5% Triton X-100) for 30 min at room temperature. Next, they were incubated with development buffer (BioRad, Hercules, CA) for 30 min at room temperature. Fresh buffer was then added and the gel was incubated overnight at 37°C. Gels were stained with a 40% methanol, 10% acetic acid and 0.5% Coomassie Blue R-250 solution for 30 min at room temperature, and destained with a 40% methanol and 10% acetic acid solution for 2 hrs. Standards for the active forms of recombinant human MMP-2 and MMP-9 were used as positive controls (Calbiochem, La Jolla, CA). Activity of the respective enzymes was observed as white bands against a blue background. Relative clearing of the blue-stained gelatin was quantitated using the gel analysis method on Image J software (NIH).

Statistical analyses:

Initially, unpaired t-tests were used to compare LM to LCM/T and OM to OCM/T. If no significant differences were observed between LM and LCM/T or between OM and OCM/T, the groups were combined for further analyses and referred to as LM and OM, respectively. To determine effects of obesity, unpaired t-tests were then used to compare LM and OM. A one-way analysis of variance (ANOVA) was used to detect differences among lean groups as well as differences among obese groups. To

analyze the overall effects of LOS, a 2X2 ANOVA with interaction was used. Main effects were diet (lean vs. obese) and LOS status (treated versus untreated). Castrated groups were excluded from these analyses. A 2X2 ANOVA with interaction was also used to determine the overall effect of testosterone, with the main effects being diet (lean vs. obese) and testosterone status (intact/testosterone supplemented vs. castrated). Losartan-treated groups were excluded from these analyses. When interaction effects were not significant, analyses were repeated using main effects only. All data are expressed as mean ± SE. Results were accepted as significant when $p \leq 0.05$.

RESULTS

Body Composition (Table 1):

After 12 wks of a high fat diet, OM were 29% heavier than LM and had a significantly higher percentage of body fat. Neither castration nor LOS treatment affected overall body weight. However, castration significantly increased body fat percentage in OCM compared with OM ($p \leq 0.01$).

Hemodynamics (Table 1):

OM exhibited significantly higher heart rate compared to LM ($p \leq 0.05$). Systolic arterial pressure (AP) was also significantly higher in OM compared to LM ($p \leq 0.05$), but mean AP and diastolic AP were not significantly different. This suggests that obesity in male rabbits resulted in an isolated systolic hypertension.

OCM exhibited significantly lower HR in comparison to OM ($p \leq 0.005$). LCM, on the other hand, exhibited no significant differences in heart rate compared to LM. Castration did not significantly alter mean, systolic or diastolic APs in lean or obese groups.

As expected, both lean and obese rabbits treated with LOS had significantly lower mean AP and diastolic AP compared with respective controls (all $p \leq 0.05$). In addition, obese rabbits treated with LOS had significantly lower systolic AP ($p \leq 0.005$).

Hormone Concentration:

As expected, castrated rabbits had significantly reduced plasma testosterone concentrations ($p \leq 0.05$; Table 1). Testosterone supplementation was effective in restoring normal testosterone concentrations. The combined OM group had 55% higher testosterone concentrations than LM (1.59 ± 0.32 vs. 1.03 ± 0.21 ng/mL, respectively), however this difference was not significant due the large variability associated with testosterone concentrations.

PRA was almost 4-fold greater in OM than LM (14.05 ±1.96 vs 3.59 ± 0.81 ng AngI/mL/hr, respectively), confirming the significant effects of obesity on the RAS (Figure 1a). Castrated groups did not have significantly different PRA than their respective controls. LOS treatment significantly increased PRA in lean rabbits as a feedback mechanism in response to blockade of Ang II effects (15.99 ± 1.75 vs. 3.59 ± 0.81 ng AngI/mL/hr, respectively; $p \leq 0.05$). This effect was not observed in obese rabbits.

OM also had significantly higher Aldo compared to LM (881 ± 200 vs. 278 ± 52 pmol/mL, respectively; $p \leq 0.05$; Figure 1b). Unexpectedly, Aldo was significantly higher in LCM/T compared to LM, but was not significantly different between OM and OCM/T. 2X2 ANOVA results revealed that Aldo tended to be higher in castrated groups ($p = 0.08$) independent of body weight ($p = 0.08$). 2X2 ANOVA results also indicated a trend for LOS treatment to reduce Aldo concentrations in obese rabbits, but not in lean rabbits (interaction, $p = 0.07$).

When Aldo concentrations were normalized to PRA, LM and OM were not significantly different (Figure 1c). LCM/T had significantly higher ratios compared with

LM ($p \leq 0.05$). LOS also significantly decreased the Aldo/PRA ratio when analyzed by 2X2 ANOVA ($p \leq 0.01$).

Structural Measurements:

Overall heart weight, LV weight, and RV weight were all significantly greater in OM compared to LM (all $p \leq 0.05$; Table 2). Castration significantly decreased LV weight and total heart weight in both lean and obese rabbits (all $p \leq 0.05$). Castration also reduced RV weights in lean rabbits ($p \leq 0.05$). 2X2 ANOVA results also indicated that LOS treatment tended to reduce LV weights ($p = 0.10$) independent of body weight.

