

THE ROLE OF NITRIC OXIDE IN TESTOSTERONE-INDUCED
VASODILATION IN PIG CORONARY ARTERIES
AND RAT THORACIC AORTA

A Senior Honors Thesis

by

JASON WILLIAM PIEFER

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment for the designation of

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOW

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Group: Biomedical Science

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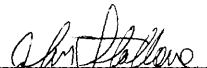
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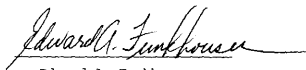
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ABSTRACT

The Role of Nitric Oxide in Testosterone-Induced
Vasodilation in Pig Coronary Arteries
and Rat Thoracic Aorta. (April 2001)

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Several studies have provided evidence that the administration of testosterone to vascular tissue causes vasodilation (Costarella, Yue). This study examines the role of nitric oxide (NO) as a potential mechanism of testosterone-induced vasodilation. This report includes a functional study that indirectly examined the role of NO and a study to determine an appropriate experimental set up to directly measure NO *in vitro*.

In the functional study, the right coronary arteries and left anterior descending arteries from exercised and sedentary, female Yucatan Mini-Swine were treated with N^o-nitro-L-arginine methyl ester (L-NAME) to inhibit nitric oxide synthase (NOS). Following blockade of NOS, seven cumulative doses (5 μ M to 300 μ M) of testosterone produced dose-dependent relaxation and resulted in maximal relaxation. This confirms that NO is not the only mediator of testosterone-induced vasodilation. In addition, this study suggests that NO plays a significant role in the mechanism of testosterone-induced

dilation in coronary arteries of exercised pigs, while it plays little or no role in the coronary arteries of sedentary pigs.

The second part of this study includes the determination of an appropriate experimental set up to directly measure NO release from the thoracic aorta of male and female Sprague-Dawley rats. The experimental set up has to account for the NO-sensitive probe being temperature sensitive, and responding to mechanical stress, acetylcholine (ACh), and background noise. An in vitro experimental set up involving the insertion of a 30 μm probe (World Precision Instruments) into the lumen of the rat thoracic aorta appears to be the most promising method. To test the viability of the set up, the response of the probe to acetylcholine was measured. A detectable response has not been measured, which indicates that the probe should not respond in the presence of tissue unless NO is released. To date, detectable measurements of NO from tissue following the administration of ACh have not been made. This indicates that the experimental set up needs further modification. In combination with functional studies, the direct measurement of NO could definitively determine the role of NO in testosterone-induced vasodilation.

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The assistance of John N. Stallone, Ph.D. (Associate Professor, Department of Veterinary Physiology and Pharmacology, Texas A&M University) is gratefully acknowledged. He provided tissues, equipment, support, and guidance that was essential for this research. As the University Undergraduate Research Fellow Advisor, his sharing of knowledge, expertise, and time resulted in an invaluable research experience for the author.

The author thanks Janet L. Parker M.D. (Professor of Medical Physiology, Texas A&M College of Medicine) for the donation of the tissues for the pig coronary artery experiments.

In addition, the author gratefully acknowledges Min Li (Doctoral Student, Department of Veterinary Physiology and Pharmacology, Texas A&M University) for providing assistance with the pig coronary artery study and for her inestimable support by preparing tissues for the rat thoracic aorta study.

Finally, the author would like to express special thanks to Dr. Wendy Baltzer DVM (Doctoral Student, Department of Veterinary Physiology and Pharmacology, Texas A&M University; Surgical Resident, Texas A&M College of Veterinary Medicine) for her support and assistance with this project.

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INTRODUCTION

The effects of sex steroids (testosterone and estrogen) on the cardiovascular system and their role in cardiovascular health and disease are important topics for scholarly inquiry. This concern has led to numerous epidemiological studies, which indicate that significant differences in cardiovascular disease exist between men and women. These studies indicate that the incidences of heart disease and hypertension are higher in men than in pre-menopausal women (1, 3). This sexual dimorphism has led to vast amounts of basic and clinical research devoted to the study of the effects of estrogen on the cardiovascular system. Most research indicates that estrogen exerts vasodilatory action on the vasculature through rapid non-genomic as well as slower genomic mechanisms (1). However, estrogen also exacerbates some cardiovascular diseases, like Raynaud's Disease and primary pulmonary hypertension, which occur at higher rates in pre-menopausal women than in men (11). The lower incidences of certain cardiovascular health problems in pre-menopausal women, such as heart attack and hypertension, have led to vast amounts of research to determine the mechanisms by which estrogen exerts its protective effects on the vasculature while testosterone was assumed to exert deleterious effects.

However, several research labs have indicated that testosterone may also exert beneficial vasodilatory effects on the vasculature (1, 3, 12). In fact, testosterone causes relaxation of the vasculature in both rat thoracic aorta and rabbit

This thesis follows the style of *American Journal of Physiology – Heart and Circulatory Physiology*.

coronary arteries and aorta (1, 12). The mechanism of this vasodilation is still uncertain. Honda *et al.* suggested that nitric oxide is not a mechanism of testosterone—induced vasodilation in the thoracic aorta of Wistar-Kyoto rats and spontaneously hypertensive rats (3). However, Costarella *et al.* indicated that nitric oxide does play a role testosterone induced vasodilation in Sprague—Dawley rats (1). These discrepancies and the limited amount of research published regarding testosterone—induced vasodilation indicate the need for further research regarding the effects of testosterone on the vasculature and the role of nitric oxide in this vasodilation mechanism.

