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THE ACUTE RESPONSES OF DIFFERENT-SIZED CORONARY ARTERIES TO TESTOSTSERONE

Sampath Madanu

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Master of Science

Cell and Molecular Biology

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ABSTRACT

Coronary arteries supply blood to the myocardium. The blood flow within the coronary arteries is altered by various compounds produced within the body. Sex hormones such as testosterone are known to cause the relaxation of large coronary arteries. But the response to testosterone is greater in *in vivo* conditions compared to *in vitro* conditions. We hypothesize that the responses of LADs (left anterior descending arteries) and its side branches to testosterone are heterogeneous and testosterone-induced vasodilation is greater in its side branches. Therefore, our study was designed to determine the effect of testosterone in different-sized coronary arteries. LADs and one of its side branches were isolated from porcine hearts and mounted in organ baths to mimic *in vivo* conditions. The coronary arteries were then preconstricted with potassium chloride (KCl) and administered increasing concentrations of testosterone to determine if the responses to testosterone vary within different regions of the coronary circulation. The testosterone caused significant relaxation in both LADs and its side branches. However the side branches showed similar responses to testosterone as compared to the larger, upstream LADs. Further studies on androgen receptor expression using real time PCR indicated that androgen receptor expression was higher in LADs than its side branches. A third group of small coronary arteries exhibited greater androgen receptor expression than the LADs and its side branches. The enhanced testosterone-induced vascular reactivity exhibited in vivo may be at the level of the small coronary arteries, not the LAD and its side branches.

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INTRODUCTION

Blood flow is the movement of blood through the heart and the blood vessels of the circulatory system. The myocardium, better known as the heart muscle, needs a constant supply of oxygen and other nutrients to meet its metabolic demands. The myocardium obtains these via a localized network of blood vessels known as the coronary arteries and the flow of blood through this network of arteries is known as the coronary circulation. The two main arteries supplying blood to the heart are the left and right coronary arteries which originate at the ascending aorta. The right coronary artery supplies blood to the right atrium and to some portions of the right ventricle while the left coronary artery supplies blood to the left atrium and ventricle. The two arteries are interconnected via anastomoses to ensure the continuous supply of blood throughout the heart.

Regulation of coronary blood flow

The cardiac muscle obtains oxygen and nutrients via the coronary circulation. Blood flow in the coronary circulation is regulated by various metabolic, endothelial, myogenic, humoral and nervous mechanisms. Metabolic regulation of coronary circulation is mediated by metabolized nutrients where oxygen acts as a significant metabolic regulator (Berne, 1963). An increase in metabolism causes an increase in the utilization of oxygen which in turn produces vasodilatory substances such as adenosine. In order to counter the increased utilization of oxygen, a balance is maintained between the oxygen supply and myocardial oxygen consumption (Berne, 1963).

This balance reflects the intracellular myocardial oxygen tension (PO_2). With an increase in

oxygen consumption, the PO_2 decreases leading to a breakdown of ATP (adenosine triphosphate) into ADP (adenosine diphosphate) and AMP (adenosine monophosphate). The released adenosine nucleotides diffuse out of the cell, migrate across the interstitial space and bind to receptors (A_1 and A_{2A}) located in smooth muscle fibers (Hein et al., 1999). Binding of adenosine to its receptors causes opening of potassium channels and efflux of potassium ions leading to the hyperpolarization of the membrane in vascular smooth muscle. This hyperpolarization results in the decrease of calcium influx through calcium channels and leads to the dilation of the coronary arteries (Komaru et al., 1991). Vasodilation increases the blood flow and delivers more oxygen to the myocardium thus returning the PO_2 to normal.

The binding of adenosine to A_1 receptors inhibits adenylyl cyclase thereby reducing cAMP levels and activates the outward current of potassium. This process of signal transduction from adenosine receptor to potassium channels is mediated by the pertusis toxin-sensitive G-protein. This shortens the duration of the action potential and finally reduces the contraction force (Hein et al., 1999).

Myogenic regulation of blood flow is carried out by the smooth muscle fibers of the blood vessel. Smooth muscle constriction and relaxation decreases and increases the arterial blood flow, respectively. Under resting conditions, coronary blood flow remains constant between mean arterial pressures of 60-140 mmHg. High arterial pressure leads to a sudden stretch of the blood vessel walls causing the smooth muscle fibers of the vessels to constrict for a few seconds. This myogenic contraction results in a stretch induced vascular depolarization which results in the activation of cationic channels enabling an influx of Ca^{+2}, Na^+, and K^+ ions. The influx of cations depolarizes the membrane of the smooth muscle and opens the voltage dependent calcium channels to increase the intracellular calcium levels. The increase in calcium activates

the actin-myosin interaction leading to smooth muscle contraction (review by Szent, 1975). Myogenic vasodilation occurs when ATP sensitive K^+ channels open to cause an efflux of K^+ ions. The efflux of K^+ hyperpolarizes the membrane which increases the threshold for cellular depolarization and finally leads to vasodilation (Haddy et al., 2006).

Endothelial regulation of coronary blood flow is controlled by the endothelium which is a single layer of cells lining the inner walls of the arteries. The endothelium modulates the activity of smooth muscle fibers by synthesizing several vasoactive substances that affect relaxation and contraction of the arterial wall in response to various stimuli. The most important vasodilator is the endothelium derived relaxing factor, nitric oxide (NO). The endothelial cells experience sheer stress as the viscous drag of blood flows rapidly along the vascular walls. This stress contorts the endothelial cells in the direction of blood flow. Stress mainly increases the intracellular calcium in the endothelium by activation of the phospholipase C pathway. Increased endothelial cell calcium activates NO synthase which induces synthesis of NO finally leading to vascular relaxation (Toda et al., 2009).

Humoral regulation involves the control of blood flow by substances secreted into body fluids. These substances include vasoactive hormones such as angiotensin, calcitonin, vasoactive -intestinal peptide and atrial natriuretic peptide. Angiotensin induces opening of calcium channels and thereby increases the calcium influx which leads to vasoconstriction of coronary arteries.

Steroids

Steroid hormones are naturally occurring organic compounds that are lipophilic in nature. Steroid hormones primarily include gonadal and adrenocortical hormones which have important physiological functions such as coordination of behavioral and physiological responses within

the organism. These hormones act on the central nervous system as well as on peripheral target tissues. Steroid hormones vary widely in their chemical structure and metabolism.

Metabolism of steroids

Steroids are primarily synthesized by the adrenal glands and the gonads (testis or ovary). Steroids are secreted into the bloodstream and transported to the target tissue where they initiate specific responses. Steroid metabolism plays an important role in the production of hormones and also in regulating the physiological and cellular actions of steroids. Steroids are derived from cholesterol, the parent compound for all steroids. In the first step of steroid synthesis, cholesterol is converted to pregnenolone in the presence of C_{20}, C_{22} lyase. Pregnenolone is the precursor for progesterone and 17 alpha hydroxyl pregnenolone. Pregnenolone is further converted to progesterone in the presence of 3 beta hydroxyl steroid dehydrogenase and 4,5 isomerase. Progesterone is converted into corticosterone in the presence of C_{21} hydroxylase and C_{11} hydroxylase. Corticosterone is converted into aldosterone in the presence of C_{18} hydroxylase and C_{18} oxidase. 17 alpha hydroxyl pregnenolone is the precursor for androgens and estrogens. Initially 17 alpha hydroxyl pregnenolone is converted to dehydroepiandrosterone in the presence of $C_{17, 20}$ lyase. Dehydroepiandrosterone is converted to androstenedione in the presence of 3 beta hydroxy steroid dehydrogenase and 4,5 isomerase. In the presence of 17 beta hydroxyl steroid dehydrogenase, androstenedione is converted to testosterone, which is further converted to estradiol in the presence of aromatase. Androstenedione is also converted to estrone in the presence of aromatase. Androgen formation occurs up to androstenedione and dehydroepiandrosterone in the adrenal glands, whereas androstenedione is further converted to testosterone only in Leydig cells. Similarly estrogen formation occurs only in ovaries because the aromatse activity is present only in these tissues.