Myocyte cross-sectional area was significantly greater in OM than LM, indicating myocyte hypertrophy (372.3 ± 19.0 vs. 305.0 ± 13.4 μm, respectively; $p \leq 0.01$; Figure 2). While castration reduced myocyte cross sectional area by 10.3% in lean rabbits and 11.9% in obese rabbits, 2X2 ANOVA results revealed only a trend for castration to reduce myocyte size ($p = 0.08$). LOS decreased myocyte cross-sectional area by 8.2% in lean rabbits (p = NS) and by 23.9% in obese rabbits ($p = 0.06$). 2 X 2 ANOVA results indicated that LOS had significant effects independent of body weight ($p \leq 0.05$),

Echocardiography:

There were no significant differences amongst groups in most of the echocardiographic measurements, including LV diameters and posterior wall thicknesses (Table 2). However, OM had significantly thicker IVSd than LM ($p \leq 0.01$). The difference was disproportionately greater than the differences in left ventricular PWd. This resulted in a significantly greater IVSd/PWd, suggestive of asymmetric hypertrophy.

Cardiac Fibrosis:

There were no significant differences in interstitial (Figure 3a) or perivascular collagen (Figure 3b) between LM and OM. Castration had no effect on either of these measurements. Unexpectedly, LOS treatment tended to increase interstitial collagen in obese groups ($p = 0.09$).

TIMP-1 protein expression was not significantly different between LM and OM (Figure 4a). Castration reduced TIMP-1 expression by 19.9% in lean and 34.4% in fat rabbits, and LOS reduced TIMP-1 expression by 59.1% in lean and 24.3% in obese rabbits. However, because of a great amount of variation between samples and small sample sizes there were no significant effects of castration or LOS on TIMP-1 expression

Zymography revealed one band of gelatinolytic activity corresponding to the active form of MMP-2 (Figure 4b). Enzymatic activity of MMP-9, however, was too low to be detected in these samples. MMP-2 activity was not significantly different between LM and OM (Figure 4c). There were no changes due to castration or LOS treatment in the lean rabbits. However, OCM and OCM/T/LOS both had significantly greater MMP-2 activity than OM (both $p \leq 0.01$).

DISCUSSION

The hypothesis of the study was that testosterone potentiated the hypertrophic and pro-fibrotic effects of obesity, acting through RAS activation to further augment LVH and cardiac fibrosis. As a result, it was hypothesized that castration would partially reverse these effects by attenuating RAS activation, while blockade of Ang II's effects at the end organ would fully reverse them by blocking both obesity-related and testosterone-related RAS activation. Obesity in male rabbits resulted in expected changes in hemodynamics, RAS activation, ventricular hypertrophy, and increased myocyte cross-sectional area. However, obesity did not result in increased cardiac collagen, TIMP-1 protein expression, or ventricular MMP-2 activity. Castration in male rabbits did not reduce PRA as expected, but reduced ventricular weights and tended to reduce myocyte cross-sectional area. Castration also significantly increased MMP-2 activity in obese rabbits and caused proportionally large decreases in TIMP-1 expression in both lean and obese rabbits. This suggests that while testosterone did not affect overall LV collagen content, it impacted regulatory factors in a direction favoring decreased collagen degradation. On the other hand, LOS treatment of castrated, testosterone-supplemented animals reduced blood pressure and myocyte hypertrophy but had no effect on ventricular collagen volume. LOS treatment increased MMP-2 activity in obese rabbits, and decreased TIMP-1 expression in lean and obese rabbits. However, the extent of change in obese rabbits due to LOS was not different than the change due to castration. Taken together, these results suggest that although testosterone augments cardiac hypertrophy and decreases collagen degradation, it does not mediate these effects via the RAS.

Effects of testosterone and obesity

We reported previously that obesity in female rabbits resulted in an increase in HR and mean AP.[40] The present results in male rabbits also show obesity-related resting tachycardia. However, only systolic AP was increased in the obese group suggesting that obese male rabbits exhibit an isolated systolic hypertension in response to a high fat diet.

Castration in obese rabbits lowered HR, but not blood pressure. This is in contrast to several animal and human studies concerning the effects of testosterone on hypertension.[5, 30, 41-43] However, most of the animal studies demonstrating reduction of blood pressure after castration were performed in spontaneously hypertensive rats.[30, 41, 42, 44] Reasons for these discrepancies may include species differences, differences in underlying disease processes, and/or genetic vs. non-genetic models of disease. In addition, the length of the feeding protocol and/or the relatively mild degree of hypertension in this model may not have been sufficient to differentiate effects of testosterone.