The present study attempted to identify the role of nitric oxide in the mechanism of testosterone—induced vasodilation. Nitric oxide is a known vasodilator that induces relaxation of the vascular smooth muscle of arteries (4). Originally, an unknown endothelium derived relaxing factor (EDRF) was deemed responsible for the relaxation of the smooth muscle of arteries (4). Later, EDRF was identified as nitric oxide released from endothelial cells and delivered to vascular smooth muscle to cause subsequent relaxation of the vasculature and vasodilation (widening of arteries). The enzyme nitric oxide synthase is responsible for the production of nitric oxide in endothelial cells. Thus, nitric oxide plays a significant role in cardiovascular health. The drug, nitroglycerine is, in fact, an NO—donor that is administered to patients suffering from angina pectoris (chest pain) or congestive heart failure because it vasodilates coronary arteries and provides subsequent relief of angina. Interestingly, testosterone was previously used in a similar manner to relieve chest pain.

This research was directed at determining the role of nitric oxide in two ways. First, functional studies were performed to determine whether nitric oxide played a role in testosterone—induced vasodilation of pig coronary arteries and to determine whether exercise influenced the vasodilation due to testosterone. These functional studies measure the ability of testosterone to cause vasodilation indirectly by monitoring the contractility of the vascular smooth muscle following testosterone administration. The use of nitric oxide synthase blockers and the subsequent response to testosterone administration are used to determine the role of nitric oxide in testosterone—induced vasodilation. The other method for determining the role of nitric oxide in vasodilation due to testosterone would be to directly measure nitric oxide release from endothelial cells. This can be accomplished using an electrode covered with a nitric oxide permeable membrane. This could be a powerful tool to definitively determine the role of nitric oxide in testosterone—induced vasodilation. The focus of this the second portion of the research presented in this study was to determine an experimental set up that would allow for the direct measurement of nitric oxide from the rat thoracic aorta.

METHODS

PIG CORONARY ARTERY FUNCTIONAL STUDY

Experimental animals. Right coronary arteries (RCAs) and left anterior descending arteries (LADs) of three exercised and two sedentary female Yucatan Mini—Swine were donated for this experiment by Dr. Janet Parker from the Texas A&M College of Medicine.

Tissue Preparation. Following the surgical excision of these arteries, they were transported in chilled Krebs—Henseleit bicarbonate buffer (KHB) on ice from Dr. Parker's laboratory to Dr. Stallone's laboratory. Upon arrival, they were immediately transferred to fresh KHB and gassed with 95% O₂/5% CO₂. The composition of KHB was (in millimolar): NaCl, 118.0; NaHCO₃, 25.0; glucose, 10.0; KCl, 4.74; CaCl₂, 2.50; MgSO₄, 1.18; and KH₂PO₄, 1.18 (pH = 7.40, osmolarity = 292 ± 1 mOsmol/kg). All adipose and connective tissue was removed from the arteries and each section of artery was cut into several rings (2 mm long). Care was taken to avoid damage to the endothelial cells lining the lumen or to the vascular smooth muscle of the arteriolar wall. Following the initial cleaning and cutting of the arteries, two rings from the RCA and two rings from the LAD were each mounted on two 25-gauge stainless steel wires. The lower wire was held stationary by a stainless steel rod, while the upper wire was connected to a force-tension transducer (Grass FT-03D; Quincy, MA) connected to a chart recorder (Gould 2600S; Cleveland, OH). This set up allowed for constant monitoring of vascular contractile tension. Following mounting, each ring was suspended in a water-jacketed tissue bath containing 15.0 ml of KHB maintained at

37° C and gassed continually with 95% O₂/5% CO₂. Passive tension was adjusted to 2.50 g over a thirty-minute period following suspension in KHB. After obtaining a stable passive tension, the coronary arteries were allowed to equilibrate for 60 minutes and their baths were changed every twenty minutes with fresh, warmed, gassed KHB. After equilibration, the tissue baths were changed to 80 mM KCl—KHB solution (NaCl exchanged for KCl) to assess maximum contractility. The bath was then changed back to regular KHB and the tissue was allowed to relax and re-equilibrate. Following re-equilibration, a second bath change to 80 mM KCl—KHB was performed in the same manner as the previous bath change. When the tissue had reached a steady maximal contractility, 1×10^{-7} mM Bradykinin (an endothelium dependent vasodilator (5)) was applied to test the endothelial integrity of the artery.

Blocking Nitric Oxide Production. After the initial stabilization and equilibration stages, one RCA and one LAD of each pair was pre-treated with KHB containing 250 μ M N⁻-nitro-L-arginine methyl ester (L-NAME) to block the synthesis of nitric oxide from L-arginine by nitric oxide synthase (NOS). The two control tissues were maintained in regular KHB.