Mechanism of action of steroid hormones

Steroid hormones act in genomic and nongenomic pathways. In genomic pathway, steroid hormones readily diffuse through the cell membrane of the target cell. Once inside, the steroid hormones bind to intracellular receptors. The hormone-receptor complex then translocates to the nucleus and interacts with specific DNA sequences known as steroid responsive elements. This interaction leads to transcription and translation of specific genes. The newly synthesized proteins may be enzymes involved in specific metabolic activities or the synthesis of other proteins. In the absence of hormone, the intracellular receptors are bound to chaperones forming receptor-chaperone complexes which are incapable of binding DNA. When the hormone binds to the receptor, the chaperone dissociates allowing the receptor to be phosphorylated. Phosphorylated receptor can bind to the steroid responsive elements in the DNA.

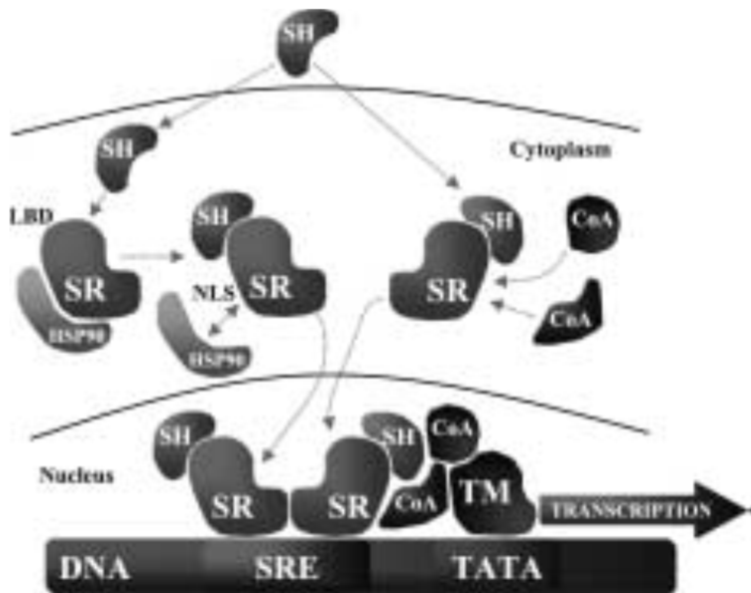

Figure 1: Genomic action of steroids: (figure adapted from Simoncini and Genazzani, 2003).

Steroids are also known to act in nongenomic pathways. Nongenomic actions of steroid hormones involve the recruitment of signaling pathways that are often associated with cell

membrane receptors such as G-protein coupled receptors, ion channels or enzyme-linked receptors. Through these pathways, steroids rapidly regulate multiple cellular functions.

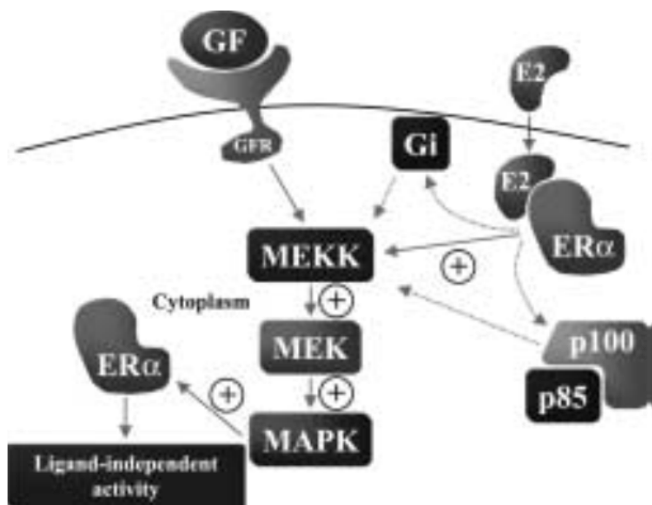

Figure 2: Nongenomic action of steroids: (figure adapted from Simoncini and Genazzani, 2003).

Coronary heart disease (CHD)

Coronary heart disease (CHD) is the most common type of heart disease caused by accumulation of cholesterol on the inner walls of arteries. It is one of the leading causes of death in the United States. Mortality data from Centers for Disease Control and Prevention have shown that the overall preliminary death rate from CHD was 195.0 per 598,607 people in 2009 (Kochanek et al., 2011).

Extensive clinical studies have identified several controllable and uncontrollable risk factors for CHD and heart attack. The controllable risk factors include high blood pressure, alcohol consumption, smoking, diabetes mellitus, obesity, lack of physical activity, and stress. Uncontrollable risk factors include heredity (children of parents with heart disease have a greater risk of heart disease than other people), gender (men have a greater risk of heart disease than woman) and increasing age (people older than 65 have a greater risk of heart attack).

The role of sex hormones such as estrogen and testosterone in cardiovascular disease has generated great interest during recent years. Sex hormones directly influence cardiovascular physiology and it has been proposed that estrogen may protect against the development of cardiovascular diseases in postmenopausal women (Grady et al., 1992). Estrogens decrease low-density lipoprotein, cholesterol and increase high-density lipoprotein (Walsh et al., 1991).

Testosterone is an androgen secreted in the testes of males and to a lesser extent in adrenal glands and ovaries of females. Testosterone induces the development of secondary sexual characteristics such as growth spurts, increased body hair, increased muscle mass, deepening of the voice, maturation of the penis, increased erectile function, spermatogenesis and bone resorption.

The mechanism of action of testosterone involves both genomic and nongenomic pathways. In genomic pathways, testosterone regulates gene expression by binding to the androgen receptor (AR). Testosterone is transported in the bloodstream bound to either sex hormone binding globulin (SHBG) or albumin. At the target cell, testosterone dissociates from the SHBG, enters the cell by endocytosis, and is converted to dihydrotestosterone. Dihydrotestosterone binds to the AR which induces a conformational change in the AR. The hormone receptor complex translocates into the nucleus where the activated AR complexed to testosterone binds to androgen response elements (AREs) present within the promoter and enhancer regions of target genes (Zhou et al., 1994). The ARE-bound AR dimer can either interact directly with components of the transcription preinitiation complex or recruit other components (transcription factors) that promote gene transcription. Testosterone also acts through a non-genomic pathway by potassium channel activation or calcium channel antagonism (Jones et al., 2003).