RAS activity was increased in all obese groups, as indicated by increased PRA and Aldo. However, the increases in PRA seen here in obese male rabbits are proportionally greater than the increases seen previously in obese female rabbits. In previous studies, PRA in obese female rabbits averaged between ~4-7 ng of Angiotensin I/ml/hr.[20, 37, 45] However, obese male rabbits in the present study had PRA values that averaged ~14 ng of Angiotensin I/ml/hr. If this greater increment were due solely to testosterone, castration would be expected to reverse it. In the current study, castration

did not attenuate PRA either in lean or obese rabbits. This suggests that factors other than testosterone exert greater control on the RAS in obesity. However, because these animals were mature when castrated, and still had very small testosterone concentrations, heightened end organ sensitivity to testosterone cannot be ruled out.

Obese rabbits exhibited increased myocyte cross-sectional area compared to lean rabbits. Obese rabbits also exhibited increased LV, RV, and total heart weights. This is most likely due to the hypertrophic influence of Ang II[9] as suggested by increased PRA, or of Aldo. Castration attenuated the hypertrophic effects, despite its lack of effect on PRA. LV weight, total heart weight, and LV myocyte cross-sectional area were all attenuated by castration in obese rabbits, suggesting that the LV is especially affected by testosterone in the face of obesity. Testosterone is known to mediate hypertrophy via its androgen receptor in cardiac myocytes.[46] Thus it is likely that the reduction in testosterone concentrations, and not testosterone's influence on the RAS, was responsible for the decrease in myocyte size and overall ventricular and heart weights.

Also, while LV chamber diameter and posterior wall thickness were increased in a previous study in female rabbits,[47] only the interventricular septum thickness was increased in the present study. This change was disproportionate to changes in the posterior wall, suggesting asymmetrical ventricular hypertrophy. The significance of this finding is not clear, and further studies are needed to confirm it. However, it may represent a gender difference in the adaptation to weight gain in this model.

The effects of obesity and testosterone on fibrosis were unexpected. Unlike previous studies in female rabbits,[48] obese male rabbits did not exhibit increased

interstitial or perivascular collagen volume compared to lean rabbits. This may represent a gender difference in the cardiac adaptation to obesity. Furthermore, castration significantly increased MMP-2 activity in obese animals, allowing for increased collagen degradation. This could be due in part to decreases in inhibition of MMP-2 activity by TIMP-1, as TIMP-1 expression was somewhat reduced in castrated animals. It can postulated that LV collagen volume did not change in castrated groups because either collagen synthesis was increased to counteract degradation, or the effects of increased degradation were not substantial enough to be measured histologically. Very few studies have examined the effects of testosterone on MMP activity in the heart. One study showed no change in gene or protein expression of MMPs upon treatment with testosterone.[49] Other studies have shown that testosterone increases cardiac fibrosis via the RAS[10, 41, 50], leading to increased intracellular TGF-β1[17] and inhibition of MMP activity.[51] The present results also indicated that testosterone inhibits MMP-2 activity, but that this occurred independently of the RAS.

Effects of RAS blockade.

We hypothesized that treatment with losartan, an angiotensin II type 1 receptor antagonist, would block both obesity-related and testosterone-related RAS influences on cardiac hypertrophy and fibrosis. LOS treatment reduced blood pressure in both lean and obese animals by 8-9 mmHg. LOS treatment also resulted in increased PRA in lean

animals, most likely due to a feedback mechanism in response to lowered blood pressure. However, this response did not occur in obese animals. This could be because PRA has reached a maximum in the obese animals, and cannot be stimulated to increase further. A loss of feedback control in the obese animals is an alternate explanation.

LOS treatment tended to reduce LV weight ($p = 0.10$) and significantly reduced myocyte cross sectional area ($p = 0.05$) independent of body weight. Myoctye cross sectional area in LOS-treated obese animals was 24% lower than in obese controls and was similar to values seen in lean controls. These data suggested that RAS activation was the major contributor to cardiac hypertrophy seen in obese male rabbits.

Perivascular collagen was not altered by LOS, however LOS treatment tended to increase interstitial collagen in obese rabbits. This contrasts with findings of many other investigators in which RAS blockade prevented cardiac fibrosis.[8, 16, 52] On the other hand, OCM/T/LOS had significantly increased MMP-2 activity. An increase in MMP-2 activity during LOS treatment is consistent with work from other laboratories.[15] This suggests that collagen synthesis must have increased to a greater extent than did MMP-2 activity in order for LOS to increase total interstitial collagen.

Effects of castration on hemodynamics and hormone concentrations.