Testosterone Concentration—Response. Following the previous bath changes, the tissues were allowed to stabilize and were then pre-contracted with 10^{-6} M PGF₂ α (a vasoconstrictor) to similar contractile tensions, which were recorded as 100% tension or 0% relaxation. The tissue was then allowed to stabilize to a steady plateau at 0% relaxation due to PGF₂ α . After the stable contractile tension had been obtained, seven doses of testosterone (5-300 μ M) (dissolved in KHB and ethanol) were administered.

The vasorelaxation response was measured as the maximum plateau response following a minimum 30—minute response period with each dose. Vascular responses to testosterone were expressed as a percentage of the contractile response induced by $\text{PGF}_2\alpha$. Previous studies have demonstrated that the dilute ethanol solvent for testosterone (<1%) has no significant effect on the arterial responses to testosterone.

DIRECT MEASUREMENT OF NITRIC OXIDE

Experimental Animals. Dr. Stallone and Min Li donated tissues from male and female Sprague—Dawley rats from Harlan Sprague—Dawley (Indiannapolis, IN). All rats were housed in standard plastic laboratory rat cages at controlled 21- 26° C and 12:12 h light—dark cycles. Purina laboratory chow (Purina Mills, St. Louis, MO) and tap water were provided ad libitum. The primary purpose of using these tissues was to measure nitric oxide release following the administration of the endothelium—dependent vasodilator acetylcholine (ACh). The sex and status of the rats used (i.e. male, female, gonadectomized, etc.) were based upon available tissue and which experiments were being performed in Dr. Stallone's lab.

Calibration of the Nitric Oxide Probes. Three chemi-selective probes sensitive to NO with different diameters and specifications were used during this research, in order to establish an experimental procedure that would allow for the direct measurement of NO from the endothelium of rat thoracic aortas. Each probe (World Precision Instruments) consisted of an electrode enclosed in a stainless steel casing. Each probe was connected to the ISONO Mark II (World Precision Instruments) a nitric oxide meter connected to a Gould chart recorder. A membrane permeable only to nitric oxide covers the electrode

at the tip of the probe. When nitric oxide diffuses through the membrane, it is oxidized at the electrode resulting in an electrical current registered on the nitric oxide meter and recorded on the chart recorder. This current is proportional to the concentration of NO surrounding the membrane. It should be noted that all probes are significantly affected by background noise, mechanical stress and temperature changes.

Probes Tested. Three different probes were tested: ISO-NOP (2mm diameter), ISO-NOP MC (microchip), and ISO-NOP30 (30 μ m diameter). Each had its advantages and disadvantages. The ISO-NOP is easily calibrated, but it is very temperature sensitive, cannot fit inside an arteriolar ring and is easily subject to membrane damage if the membrane contacts anything solid. The ISO-NOP MC is the most sensitive to NO and is only moderately temperature sensitive, but it cannot fit into an intact arteriolar ring. The ISO-NOP30 has a small enough diameter to fit into an arteriolar ring, but it is very fragile and more difficult to calibrate. The ISO-NOP30 is the most reliable probe of the three and thus will be used in future experiments to measure NO release.

Calibration of the ISO-NOP30 nitric oxide probe. First, the probe is suspended from a micromanipulator secured to a steel clamp rod and the probe is lowered into 10 ml of a saturated solution of cupric chloride (approximately 2.4-mM) at 37⁰ C. The cupric chloride solution is maintained at 37⁰ C by installation of the beaker into a water-jacketed glass petri dish with 39.4⁰ C tap water circulated through it. A magnetic stir bar serves the dual purpose of keeping the cupric chloride solution at a constant uniform temperature while also ensuring proper mixing of calibration solutions. A solution of 100.1 μ M S-nitroso-N-acetyl-D,L-penicillamine (SNAP) is made. SNAP is a NO donor

that rapidly decomposes to NO and a disulfide by-product in the presence of cupric chloride (CuCl_2). The decomposition of SNAP will generate known concentrations of nitric oxide in the calibration solution allowing for calibration of the nitric oxide probe. In order to make the SNAP solution, first, 5 mg of EDTA are added to 250 ml of double distilled water and the pH is adjusted to 9.0 using dilute NaOH. Then, 5.6 mg of >98% purity SNAP is added and stirred until dissolved to make the final solution of 100.1 μM SNAP. Three aliquots (5 μl , 10 μl , 20 μl) of SNAP are then added to the 37° C cupric chloride causing changes in the concentration of NO in the calibration beaker, stimulating the probe and causing the subsequent responses on the chart recorder. Following the addition of each aliquot of SNAP, a plateau is reached and the next aliquot is added. The change in the concentration of NO following each addition results in a current in the electrode measured in picoamps on nitric oxide meter and recorded on the chart recorder. The plateau recorded on the chart recorder represents the picoamp (pA) current change in the electrode during the period of time following the administration of SNAP. Each administration of SNAP: 5 μl , 10 μl , and 20 μl results in the generation of a change in NO concentration change of 27 nM, 54 nM and 107.7 nM. The pA response measured from the beginning of each plateau to its maximum peak corresponds to the nM concentration changes previously listed. Thus, the pA response can be graphed as a function of nM NO concentration changes. The linear regression line is the calibration curve. The slope (measured in picoamps/nanomol (pA/nM)) of the calibration curve determines the sensitivity of the NO probe to changes in NO concentration.