The putative role of testosterone on vascular reactivity of coronary arteries has received great

interest during recent years since testosterone directly influences cardiovascular physiology (Deenadayalu et al., 2001). Men suffering from coronary artery disease (CAD), and myocardial infarction have lower levels of testosterone than healthy men (English et al., 2000b) and the low plasma testosterone levels may be associated with increased risk of coronary artery disease in men (Rosano et al., 2007). The lower testosterone levels are also associated with adverse cardiovascular risk factors such as premature atherosclerosis, increased visceral adipose tissue, hyperinsulinemia and increased fibrinogen in humans (Shabsigh et al., 2005). Evidence suggests that testosterone has beneficial effects on atherogenic blood lipid profile (high amount of low density lipoprotein, low amount of high density lipoprotein and high triglycerides) and against atheroma formation (accumulation of fat on inner lining of an artery). Testosterone causes decreases in the LDL and triglycerides and increases HDL (Rosano and Mercuro, 2001). Intracoronary infusions of testosterone provide improvement in myocardial ischemia (reduced blood supply to myocardium) and angina (lack of oxygen supply to myocardium) by inhibiting plaque formation in blood vessels (Rosano et al., 1999). Administration of testosterone caused coronary artery dilation and increased coronary blood flow in men suffering from coronary artery disease (Webb et al., 1999a).

The direct coronary action of testosterone was revealed from studies performed by Webb et al (1999b), who demonstrated that an intra-coronary infusion of physiological concentrations of testosterone increased coronary artery diameter and coronary blood flow in men with CAD. Previous studies on animals demonstrated that testosterone caused vasodilation in rabbit coronary arteries and aorta (Yue et al., 1995), rat left anterior descending artery (English et al., 2000a), rat thoracic aorta (Ding and Stallone, 2001), canine coronary artery (Chou et al., 1996),

porcine left anterior descending artery (Deenadayalu et al., 2001) and in human coronary arteries (Webb et al., 1999b).

Testosterone is known to be an acute vasodilator. Testosterone causes the dilation of arteries by inducing the release of nitric oxide which signals the surrounding smooth muscle to relax causing vasodilation and finally increasing blood flow. Testosterone also causes vasodilation by directly acting on vascular smooth muscle through potassium channel activation or calcium channel antagonism (Yue et al., 1995).

Testosterone shows greater response *in vivo* compared to *in vitro* because SHBG is involved in presentation of testosterone to effector proteins. In *in vitro* condition absence of SHBG in target cells may contribute to loss of potency (Webb et al., 1999a). Testosterone acts on large and small coronary arteries in *in vivo* conditions, however small coronary arteries may exhibit greater responses to testosterone due to higher expression of AR in them. The purpose of this study is to investigate the response of LAD and its side branches to testosterone. We hypothesize that the regulation of blood flow in the coronary circulation, including vascular responses to testosterone is heterogeneous and testosterone-induced vasodilation may be greater in small arteries than large arteries.

Materials and Methods

Preparation of arterial rings

Fresh porcine hearts (n=13) were obtained from a local abattoir and they were kept on ice during their transport to the laboratory. The left anterior descending (LAD) artery and its side branches were dissected from the hearts and cleaned of fat and connective tissue and placed in Krebs-Henseleit solution.

The arterial rings (length=5mm) were cut from LADs or its side branches and mounted onto hooks in organ baths (Figure 2) containing 25 ml of Krebs Henseleit solution (pH of 7.4) with the following composition (in mM): 118.0 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 11.0 glucose. The lower hook was fixed in position and the upper hook was connected to a force transducer which was coupled to an amplifier and computer-based data acquisition system for the continuous measurement of arterial tension. A change in arterial tension due to arterial contraction or relaxation was measured by the transducer and recorded on a multichannel recorder using Labscribe (2.0) software.

Initially Potassium Chloride (KCl) optimization experiments were performed to determine the concentration of KCl that can be used for assessing the viability of coronary arteries. This was done by administering increasing concentrations of KCl (10mM-120mM KCl) to organ baths containing coronary arteries. The arterial rings were equilibrated for 60 minutes in Krebs-Henseleit solution with a passive tension of 7.0 grams for LADs or 1.5 grams for side branches and all arteries were maintained at 37°C to mimic *in vivo* conditions. The experimental groups included LAD vehicle (control), LAD testosterone (treatment), side branch vehicle (control) and side branch testosterone (treatment). The organ baths were continuously gassed with 95% O_2 and 5% CO_2. During the period of equilibration, rings were rinsed once every 15 minutes by

replacing the Krebs-Henseleit solution to remove metabolic wastes produced by the arteries. Following equilibration, viability of arterial rings was assessed via addition of a known vasoconstrictor, KCl (60 mM). Only viable arteries (arteries that constricted upon KCl treatment) were selected for further experimentation. Following the viability test, Krebs-Henseleit solution (containing 60mM KCl) was replaced with fresh Krebs-Henseleit solution in the organ bath. The arteries were incubated for 5 minutes in the Krebs-Henseleit solution which allowed the arteries to return to a normal relaxed state. Then, KCl (15, 30, 45, 60 mM) was added cumulatively to induce contraction in the vessels followed by induction of relaxation by the cumulative addition of sodium nitroprusside (SNP10^{-7}-10^{-3} M). The arterial rings were then exposed to increasing concentrations of KCl (15, 30, 45, 60mM), followed by testosterone (10^{-7}M-10^{-3}M) or methanol (the vehicle for dissolving testosterone).

RNA isolation

Fresh porcine hearts were obtained from local abattoir and they were kept on ice during their transport to the laboratory. Left anterior descending coronary artery (LAD) and its side branches were dissected from 3 different porcine hearts and cleaned of fat and connective tissue and then placed in Krebs-Henseleit solution. LAD was categorized as large artery, the branch of LAD was categorized as medium artery and the branch of medium sized artery was categorized as small artery. Total ribonucleic acid (RNA) was extracted from large, medium and small coronary arteries using TRIzol® reagent (Invitrogen, CA, USA). TRIzol® is a monophasic solution of phenol and guanidine isothiocyanate, which facilitates the isolation of RNA from tissues by disrupting cells. Initially large, medium and small coronary arteries were homogenized in 2ml of TRIzol® and then sonicated mildly using sonifier (Brasnon sonifier 150, CT, USA). The homogenized samples were then incubated at 25^0 C for 5 minutes to facilitate the

dissociation of nucleoprotein complexes. Chloroform (Fisher Scientific, NJ, USA) was added to each of the sonicated arteries and incubated for 5 minutes at room temperature. The samples were then centrifuged at 12,000g for 15 minutes at 4^0 C. Following centrifugation, the mixture separated into a colorless upper aqueous phase containing RNA, an interphase containing DNA and lower red phenol-chloroform phase containing proteins. The aqueous phase (25 ul) containing RNA was transferred into new RNAse free eppendorf tubes and RNA was precipitated from aqueous phase by adding 1ml of isopropanol (Sigma Aldrich, MO, USA). The samples were incubated at room temperature for 10 minutes and were centrifuged at 15,000g for 15 minutes. The supernatant was removed and the pellet was washed with 80% ethanol (Sigma Aldrich, MO, USA). The RNA pellet was air dried completely and resuspended in 100 µl of nuclease free water. 10 µl of DNAse buffer (Applied Biosystems, CA, USA; that is 1/10 volume of RNA solution) was added to the RNA samples, followed by 2 ml of RNAse inhibitor (Applied Biosystems, CA, USA) and 2 µl of DNAse 1 (Applied Biosystems, CA, USA). RNA samples were then incubated for 20 minutes at 37^0 C in a water bath. 1 µl of 2 mercaptoethanol (Sigma Aldrich, MO, USA) was added to 700 ul of lysis buffer RLT (Quiagen, MY, USA; 4 M guanidinium thiocyanate, 10 mM Tris-Cl [pH 8.0], 1 mM EDTA). 350ul of 2 mercaptoethanol and buffer RLT mixture were added to the RNA solution to denature RNase enzymes. Then 250ml of 100% ethanol were added to the RNA samples to promote the selective binding of RNA to Quiagen column. These samples were loaded onto Quiagen columns and centrifuged at 5,000 rpm for 15 sec at room temperature. The columns were washed twice with 500ul of wash buffer RPE (Quiagen, MY, USA; 10 mM Tris-Cl [pH 8.0], 1 mM EDTA, 80% ethanol) and centrifuged at 10,000 rpm for 15 seconds at room temperature. The columns were placed in 2 ml collection tubes, centrifuged for 1 minute at 15,000 rpm and then placed in 1.5 ml collection