Unexpectedly, LCM/T exhibited greater Aldo concentrations than LM, and OCM/T exhibited lower heart rate and systolic pressure than OM. This suggests that orchiectomy may remove testicular influences not restored by testosterone replacement alone. Possible candidates include other testicular hormones such as dihydrotestosterone (DHT),

estradiol, and inhibin. Though extra-testicular tissues routinely convert testosterone into both estradiol and DHT, the loss of the testicular contributions may have subtle and yet unappreciated effects. Likewise, both follicle-stimulating hormone (FSH) and leutinizing hormone (LH) rise dramatically after castration, but testosterone replacement preferentially suppresses LH. In contrast, testicular inhibin preferentially regulates pituitary FSH.[53] Despite testosterone replacement, the continued absence of inhibin after castration would presumably change the steroid feedback dynamics at the pituitary and increase the secretion of FSH relative to LH. The data also do not rule out a missing non-humoral testicular influence. Thus, the LCM/T and OCM/T groups are hemodynamically and hormonally different from their respective controls. They should have less inhibin, more FSH and perhaps subtle differences in the mix of circulating DHT and estradiol. How these hormonal changes affect Aldo or hemodynamics is unclear. Inhibin production has been identified in adrenocortical tissue[54], but its effects on Aldo production have not been reported. Resolving these complex effects of castration in the current rabbit model will clearly require additional studies.

PERSPECTIVES

The results of this study support the hypothesis that testosterone contributes to obesity-related cardiac hypertrophy. In contrast to the hypothesis, testosterone did not appear to exert these effects via activation of the RAS. Testosterone and Ang II, in the presence of obesity, appear to decrease the degradation of collagen, but not enough to increase total measurable LV collagen content. This could be due to a decrease in collagen synthesis, however factors regulating collagen synthesis were not measured.

Further study must be done to elucidate all of the mechanisms involved in the relationship between testosterone, obesity and cardiac fibrosis. Specifically, the rate of formation of collagen needs to be measured to identify the chief factors by which testosterone and Ang II exert their fibrotic effects. It would also be of interest to develop a more specific time frame for when hypertension begins to result in increased transcription of fibrotic factors and increased collagen deposition.

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FIGURE LEGENDS:

Figure 1. A. Plasma renin activity (PRA). B. Aldosterone. C. Aldosterone/PRA ratio. LM = lean, LCM/T = lean castrated supplemented with testosterone, LCM = lean castrated, LCM/T/LOS = lean castrated supplemented with testosterone and losartan-treated, OM = obese, OCM/T = obese castrated supplemented with testosterone, OCM = obese castrated, OCM/T/LOS = obese castrated supplemented with testosterone and losartan-treated. * $p \leq 0.05$ vs. LM, unpaired t-test. † $p \leq 0.05$ vs. LM, one-way ANOVA. ‡ $p \leq 0.01$, vs. untreated groups, 2X2 ANOVA. Numbers in parentheses indicate sample sizes.

Figure 2. Myocyte cross-section area. LM = combined group: lean + lean castrated supplemented with testosterone, LCM = lean castrated, LCM/T/LOS = lean castrated supplemented with testosterone and losartan-treated, OM = combined group: obese + obese castrated supplemented with testosterone, OCM = obese castrated, OCM/T/LOS = obese castrated supplemented with testosterone and losartan-treated. * $p \leq 0.05$ vs. LM, unpaired t-test. † $p \leq 0.05$ vs. untreated groups, 2X2 ANOVA. Numbers in parentheses indicate sample sizes.

Figure 3. A. Interstitial percent collagen volume. B. Perivascular collagen (percent volume collagen/percent volume vessel area). LM = combined group: lean + lean castrated supplemented with testosterone, LCM = lean castrated,

LCM/T/LOS = lean castrated supplemented with testosterone and losartan-treated, OM = combined group: obese + obese castrated supplemented with testosterone, OCM = obese castrated, OCM/T/LOS = obese castrated supplemented with testosterone and losartan-treated. Numbers in parentheses indicate sample sizes.

Figure 4. A. TIMP-1 protein expression. B. Representative zymogram of MMP-2 activity. Lane 1 = recombinant MMP-2, Lanes 2 & 3 = LM, Lanes 4 & 5 = LCM/T, Lanes 6 & 7 = LCM, Lanes 8 & 9 = LCM/T/LOS, Lanes 10 & 11 = OM, Lanes 12 & 13 = OCM/T, Lanes 14 & 15 = OCM, Lanes 16 & 17 = OCM/T/LOS C. Average group values of left ventricular MMP-2 activity. LM = combined group: lean + lean castrated supplemented with testosterone, LCM = lean castrated, LCM/T/LOS = lean castrated supplemented with testosterone and losartan-treated, OM = combined group: obese + obese castrated supplemented with testosterone, OCM = obese castrated, OCM/T/LOS = obese castrated supplemented with testosterone and losartan-treated. * $p \leq 0.01$ vs. OM, one-way ANOVA. Numbers in parentheses indicate sample sizes.