Tissue measurements. Following calibration, the probe is immersed into double distilled water for 2 seconds and then transferred to 37° C KHB gassed with 95%O₂/5%CO₂. It is allowed to reach a steady baseline in this solution while the tissue is prepared.

Tissue preparation. Following the harvest of the rat thoracic aorta, it is carefully cleaned and cut (to avoid damage to endothelial cells) into 3mm rings. The rings are placed into cold, gassed KHB. One ring is removed and mounted on two 25 gauge stainless steel wires that are mounted on a nylon base that fits into the bottom of a 10 ml beaker. Following mounting, the beaker is filled with 10 ml of warm, gassed KHB and placed into the tissue bath to ensure a steady 37° C temperature. At this point, the probe is removed from the original KHB and placed into the warm, gassed KHB with the tissue mounted in it. The probe is initially placed away from the tissue to determine the baseline. The probe is then moved and lowered into the lumen of the ring, taking special care to avoid making contact between the ring and the tissue or the stainless steel support rods. Contact with either one could result in damage to the membrane or electrode tip. Then, a steady baseline NO signal is measured. Following the acquisition of a steady baseline, 100 µl aliquots of ACh solutions (10⁻⁵, 10⁻⁴, 10⁻³ M) are added to the buffer. ACh is a known endothelium—dependent vasodilator that causes nitric oxide release. The nitric oxide released from the endothelial cells lining the lumen of the thoracic aorta ring should cause a NO concentration change in the buffer around the probe, and that NO should diffuse through the membrane of the detection probe resulting in a current increase recorded on the chart recorder.

RESULTS

PIG CORONARY ARTERY FUNCTIONAL STUDY

Relaxation due to testosterone. Testosterone administration resulted in total relaxation of all arteries. Seven doses of testosterone were administered to the tissue bath resulting in corresponding concentration changes of 5 μM , 10 μM , 25 μM , 75 μM , 150 μM , and 300 μM , respectively. Both L-NAME and vehicle—treated RCAs and LADs reached maximal relaxation following these seven doses (Tables 1 & 2). However, L-NAME—treated specimens averaged less response at the threshold concentration of 5 μM testosterone (Tables 1 & 2). The variability (standard error (SE)) for these responses was relatively high, probably due to the small number of samples tested ($n = 2-3$). The EC-50, defined as the concentration (μM) at which tissues reached 50% relaxation, provides valuable insight into the role of NO in testosterone—induced vasodilation, as well as the effects of exercise on the vasodilatory response to testosterone (Tables 1 & 2). The mean EC-50 values for L-NAME—treated exercised pigs are higher than those of vehicle—treated exercised pigs, while little difference exists in the mean EC-50 values for sedentary pigs.

Right coronary arteries. RCAs attained total relaxation following the seven treatments of testosterone (Table 1 & Figure 1). Inhibition of NOS in RCAs from exercised pigs resulted in lower response at threshold (Table 1), and decreased relaxation following the first three doses of testosterone (Figure 1). However, inhibition of NOS in RCAs from sedentary pigs did not result in any noticeable difference in the average threshold response (Table 1) or in responses at the lowest three doses of testosterone (Figure 1).

Table 1. *Right Coronary Arteries*

Group	Treatment	Maximal Response	Threshold Response	EC-50
		Mean \pm SE	Mean \pm SE	Mean \pm SE
Exercised	vehicle	101 \pm 0 %	29 \pm 12 %	11.4 \pm 2.9
	L-NAME	101 \pm 1 %	10 \pm 6 %	18.8 \pm 2.5
Sedentary	vehicle	101 \pm 1 %	6 \pm 10 %	25.9 \pm 9.6
	L-NAME	101 \pm 0 %	4 \pm 9 %	23.3 \pm 5.9

Values are means \pm SE (standard error) from 3 exercised pigs and 2 sedentary pigs. Maximal response was measured following the seventh testosterone administration. Threshold response was measured following the first testosterone administration. The EC-50 value represents the average concentration (μ M) at which tissues reached 50% relaxation.

Table 2. *Left Anterior Descending Arteries*

Group	Treatment	Maximal Response	Threshold Response	EC-50
		Mean \pm SE	Mean \pm SE	Mean \pm SE
Exercised	vehicle	101 \pm 1 %	18 \pm 5 %	11.0 \pm 1.9
	L-NAME	101 \pm 0 %	12 \pm 6 %	16.1 \pm 2.2
Sedentary	vehicle	101 \pm 0 %	12 \pm 14 %	18.4 \pm 6.6
	L-NAME	100 \pm 0 %	6 \pm 5 %	26.3 \pm 6.7

Values are means \pm SE from 3 exercised pigs and 2 sedentary pigs. Maximal response was measured following the seventh testosterone administration. Threshold response was measured following the first testosterone administration. The EC-50 value represents the average concentration (μ M) at which tissues reached 50% relaxation.