tubes. RNA was eluted by adding 50 µl of nuclease free water (Qiagen, MY, USA) to the RNeasy Minispin columns (Qiagen, MY, USA) followed by centrifugation of columns at 10,000 rpm for 1 minute at room temperature. The RNA elution step was repeated and the resulting RNA was quantified using nanovue spectrophotometer (GE healthcare, NA, USA). The amount of RNA isolated from each elution step was shown in Table1.

cDNA generation

cDNA was generated from total RNA using reverse transcriptase enzyme. The reverse transcription reaction was performed in 20 µl reaction volume containing 3.2 µl of nuclease free water, 2 µl of RT buffer, 0.8 µl of dNTPs, 2 µl of random primers, 1 µl of reverse transcriptase, 1 µl of RNAse inhibitor and 10 µl of total RNA. The reactions were incubated for 10 minutes at 25^0 C, 2 hours at 37^0 C, 5 seconds at 85^0 C and 4^0 C overnight. The cDNA samples were then stored at -20^0 C until their use.

Real-time PCR

The steady state levels of mRNA in large, medium and small coronary arteries were analyzed by performing real-time PCR (Stratgene MX 3000 P^{Tm}, Agilent Technologies, CA, USA) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as control. Real time PCR of AR and GAPDH genes were performed using Go Taq Q PCR master mix (Promega, WI, USA). Primers for GAPDH and AR were designed using Primer 3 Software. The primer sequence for GAPDH was forward primer 5' ACCAGGTTGTGTCCTCTGACTT 3' and reverse primer 5' TGCTGTAGCCAAATTCATTGTC 3' whereas primer sequences for AR were forward primer 5' CATATTGAAGGCTATGAGTG 3' and reverse primer 5' CCCATCCAGGAATACTGAAT 3'. The 20 µl of cDNA were incubated for 2 minutes at 95^0 C followed by 1 minute at 95^0 C, 30 seconds at 55^0 C, 30 seconds at 72^0 C, 1 minute at 95^0 C, 30

seconds at 55^0 C and 30 seconds at 95^0 C. The products were separated using agarose gel electrophoresis (1.5% of agarose, run at 125 V) and visualized by ethidium bromide staining and photographed using Image J software. The flow chart for designing and implementing real-time PCR assays was shown in Figure3.

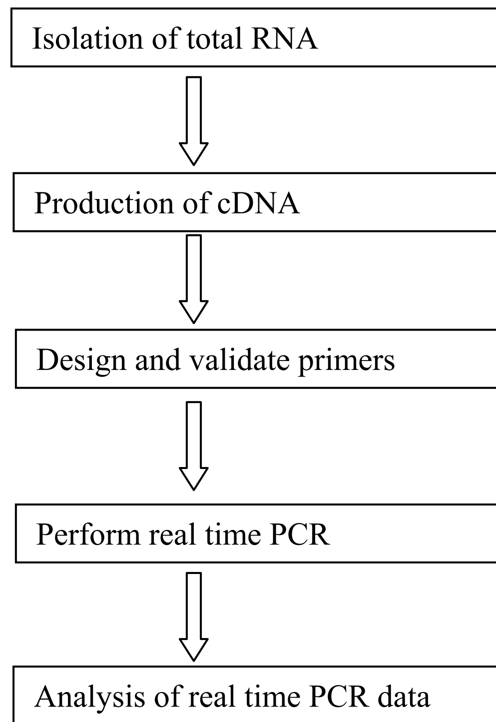

Figure 3: Flow chart for designing and implementing real time PCR assays.

Data analysis

Statistical analysis was performed using Sigma Stat (3.1). Since the arterial rings were subjected to repeated doses of increasing concentrations of KCl, SNP and testosterone in each treatment group and dose responses recorded as changes in the tension (measured in grams), we used one-way Repeated Measures ANOVA to analyze the data. Responses of LADs to different concentrations of KCl were compared by one way Repeated Measures ANOVA. The differences were considered statistically significant at $p<0.05$. The changes in gene expression levels of AR were analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Results

KCl optimization

The KCl optimization experiments indicate that increasing concentrations of KCl caused the contraction of the arteries (fig.5). Artery contraction increased significantly up to a concentration of 60 mM KCl, with no significant increase beyond that point. Hence 60mM KCl was used as an optimum concentration for testing the viability of coronary arteries since this concentration was sufficient to produce a maximal contraction.

Arterial tension studies

The LADs and side branches displayed significant contractions in response to KCl (fig.6). However, the contraction was greater in LADs compared to the side branches.

Effect of sodium nitroprusside on coronary arteries

The LADs and side branches displayed a significant relaxation in response to sodium nitroprusside (SNP; fig.7). SNP caused relaxation of both LADs and side branches equally and there was no significant difference in SNP induced relaxation between the LADs and side branches.

Effect of Testosterone on coronary arteries

The LADs and side branches exhibited a significant relaxation in response to testosterone (fig.8). Testosterone caused relaxation of both LADs and side branches at higher concentrations and there was no significant difference in testosterone-induced relaxation between LADs and side branches. The relaxation was observed only in the treated arteries whereas there was no relaxation in control arteries. This indicates that the observed relaxation was caused by testosterone, and not as a result of the methanol.

Expression of androgen receptor in coronary arteries

The relative changes in androgen receptor expression from real time PCR experiments were analyzed by $2^{-\Delta\Delta Ct}$ method. Using this method, data were presented as fold change in gene expression normalized to endogenous reference gene (GAPDH) and relative to LAD (fig 9). Thus the depicted fold change in small arteries is high relative to LAD. The fold change in androgen receptor expression of medium artery was approximately 25% of LAD.