Table 1: Group Characteristics

	LM (n=20)	LCM (n=10)	LCM/T/LOS (n=11)	OM (n=9)	OCM/T (n=10)	OCM (n=10)	OCM/T/LOS (n=9)
Body Weight (kg)	3.8 ± 0.1	3.7 ± 0.0	3.8 ± 0.0	4.9 ± 0.2*	5.1 ± 0.2	5.1 ± 0.2	4.8 ± 0.2
Body Fat (%)	11.3 ± 2.7	12.6 ± 1.3	15.5 ± 2.2	28.0 ± 1.4*	33.0 ± .2	36.2 ±2.9**	29.5 ± 2.2
Testosterone (ng/mL)	0.74 ± 0.27	0.09 ± 0.02‡	1.08 ± 0.14	1.86 ± 0.60	1.34 ± 0.21	0.10 ± 0.04**	1.76 ± 0.18
Heart Rate (beats/min)	168 ± 7	163 ± 5	185 ± 8	245 ± 10*	205 ± 7†	211 ± 6**	243 ± 13
Mean AP (mmHg)	83 ± 2	81 ± 2	75 ± 2‡	89 ± 4	86 ± 1	92 ± 1	77 ± 2**
Systolic AP (mmHg)	102 ± 2	98 ± 3	94 ± 3	117 ± 4*	105 ± 3†	116 ± 3	100 ± 3**
Diastolic AP (mmHg)	75 ± 2	72 ± 2	66 ± 2‡	77 ± 3	76 ± 2	81 ± 1	65 ± 2**

Data are mean ± standard error. * $p \leq 0.05$ vs. LM, unpaired t-test †$p \leq 0.05$ vs. OM, unpaired t-test ‡$p \leq 0.05$ vs. LM, one-way ANOVA **$p \leq 0.005$ vs. OM, one-way ANOVA. AP = arterial pressure, LM = combined group: lean + lean castrated supplemented with testosterone, LCM = lean castrated, LCM/T/LOS = lean castrated supplemented with testosterone and losartan-treated, OM = obese, OCT/T = obese castrated supplemented with testosterone, OCM = obese castrated, OCM/T/LOS = obese castrated supplemented with testosterone and losartan-treated.

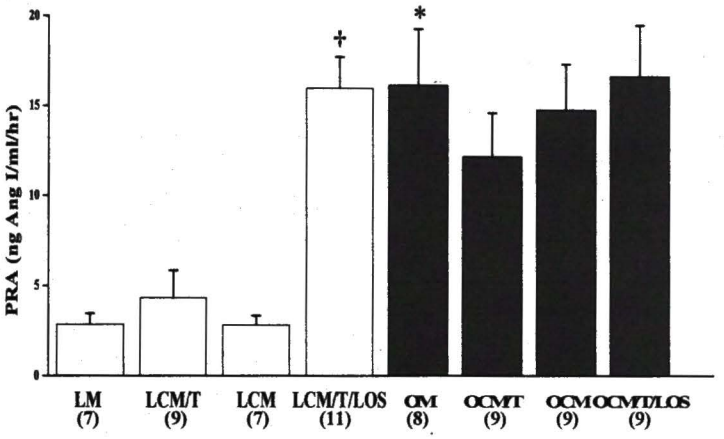
Table 2: Cardiac Structure and Function

	LM (n=20)	LCM (n=10)	LCM/T/LOS (n=11)	OM (n=19)	OCM (n=10)	OCM/T/LOS (n=9)
Heart Weight (g)	7.57 ± 0.14	6.62 ± 0.19†	7.26 ± 0.17	9.35 ± 0.24*	8.25 ± 0.39†	8.51 ± 0.35
LV Weight (g)	4.75 ± 0.10	4.26 ± 0.14†	4.56 ± 0.12	6.05 ± 0.16*	5.31 ± 0.26†	5.52 ± 0.23
RV Weight (g)	1.72 ± 0.04	1.40 ± 0.08†	1.72 ± 0.06	2.05 ± 0.06*	1.94 ± 0.11	1.93 ± 0.10
LV IDd (mm)	15.7 ± 0.5	14.6 ± 0.4	16.0 ± 0.9	14.8 ± 0.6	15.9 ± 0.9	15.1 ± 0.8
LV IDs (mm)	10.6 ± 0.6	9.9 ± 0.4	11.1 ± 0.7	10.4 ± 0.6	11.8 ± 0.9	10.4 ± 0.7
LV PWd (mm)	2.3 ± 0.1	2.6 ± 0.2	2.3 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.5 ± 0.2
LV PWs (mm)	3.3 ± 0.1	3.4 ± 0.3	3.4 ± 0.4	3.5 ± 0.2	3.4 ± 0.2	3.4 ± 0.1
IVSd (mm)	2.2 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	2.8 ± 0.1*	2.5 ± 0.2	2.7 ± 0.3
IVSs (mm)	3.4 ± 0.1	3.1 ± 0.2	3.2 ± 0.3	3.7 ± 0.2	3.5 ± 0.2	3.4 ± 0.2
IVSd/PWd	0.94 ± 0.1	1.00 ± 0.1	0.97 ± 0.1	1.21 ± 0.1*	0.97 ± 0.1	1.08 ± 0.1
Fractional Shortening (%)	33.6 ± 1.9	32.3 ± 2.0	30.8 ± 1.5	30.2 ± 1.9	26.3 ± 2.7	31.8 ± 1.9
Ejection Fraction	0.55 ± 0.03	0.54 ± 0.03	0.52 ± 0.02	0.51 ± 0.03	0.45 ± 0.04	0.53 ± 0.03