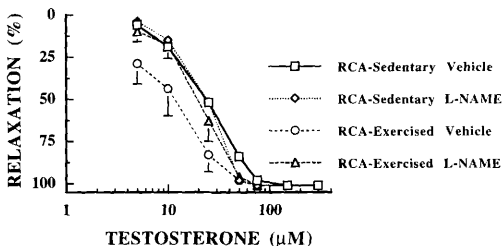


Fig. 1. Concentration-response effects of testosterone in right coronary arteries (RCA) of three exercised pigs and two sedentary pigs. Mean percentage relaxation (RELAXATION (%)) is plotted as a function of testosterone concentration levels (TESTOSTERONE (μM)) at seven different concentration levels for each tissue. Each tissue is an exercised or sedentary RCA treated with L-NAME or the Vehicle (KHB). L-NAME treatment appears to reduce mean relaxation in RCA of exercised pigs, but does not appear to reduce mean relaxation in RCA of sedentary pigs at the first three concentrations (5 μM , 10 μM , 25 μM). Each point represents the mean \pm SE; for the sake of clarity, SE bars were eliminated from some data points.

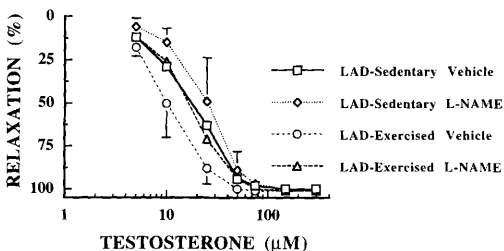


Fig. 2. Concentration-response effects of testosterone in left anterior descending arteries (LAD) of three exercised pigs and two sedentary pigs. Mean percentage relaxation (RELAXATION (%)) is plotted as a function of testosterone concentration levels (TESTOSTERONE (μM)) at seven different concentration levels for each tissue. Each tissue is an exercised or sedentary LAD treated with L-NAME or the Vehicle (KHB). L-NAME treatment appears to reduce mean relaxation in Exercised LAD, and may reduce mean relaxation in Sedentary LAD at the first three concentrations (5 μM , 10 μM , 25 μM). Each point represents the mean \pm SE; for the sake of clarity, SE bars were eliminated from some data points.

Interestingly, the average response to testosterone is similar between L-NAME treated exercised RCAs and both L-NAME and vehicle treated sedentary RCAs.

Left anterior descending arteries. LADs, like RCAs, attained total relaxation following the seven doses of testosterone (Table 2). Inhibition of NOS in LADs from both exercised and sedentary pigs resulted in lower average response at threshold (Table 2) and decreased relaxation following the lowest three doses of testosterone (Figure 2).

This finding differs from that of RCAs, in which there was not a significant difference in response between L-NAME treated and vehicle treated sedentary pigs (Figure 1). The variability (standard error) of vehicle—treated sedentary RCAs and LADs is significantly higher than the variability of other groups (Tables 1 & 2). This may account for the discrepancy between the LADs and RCAs.

Exercised vs. Sedentary. In addition to determining the role of NO in testosterone—induced vasodilation, these experiments allowed for a comparison of the response to testosterone administration between exercised and sedentary pigs. The inhibition of NOS in sedentary tissues did not produce the same results as observed in exercised tissues (Figures 3 & 4). In general, the inhibition of NOS did not have a significant effect on sedentary tissues (Figure 3). Each tissue still reached total relaxation (Table 3) following the seven testosterone administrations. In exercised tissues, the inhibition of NOS resulted in less relaxation response to testosterone than in vehicle—treated exercised tissues (Figure 4). However, all exercised tissue samples reached maximal relaxation following the seven treatments of testosterone (Table 4). The mean response at threshold in exercised tissues was greater in vehicle—treated samples than in

Table 3. *Sedentary Tissue Specimens*

Tissue	Treatment	Maximal Response	Threshold Response	EC-50
		Mean \pm SE	Mean \pm SE	Mean \pm SE
RCA	vehicle	101 \pm 1 %	6 \pm 10 %	25.9 \pm 9.6
RCA	L-NAME	101 \pm 0 %	4 \pm 9 %	23.3 \pm 5.9
LAD	vehicle	101 \pm 0 %	12 \pm 14 %	18.4 \pm 6.6
LAD	L-NAME	100 \pm 0 %	6 \pm 5 %	26.3 \pm 6.7

Values are means \pm SE from the RCAs and LADs of 2 sedentary pigs. Maximal response was measured following the seventh testosterone administration. Threshold response was measured following the first testosterone administration. The EC-50 value represents the average concentration (μ M) at which tissues reached 50% relaxation.

Table 4. *Exercised Tissue Specimens*

Tissue	Treatment	Maximal Response	Threshold Response	EC-50
		Mean \pm SE	Mean \pm SE	Mean \pm SE
RCA	vehicle	101 \pm 0 %	29 \pm 12 %	11.4 \pm 2.9
RCA	L-NAME	101 \pm 1 %	10 \pm 6 %	18.8 \pm 2.5
LAD	vehicle	101 \pm 1 %	18 \pm 5 %	11.0 \pm 1.9
LAD	L-NAME	101 \pm 0 %	12 \pm 6 %	16.1 \pm 2.2

Values are means \pm SE from the RCAs and LADs of 3 exercised pigs. Maximal response was measured following the seventh testosterone administration. Threshold response was measured following the first testosterone administration. The EC-50 value represents the average concentration (μ M) at which tissues reached 50% relaxation.