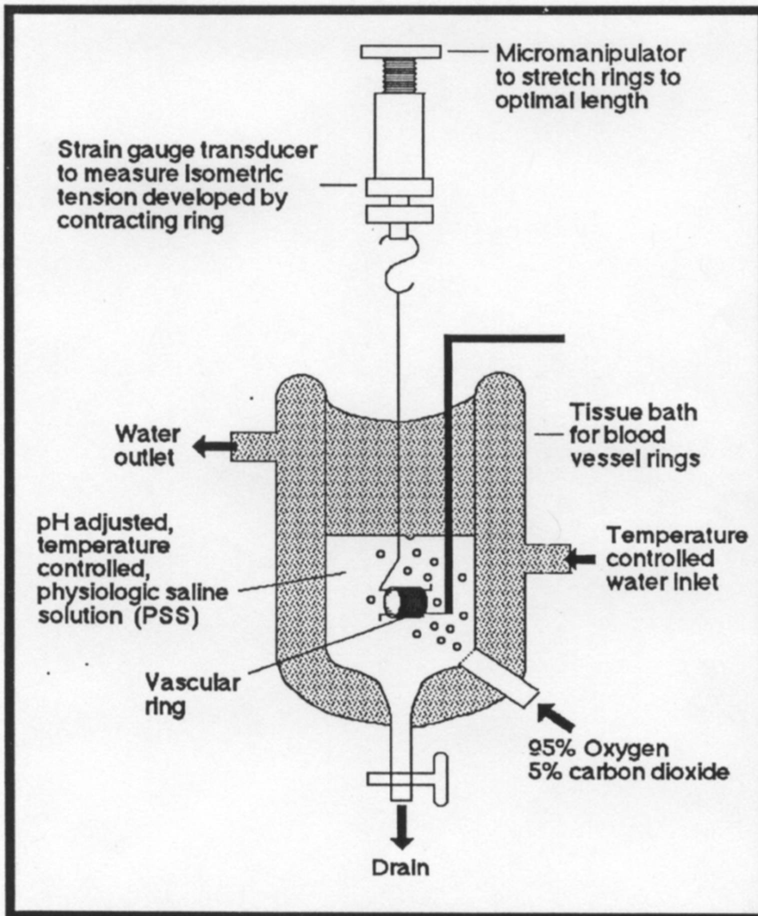

Figure 4: Schematic diagram of experimental set-up used to perform experiments with coronary arteries. Created by Donald B.Stratton, Ph.D. (Drakes University, Des Moines, Iowa).

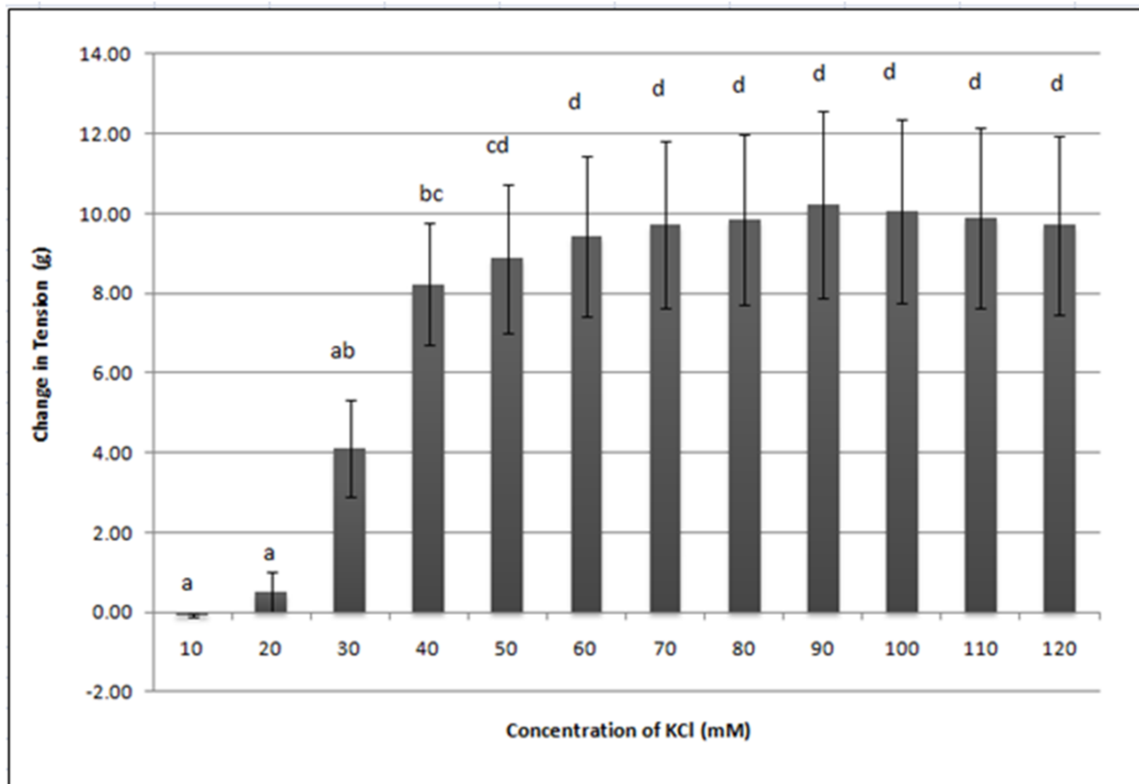

Figure 5: Histogram depicting the effect of increasing concentrations of KCl on left anterior descending arteries (n=13) expressed as change in the tension. (**d** is significantly different from **a** and **ab**, where as **bc** and **cd** are significantly different from **a**) Data are expressed as the mean±SEM ($p<0.05$).

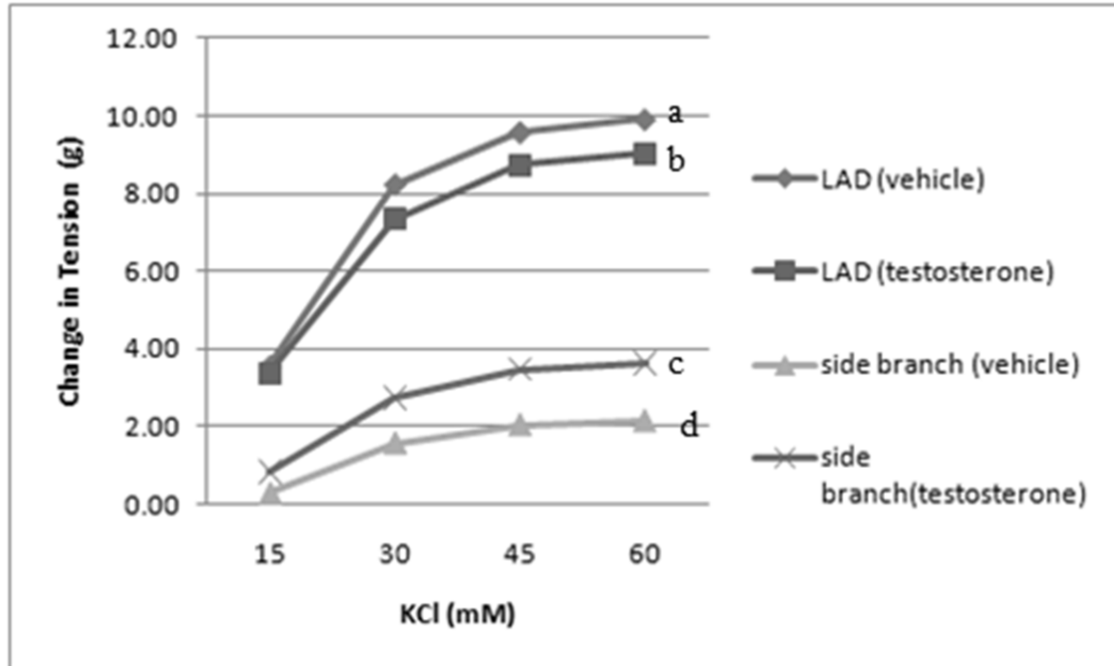

Figure 6: The effect of increasing concentrations of KCl on left anterior descending artery and side branches (n=13). (**a** and **b** are significantly different from **c** and **d**). Data are expressed as the mean±SEM ($p < 0.05$).