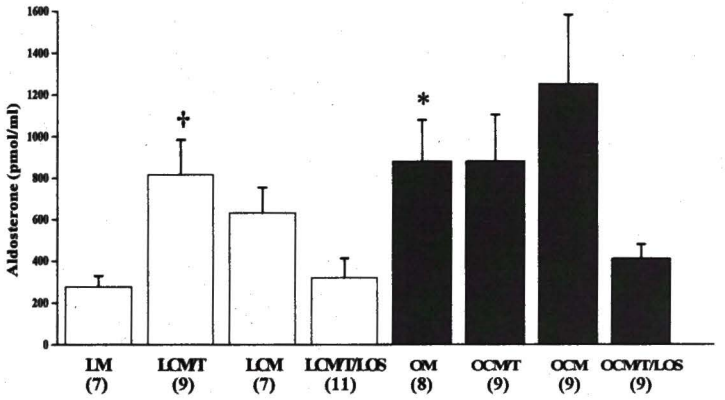
Data are mean ± SE. *$p \leq 0.05$ vs. LM, unpaired t-test; †$p \leq 0.05$ vs. respective LM or OM control, one-way ANOVA. LV = left ventricle, RV = right ventricle, IDd = end diastolic diameter, IDs = end systolic diameter, PWd = posterior wall in diastole, PWs = posterior wall in systole, IVSd = interventricular septum in diastole, IVSs = interventricular septum in systole. LM = combined group: lean + lean castrated supplemented with testosterone, LCM = lean castrated, LCM/T/LOS = lean castrated supplemented with testosterone and losartan-treated, OM = combined group: obese + obese castrated supplemented with testosterone, OCM = obese castrated, OCM/T/LOS = obese castrated supplemented with testosterone and losartan-treated.

Figure 1.

A.


PRA (ng Ang I/ml/hr)
20
15
10
5
0
†
*
LM (7)
LCM/T (9)
LCM (7)
LCM/T/LOS (11)
OM (8)
OCM/T (9)
OCM (9)
OCM/T/LOS (9)

B.


Aldosterone (pmol/ml)
1600
1400
1200
1000
800
600
400
200
0
†
*
LM (7)
LCM/T (9)
LCM (7)
LCM/T/LOS (11)
OM (8)
OCM/T (9)
OCM (9)
OCM/T/LOS (9)

C.

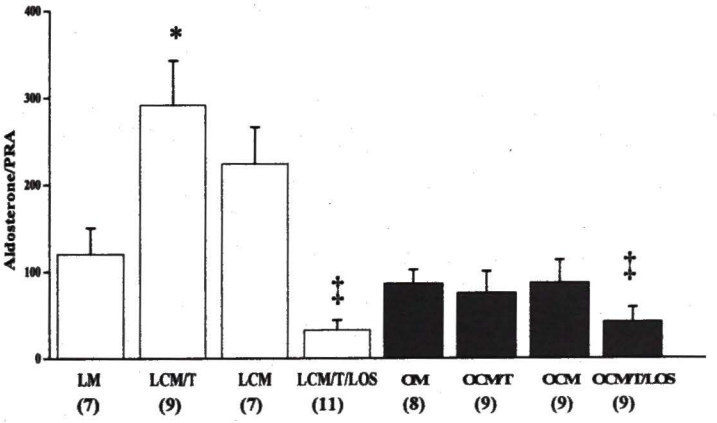

Aldosterone/PRA
400
300
200
100
0
*
‡
‡
LM (7)
LCM/T (9)
LCM (7)
LCM/T/LOS (11)
OM (8)
OCM/T (9)
OCM (9)
OCM/T/LOS (9)

Figure 2.