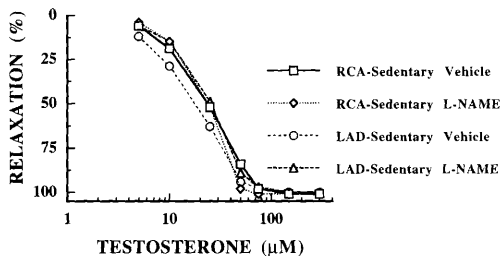


Fig. 3. Concentration-response effects of testosterone in right coronary arteries (RCA) and left anterior descending arteries (LAD) of two sedentary pigs relax. Mean percentage relaxation (RELAXATION (%)) is plotted as a function of testosterone concentration levels (TESTOSTERONE (μM)) at seven different concentration levels for each tissue. Each tissue is an RCA or LAD treated with L-NAME or the Vehicle (KHB). L-NAME does not significantly reduce mean relaxation in RCA or LAD at the first three concentrations ($5 \mu\text{M}$, $10 \mu\text{M}$, $25 \mu\text{M}$). Each point represents the mean \pm SE; for the sake of clarity, SE bars were eliminated.

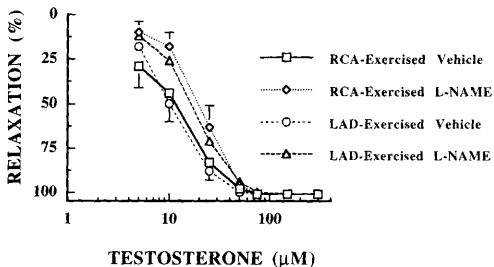


Fig. 4. Concentration-response effects of testosterone in right coronary arteries (RCA) and left anterior descending arteries (LAD) of three exercised pigs. Mean percentage relaxation (RELAXATION (%)) is plotted as a function of testosterone concentration levels (TESTOSTERONE (μM)) at seven different concentration levels for each tissue. Each tissue is an exercised RCA or LAD treated with L-NAME or the Vehicle (KHB). L-NAME reduces mean relaxation in RCA and LAD at the first three concentrations ($5 \mu\text{M}$, $10 \mu\text{M}$, $25 \mu\text{M}$). Each point represents the mean \pm SE; for the sake of clarity, SE bars were eliminated from some data points.

L-NAME treated samples (Table 4) and the response to the first lowest three testosterone treatments was also greater in vehicle—treated tissues as opposed to L-NAME treated tissues (Figure 4). Although the mean response at threshold for sedentary vehicle—treated LADs is lower than that of the other three groups (Table 3), the variability (SE) for both sedentary vehicle—treated LADs and RCAs suggests that these groups do not differ significantly. It should be noted that the mean EC-50 values for exercised pigs (Table 4) are lower than the mean EC-50 values of sedentary pigs (Table 3). In addition, L-NAME—treated exercised tissues reached 50% relaxation at higher concentration than vehicle—treated tissues (Table 4). Whereas, L-NAME—treated sedentary tissues reached 50% relaxation at similar concentrations to vehicle—treated sedentary tissues (Table 3).

DIRECT MEASUREMENT OF NITRIC OXIDE

2 mm probe and microchip probe. The ISONOP 2mm and ISONOP MC (micro chip) probes were the first probes used in an attempt to directly measure nitric oxide release. The major problems with these two probes were their sizes. Neither probe would fit into an intact rat thoracic aorta. Thus, following removal and cleaning of the thoracic aorta, 2mm rings were cut and then cut longitudinally in order for the ring to be laid open with the endothelial surface facing upward. In these experiments, each probe was lowered to within 1 mm of the endothelial cells. However, neither probe provided reproducible results. The 2 mm probe reacted inappropriately ACh, was highly sensitive to temperature, and required a 12 to 16 hour charging time for the electrode if the

membrane was broken and had to be replaced. The microchip probe, although highly sensitive to NO (World Precision Instruments claim it is the most sensitive NO probe on the market) and only moderately sensitive to temperature, was too bulky to get close enough to make nitric oxide measurements from the endothelial cells of tissue specimens.

30 μm probe. The ISONOP 30 μm probe (World Precision Instruments) was the most functionally reliable for the experimental set up. It was calibrated daily using the cupric chloride calibration solution and SNAP to generate calibration curves (Figure 5). At 37° C, the probe sensitivity is generally between 8 and 11 picoamps/nanomolar concentration change. Without tissue present, the ISONOP 30 μm did not respond inappropriately to ACh (Figure 6). Background noise was considerable with all probes tested. Although the background noise seems considerable at 37° C, it is greatly reduced when the probe is lowered into the tissue (Figure 7).

Although the isolated rat aorta should produce detectable amounts of nitric oxide when treated with acetylcholine, it has not yet yielded positive results (Figure 7). This could be due to endothelial damage, lack of time for the tissue to equilibrate to the KHB environment, or decreased sensitivity. In rat superior mesenteric arteries, a 10-16 nM NO concentration change occurred with the addition of acetylcholine (3 μM) and a 17 \pm 5 nM NO concentration change occurred in response to 1 μM acetylcholine (10).