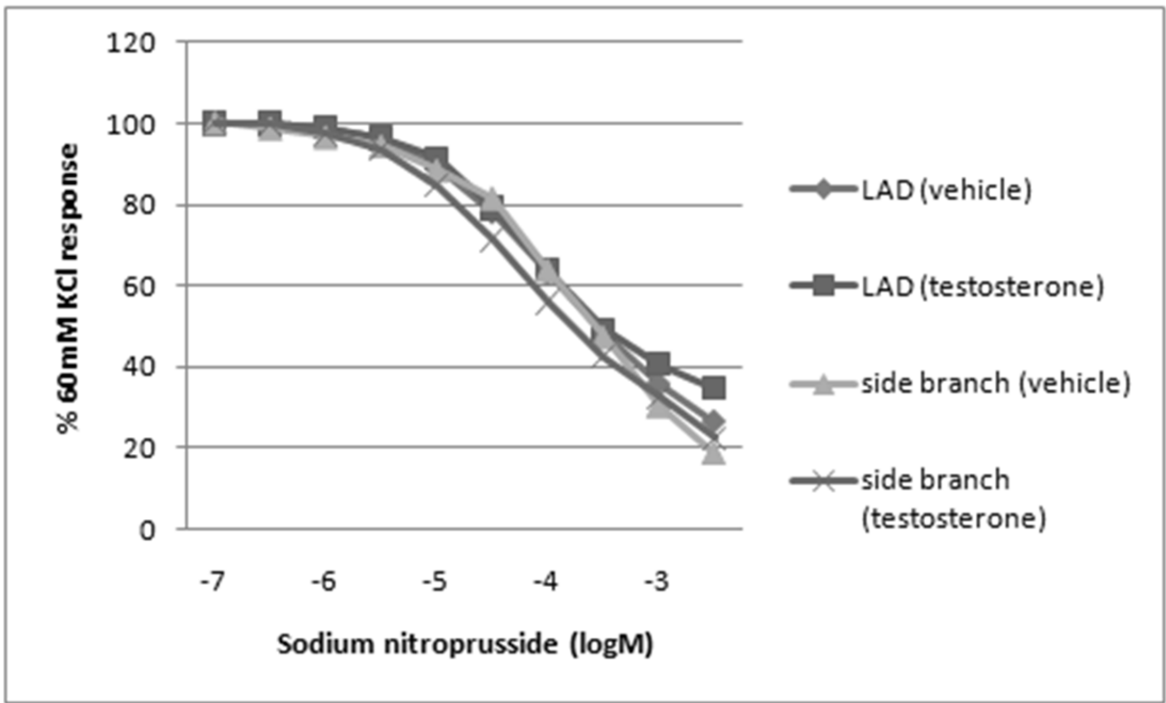

Figure 7: The effect of increasing concentrations of sodium nitroprusside on anterior descending artery and side branches (n=13). Data are expressed as the mean±SEM ($p<0.05$).

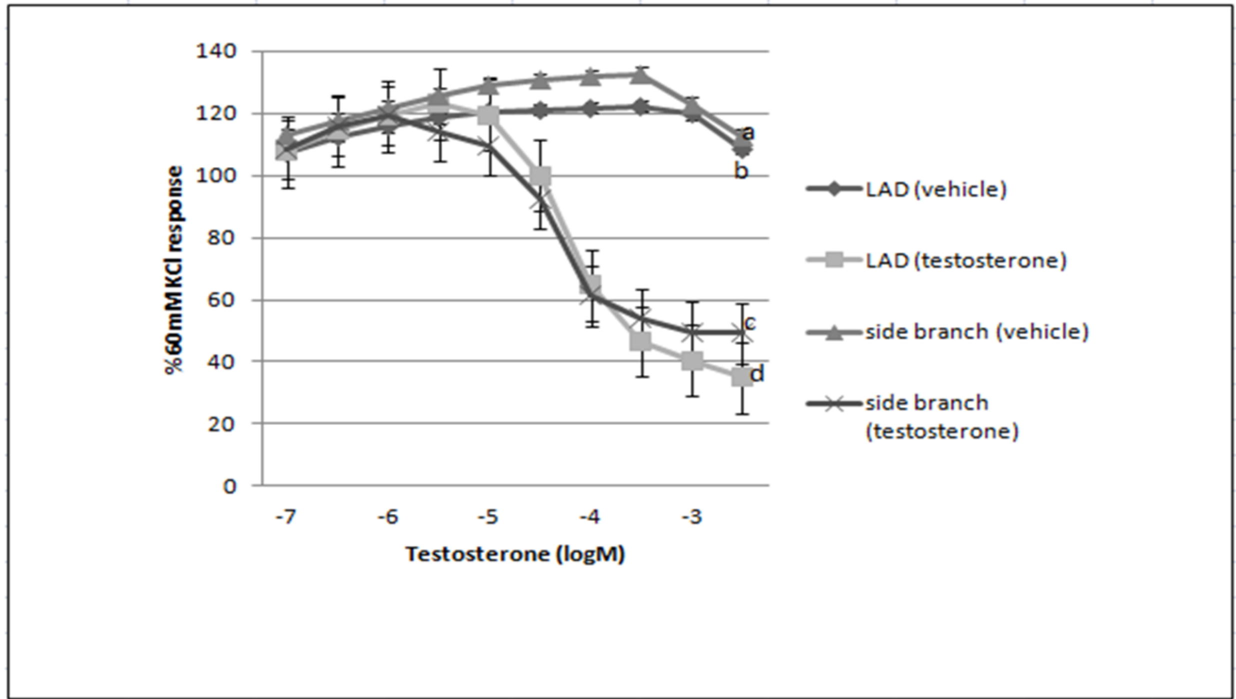

Figure 8: The effect of increasing concentrations of testosterone on anterior descending artery and side branches (n=13). (a, b are significantly different from c, d.). Data are expressed as the mean±SEM ($p<0.05$).