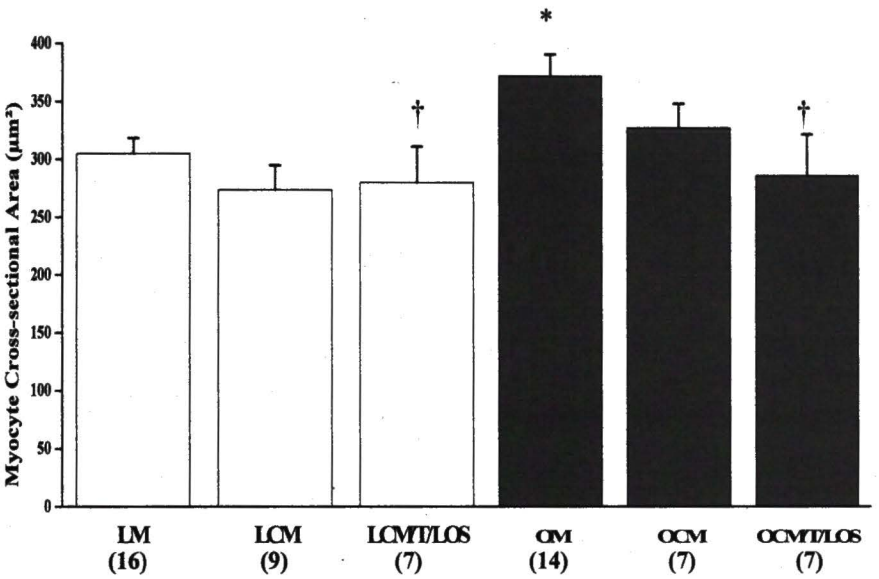
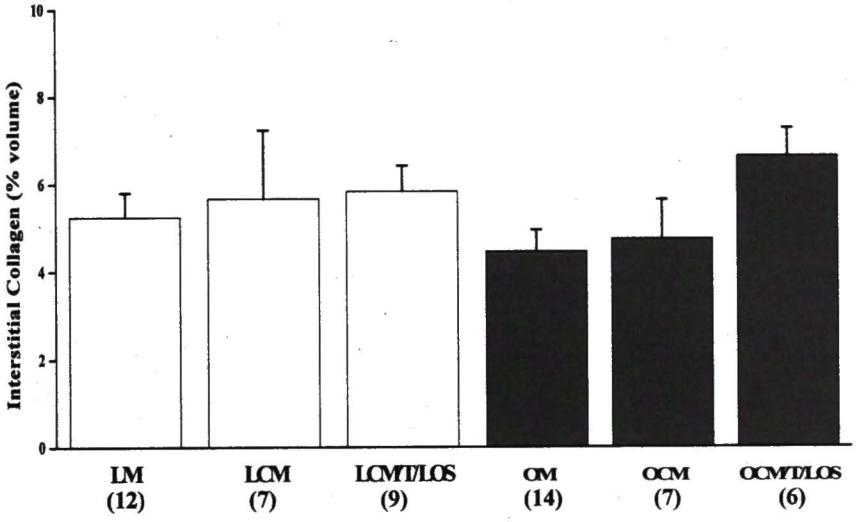

Myocyte Cross-sectional Area (μm²)
400
350
300
250
200
150
100
50
0
*
†
†
LM
(16)
LCM
(9)
LCMT/LOS
(7)
OM
(14)
OCM
(7)
OCMT/LOS
(7)

Figure 3.

A.


Interstitial Collagen (% volume)
10
8
6
4
2
0
LM (12)
LCM (7)
LCM/T/LOS (9)
OM (14)
OCM (7)
OCM/T/LOS (6)

B.

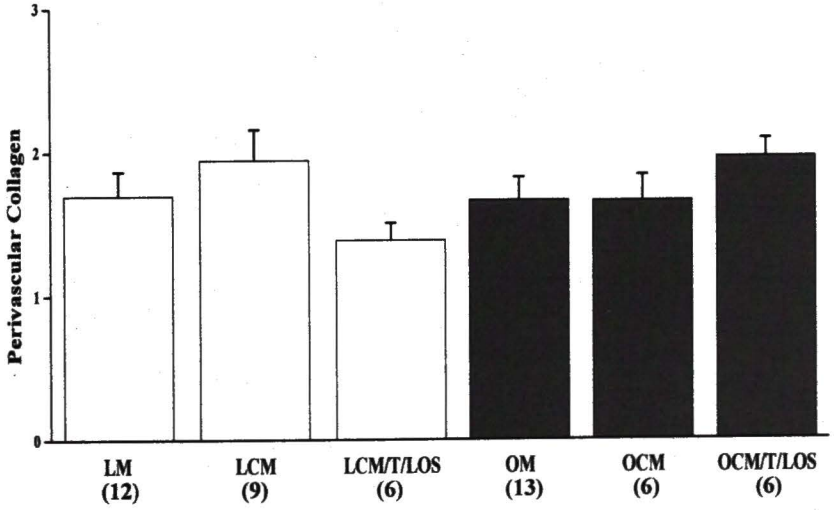
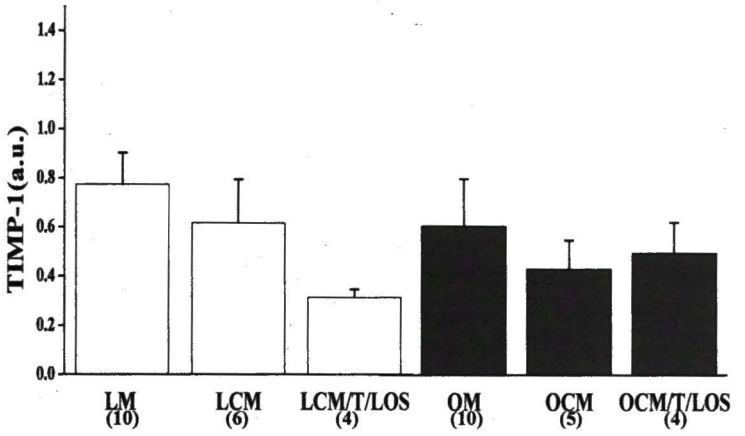
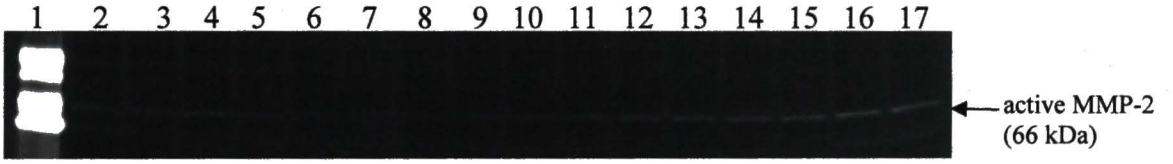