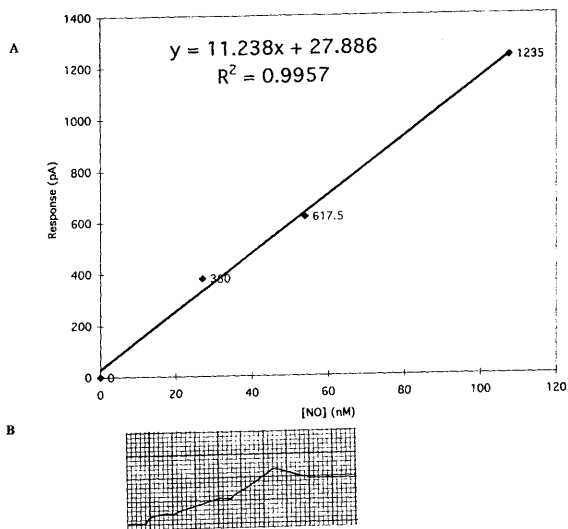


Figure 5. (A) Sample calibration curve following the addition of SNAP to saturated cupric chloride calibration solution. The slope of the curves represents the sensitivity of the probe in (picoamps response)/(nanomolar NO concentration change). (B) Tracing yielding the above calibration curve. Each plateau is followed the addition of 5 μ l, 10 μ l, and 20 μ l of SNAP solution causing known NO concentration changes of 27 nM, 54 nM, and 107.7 nM.

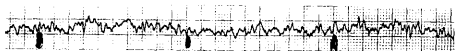


Figure 6. Tracings showing no sensitivity to acetylcholine without tissue present. Solid lines mark the addition of acetylcholine in concentrations of 10^{-5} , 10^{-4} , and 10^{-3} M from left to right.

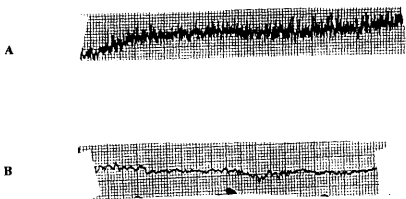


Figure 7. Tracings of responses to the probe in the tissue bath. (A) Probe away from tissue; (B) probe inside lumen of aorta

DISCUSSION

PIG CORONARY ARTERY FUNCTIONAL STUDY

Several research laboratories have demonstrated experimentally that testosterone affects the vasculature in a beneficial manner. Researchers have shown that testosterone relaxes the thoracic aorta in rats (1, 3) and in the rabbit coronary arteries and aorta (12). In addition, the lack of testosterone in genetically engineered mice relates directly to the occurrence of hypertension (7). Furthermore, research indicates that endothelium derived NO may act as a partial mechanism of vasodilation due to the effects of gonadal steroid hormones (11). This research indicates that the dogma that associates estrogen with beneficial vascular effects and testosterone with deleterious vascular effects may be false.

Unlike testosterone, estrogen has received considerable attention due to sexual dimorphism in cardiovascular disease in humans. Pre-menopausal women are at less risk than men for cardiovascular diseases like hypertension and coronary heart disease. In response to these differences, researchers have focused on the effects of estrogen on the vasculature. In addition to being a vasodilator, estrogen also enhance the beneficial response of Ach--induced vasodilation. Studies indicate that NO and calcium--activated potassium channels are the proposed mechanism of estrogen--induced vasodilation (9). This is confirmed by other research indicating NO and cyclic GMP as mediators of vasorelaxation in coronary arteries (2).

This research was approached with these studies involving testosterone and estrogen in mind. The apparent beneficial effects of estrogen on the vasculature and the

apparent beneficial effects of testosterone on the vasculature don't fit into the dogma that views estrogen as beneficial and testosterone as deleterious. Although the mechanism of estrogen—induced vasodilation seems to have been elucidated, research labs studying testosterone differ on their view as to whether NO is a mediator of testosterone—induced vasodilation. Some researchers claim that NO plays no role (3, 8), while others propose that NO may play a significant role (1, 11). These conflicting findings with regard the role of NO as a mechanism of testosterone—induced vasodilation indicate that further research is necessary in order to determine the mechanism of testosterone—induced vasodilation.

In the present investigation, the role of NO in the mechanism of vasodilation following acute testosterone administration was examined in exercised and sedentary pig coronary arteries. This research allowed for an examination of the effects the nitric oxide synthase (NOS) blocker L-NAME on testosterone—induced vasodilation in exercised and sedentary pig coronary arteries.

Role of nitric oxide in exercised pigs. The results of this study suggest that NO plays a partial role in testosterone-induced vasodilation in exercised pigs. The inhibition NOS by L-NAME resulted in a significant decrease in relaxation in the RCAs and LADs of exercised pigs (Figure 4). This finding indicates that nitric oxide participates in testosterone-induced vasodilation; however, the fact that all tissues, both vehicle—treated and L-NAME—treated, reached total relaxation indicates that nitric oxide is not the only mechanism by which testosterone induces vasodilation in pig coronary arteries.

Role of nitric oxide in sedentary pigs. The inhibition of NO did not significantly affect the response of RCAs of sedentary pigs (Figure 3). Interestingly, the inhibition may have affected the response of LADs these tissues (Figure 3). This finding indicates that nitric oxide either plays little or no role in testosterone-induced vasodilation of coronary arteries of sedentary pigs.