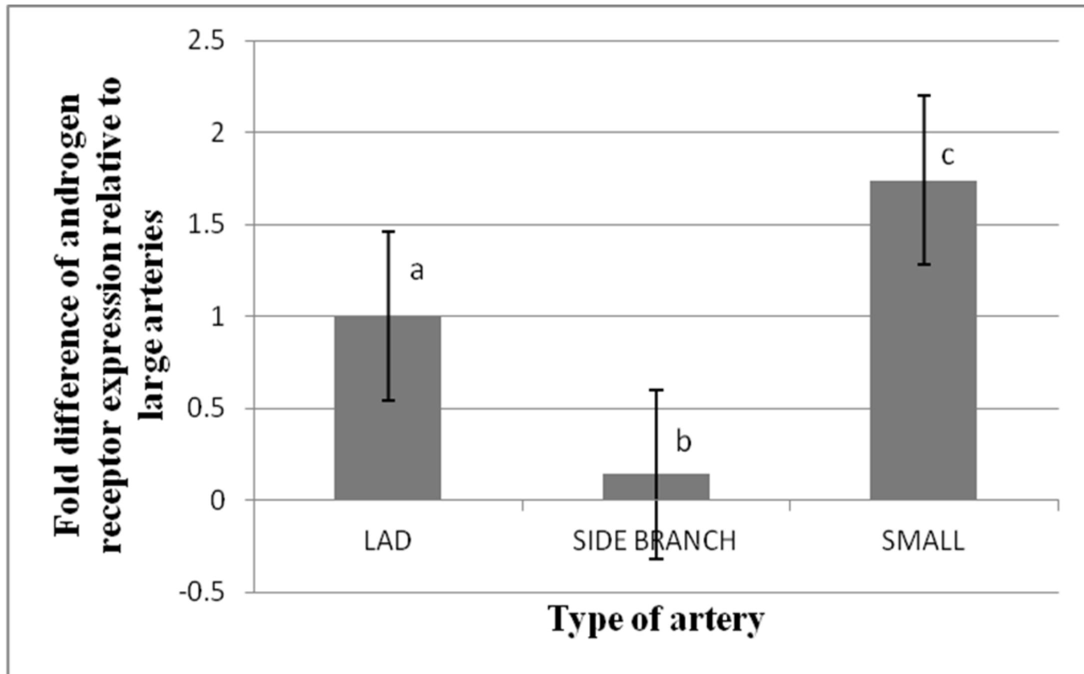

Figure 9: Expression of androgen receptor in coronary arteries (n=3). The changes in mRNA expression for androgen receptor were measured using Ct method. The Ct method compares the fold change in gene expression in control to that of LAD medium and small arteries. Androgen receptor expression was plotted as averages of changes of fold changes for 3 animals in each treatment group. **a** and **c** are not significantly different from each other, however they are significantly different from **b**.

Table 1. The concentrations of RNA isolated from different-sized coronary arteries from different porcine hearts.

Artery	**Concentration of RNA**
Coronary arteries isolated from first porcine heart	
Left anterior descending artery (Large artery)	3.6 µg/ml
Medium-sized coronary artery	3.6 µg/ml
Small-sized coronary artery	4.7 µg/ml
Coronary arteries isolated from second porcine heart	
Left anterior descending artery	4.5 µg/ml
Medium-sized coronary artery	3.7 µg/ml
Small-sized coronary artery	3.3 µg/ml
Coronary arteries isolated from third porcine heart	
Left anterior descending artery	2.6 µg/ml
Medium-sized coronary artery	2.4 µg/ml
Small-sized coronary artery	1.6 µg/ml

Discussion

The present study reports that testosterone induces relaxation in porcine coronary arteries. The results are consistent with those from previous studies which have demonstrated that testosterone causes vasodilation in rabbit coronary arteries and aorta (Yue et al, 1995), rat left anterior descending artery (English et al, 2000), rat thoracic aorta (Ding et al, 2001), canine coronary artery (Chou et al, 1996), porcine left anterior descending artery (Deenadayalu et al, 2001) and in human coronary arteries (Webb et al, 1999b).

The optimization studies indicated that 60mM KCl caused the greatest vasoconstriction in porcine coronary arteries. Although not statistically different from other KCl concentrations, the maximum response was observed at 60mM KCl concentration. Hence we used 60mM KCl as an optimum concentration for testing the viability of coronary arteries. KCl induced contraction in both LADs and medium sized coronary arteries. The KCl induced contraction was greater in LADs compared to medium sized coronary arteries. This may be due to difference in the passive tensions applied on the LADs and medium sized coronary arteries.

In the resting condition, the concentration of potassium is high while the concentration of calcium is low within the intracellular environment. Potassium ions move along their concentration gradient into the extracellular environment through potassium channels and generate a resting potential of -60 mv (Jones et al, 2004). However, the addition of external KCl inhibit the movement of potassium ions by disrupting the potassium gradient, which leads to depolarization of membrane potential and causes activation of voltage dependent calcium channels and an influx of Ca^{2+} ions. Increased intracellular calcium level facilitates actin-myosin interaction leading to smooth muscle contraction.

Similarly sodium nitroprusside (SNP) caused dilation in both LADs and side branches (fig 7).

These results are consistent with Cogolludo et al, (2001) who reported that SNP induces vasodilation by activating soluble guanylate cyclase thereby causing a subsequent increase in cGMP levels. The cGMP further activates SERCA (sarcoplasmic reticulum Ca^{2+}–ATPase) and Na^+/K^+ ATPase causing the opening of potassium channels. cGMP may also inhibit the L-type Ca^{2+} channels and prevent the Ca^{2+} influx through these channels and decrease the intracellular Ca^{2+} and contractile force in arteries (Cogolludo et al, 2001).

We have investigated the response of LAD and medium sized coronary arteries to testosterone. Testosterone induced relaxation of both LADs and medium sized branches (fig 8). When the LADs and side branches were exposed to testosterone there was a gradual increase in the relaxation with the increasing testosterone concentrations.

Testosterone may regulate cellular functions via interaction with an androgen receptor that is present on the target cell. However, most of the testosterone may get converted to dihydrotestosterone at the target cell. The metabolism of testosterone is described in the following flow chart.

Metabolism of testosterone:

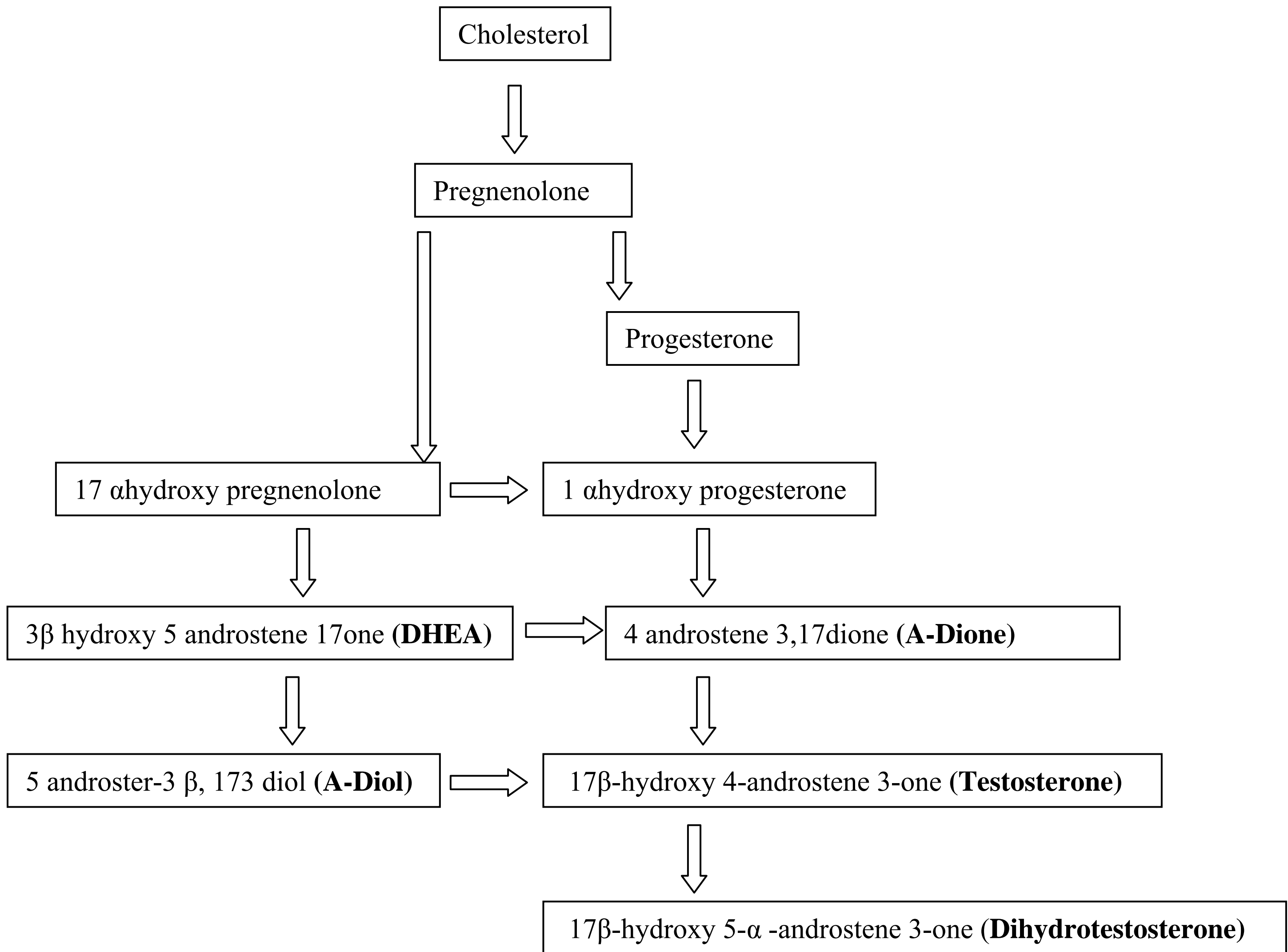

Figure 10: Flow chart describing different steps involved in metabolism of testosterone.

The androgen receptor is a protein of approximately 110 kDa with several domains for androgen binding, nuclear localization, dimerization, DNA binding and transactivation (review by Zhou et al., 1994). The predominant site of localization of AR in the absence of androgen is in the cytoplasm, while ligand presence induces the import of AR into the nuclei. Androgen receptors are also expressed in blood vessels, especially in myocardium (Lin et al., 1990), aorta

(Tamaya et al., 1993) and vascular smooth muscle (Higashiura et al., 1997). Arterial sex hormone receptors are probably involved as part of genomic actions of steroids on the level of the arterial vessel wall. Since LADs showed a significant dilator response to testosterone there might be high expression of androgen receptor in LADs. The expression of androgen receptor in LADs and other coronary arteries was investigated by performing Q-PCR. Data indicated high expression of androgen receptor in LADs compared to medium sized coronary arteries. LADs responded greatly to testosterone compared to the medium sized coronary arteries, this may be due to the high expression of androgen receptors in LADs.

Testosterone may act through nongenomic mechanisms. Non-genomic effects can be indicated as: (i) actions that are too rapid to be compatible with RNA and protein synthesis (i.e. ensue within seconds to minutes from the challenge with the hormone); (ii) actions that can be reproduced in the presence of inhibitors of RNA or protein synthesis; (iii) actions that can be reproduced by using steroid hormones coupled to cell membrane-impermeable molecules; (iv) actions that steroid hormones induce in cells with highly compacted chromatin, in which RNA and protein synthesis are absent (such as spermatozoa); and (v) actions that are elicited by steroid hormones via binding to receptors containing mutations which make them incapable of activating transcriptional processes (Simoncini and Genazzani, 2003).

Thus if the testosterone induced dilation is through nongenomic pathway the effect might be fast and not reduced by flutamide (nuclear androgen receptor blocker). Experiments indicated that testosterone activity is not reduced in presence of flutamide indicating that testosterone acts via nongenomic pathway (Yue et al., 1995). In nongenomic mechanism testosterone might be acting on specific binding sites within the smooth muscle cell. Testosterone might be acting by inhibiting calcium channels and opening potassium channels. Opening of K^+ATP channels

causes the outward flux of potassium, which causes hyperpolarization of membrane, which increases the threshold for cellular depolarization finally causing vasodilation. However, testosterone is also known to induce vasodilation by inhibiting voltage gated calcium channels (VGCC) and store operated calcium channels (SOCC). By acting on these channels testosterone inhibits the entry of calcium into the cell which is necessary for contraction (Jones et al., 2004).

Testosterone induces vasodilation at very low concentration *in vivo* (10-50 nm, Webb et al., 1999). *In vitro* conditions need higher concentrations (10-100 um, Jones et al., 2003) of testosterone to induce vasodilation, where as in isolated cell studies concentration of 1 um was sufficient (Jones et al., 2004). We hypothesized that *in vitro* conditions need higher concentrations of testosterone to induce vasodilation in large arteries but not small arteries. The small coronary arteries may be responding greatly to testosterone due to higher expression of androgen receptors. However, Q-PCR data indicated that there was no difference in expression of androgen receptor in LADs and small coronary arteries. Studies by Webb et al 1999, demonstrated that the response is high in isolated cells of coronary arteries in *in vivo* condition because SHBG is involved in presentation of testosterone to effector proteins. In *in vitro* condition absence of SHBG in target cells contributes to loss of potency (Webb et al., 1999a).

SHBG is a carrier protein which plays an important role in regulating the amount of unbound steroid in the blood. It is a homodimer of subunits that is bound noncovalently and contains one steroid binding site (Petra, 1991). SHBG binds to 17 beta hydroxyl steroids (testosterone and dihydroxytestosterone) in a 1:1 ratio. The majority of the testosterone and dihydroxytestosterone (60%) are bound to SHBG and only 4% remain in unbound state in the serum (Heinlein and Chang, 2002).

SHBG has high affinity for dihydrotestosterone and testosterone and low affinity for

estradiol; however, SHBG binds to dihydrotestosterone twice as strongly as testosterone (Pearlman and Crepy, 1996). SHBG is mainly involved in transportation of testosterone to the target tissue. It is responsible for correct orientation of testosterone within the target membrane. This orientation of testosterone to the smooth muscle cell is critical in determining the potency (Heinlein and Chang, 2002). At the target tissue, the SHBG maintain the steady state concentrations of free hormone, which is necessary for diffusion of steroids across the cell membranes (Mendel, 1992). This passive diffusion of SHBG across the cell membrane is enhanced by SHBG receptors (Rosner, 1990). SHBG receptors promote uptake by either sequestration (Avvakumov, 1991) or endocytosis of SHBG-steroid complex (Porto et al, 1995).

SHBG plays an important role in correct orientation of testosterone within the target membrane thus increasing the potency of testosterone. The response of coronary arteries to testosterone is greater in *in vivo* condition because of the following reasons: 1) SHBG which increases the potency of testosterone is absent in *in vitro* conditions, 2) AR might be inactive and 3) Testosterone is converted to dihydrotestosterone in *in vivo* conditions where as the experiments performed in *in vitro* conditions used testosterone rather than dihydrotestosterone. However, studies in presence of AR blocker and SHBG might be helpful in understanding the mechanism of action of testosterone in detail.

In summary, the goal of this study was to determine the response of different sized coronary arteries to testosterone. Testosterone induces vasodilation in both LADs and side branches. LADs and side branches exhibited similar responses to testosterone. Testosterone might be causing the vasodilation via a nongenomic pathway by acting through the AR. This study was a crucial attempt to describe the relaxation effect of testosterone in smaller coronary arteries. The results obtained form an important foundation for future research in determining whether

testosterone acts differentially throughout the coronary circulation. Further studies involving large, medium and small coronary arteries in the presence of AR blocker and SHBG may be helpful in determining the mechanism of action of testosterone in coronary arteries.

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