Perivascular Collagen
3
2
1
0
LM (12)
LCM (9)
LCM/T/LOS (6)
OM (13)
OCM (6)
OCM/T/LOS (6)

Figure 4.

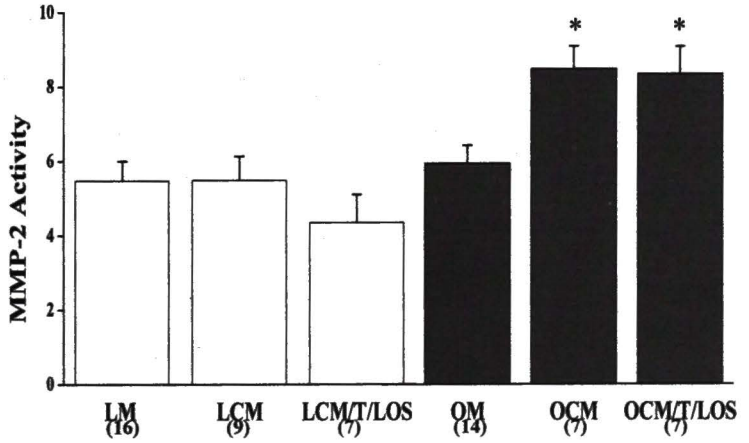
A.


TIMP-1 (a.u.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LM (10)
LCM (6)
LCM/T/LOS (4)
OM (10)
OCM (5)
OCM/T/LOS (4)

B.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
active MMP-2
(66 kDa)

C.


MMP-2 Activity
10
8
6
4
2
0
*
*
LM (16)
LCM (9)
LCM/T/LOS (7)
OM (14)
OCM (7)
OCM/T/LOS (7)

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CHAPTER III

SUMMARY AND CONCLUSIONS

Effects of obesity:

- Increased systolic blood pressure and resting heart rate
- Increased plasma renin activity and aldosterone
- Increased heart and LV weights
- Increased myocyte cross-sectional area
- No effect on interstitial or perivascular collagen
- No effect on MMP-2 activity or TIMP-1 expression

Effects of testosterone:

- No effect on blood pressure
- No effect on plasma renin activity
- Increased heart and LV weights
- Increased myocyte cross-sectional area
- No effect on interstitial or perivascular collagen
- Decreased MMP-2 activity in obese rabbits only

Effects of the renin-angiotensin system:

- Increased blood pressure
- Tended to increase aldosterone
- Tended to increase LV weight
- Increased myocyte cross-sectional area
- No effect on interstitial or perivascular collagen
- Decreased MMP-2 activity in obese rabbits only

Overall Conclusions:

Twelve wks of high fat diet was sufficient to create hypertension, activate the renin-angiotensin system, and cause myocyte hypertrophy. Castration did not alter RAS activity but tended to attenuate myocyte cross-sectional area, suggesting that testosterone acts independently of the RAS to mediate myocyte size. Blockade of the RAS attenuated both blood pressure and myocyte hypertrophy. Thus, testosterone and obesity-mediated RAS activity act independently to increase LVH.

After 12 wks of high-fat diet, overall fibrosis was not increased. This may be due to the mild degree of hypertension in this model. However, the data indicated that testosterone and the RAS both decreased MMP-2 activity but only in obese animals. Proportionally large increases in TIMP-1, together with decreases in MMP-2 activity suggest a trend towards decreased collagen degradation. Depending upon the rate of collagen synthesis this could lead to increased collagen accumulation, but in the present study no changes were observed.

APPENDIX

EXPERIMENTAL PROBLEMS

I attempted to measure collagens type I and III, TGF-β1, and decorin in left ventricular samples by western blot. However, after trying many different blocking techniques, these immunoblots continued to produce a high degree of non-specific binding, such that the band of interest could not be identified with confidence. The following mouse monoclonal antibodies were obtained from Calbiochem (La Jolla, CA):

Collagen type I – Cat. No. CP17

Collagen type III – Cat. No. CP19

TGF-β1 – Cat. No. GF33L, clone # 9016.2

Decorin – Cat. No. PC673 (goat polyclonal antibody)

Varying concentrations and types of blocking buffers were use (non-fat milk, BSA), as well as various lengths of incubation time in the blocking buffer. Non-fat milk was also added to the primary and secondary antibody dilutions, however, non-specific binding persisted.

We hypothesize that the antibodies were not specific for the rabbit target proteins.


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