Significance. The present findings suggest that nitric oxide acts as a mediator of testosterone-induced vasodilation in pig coronary arteries; however, there must be other mechanisms by which acute testosterone administration causes vasodilation in these arteries. Possible mechanisms include altering potassium channel permeability or direct interaction with receptors on endothelial cells or vascular smooth muscle cells (3). In addition, this study also suggests that nitric oxide production in response to acute testosterone administration is upregulated in exercised pigs as compared to the minor role it may play in sedentary pigs. Due to the small sample size of the present study, these data are not conclusive and will require further testing to definitively determine the effects of exercise as it relates to testosterone-induced vasodilation.

Exercise vs. Sedentary. This study offered the unique opportunity to examine the effects of NO on coronary tissue, as well as the response of tissues from exercised and sedentary pigs to testosterone. Interestingly, these data suggest differences in the response to testosterone between exercised and sedentary pigs. Although both exercised and sedentary tissues attained maximal relaxation following the seven doses of testosterone, in general the sensitivity to testosterone (EC-50 values) of exercised pigs was higher than that of sedentary pigs (Figures 3 & 4). This indicates that the

vasodilatory effects of testosterone are upregulated in exercised pigs. In addition, these data suggest that nitric oxide plays a role in exercised pigs, but not sedentary pigs, and that NO increases the sensitivity of exercised pigs to testosterone.

DIRECT MEASUREMENT OF NITRIC OXIDE

Although not as fruitful as the pig coronary artery study, the results of this study will provide very useful for future research. The experimental set up that resulted from this research should provide a strong foundation for future direct measurements of NO released from the endothelial cells of intact rat thoracic aortas. Some possible solutions to the current problems include: shielding the experimental set up with a Faraday cage, redesigning the experiment to incorporate a magnetic stirrer to ensure rapid and uniform mixing of solutions, or purchasing a new chart recorder that can allow for larger scale changes. However, even if none of these adjustments are implemented, the experimental set up should theoretically work. Thus, human error may play a significant role in the failure of this experimental set up to yield reliable, reproducible results.

CONCLUSION

PIG CORONARY ARTERY FUNCTIONAL STUDY

Primary findings. The primary findings of this study suggest that (1) testosterone does cause concentration dependent vasodilation of pig coronary arteries (2) NO plays a partial role in testosterone-induced vasodilation in exercised pigs; (3) NO plays little or no role in testosterone-induced vasodilation in sedentary pigs; and (4) NO is not the sole mediator of testosterone-induced vasodilation in either sedentary or exercised pigs.

Given the clinical importance of exercise on cardiovascular health, it is significant that nitric oxide release due to testosterone may be upregulated in exercised subjects. An increased production of nitric oxide due to testosterone administration in exercised tissues suggests that testosterone treatments given to patients will have beneficial effects on their cardiovascular, possibly reducing blood pressure and decreasing the risk of hypertensive crises. Thus, physicians with patients in need of hormonal treatments may prescribe a parallel daily exercise regimen to help reduce the risk of hypertensive problems. In addition, physicians may prescribe testosterone treatments to healthy, exercising patients without worrying about hypertensive complications.

DIRECT MEASUREMENT OF NITRIC OXIDE

The experimental set up proposed may need some minor modifications. However, the majority of work to set up a reliable protocol for an experiment to directly measure nitric oxide from rat thoracic aortas is now complete. Experiments using both functional studies and studies implementing the direct measurement of nitric oxide should be able to definitively determine the role of NO in testosterone—induced vasodilation.

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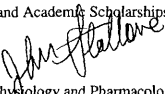
APPENDIX

APPENDIX

MEMORANDUM

DATE: April 12, 2001

TO: Mary Scott
Administrative Assistant
Office of Honors Program and Academic Scholarships
MS# 4233 TAMU

FROM: John N. Stallone, Ph.D. 
Associate Professor
Department of Veterinary Physiology and Pharmacology
College of Veterinary Medicine
MS#4466

SUBJECT: Animal Use Approval - Undergraduate Research Fellows Program

This memo addresses approval of animal use by Jason W. Piefer, for his project as a Texas A & M University Undergraduate Research Fellow. Mr. Piefer is performing his research project in my laboratory, and is working to determine the mechanism by which the gonadal steroid hormone testosterone produces arterial vasodilation. Most of the experiments he performed utilized vascular tissues obtained from our experimental rats. The methods for Jason's experiments are identical to those which we routinely use in our laboratory and which have been approved by the University Laboratory Animal Care Committee (ULACC; please see attached memo of approval). Several additional experiments were also performed using sections of coronary arteries obtained from exercise-trained pigs, which were provided by Dr. Janet Parker, in the Department of Medical Physiology (Dr. Parker is also a sponsor in the Undergraduate Research Fellows Program), and she graciously provided us with tissues from exercise-trained and sedentary-control pigs, in order to examine the effects of exercise training on testosterone-induced vasodilation. Dr. Parker's experiments have also been approved by ULACC and her experiments involve identical methods to those used by Jason in his experiments. Thus, all of Jason's experiments involve ULACC-approved methods and animals.

VITA

PERSONAL INFORMATION

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I am currently a Senior majoring in Biomedical Science in the College of Veterinary Medicine at Texas A&M University. I expect to graduate with a Bachelor of Science in Biomedical Science in May of 2001. I expect to graduate with the following honors: University Undergraduate Research Fellow, University Honors, and Suma Cum Laude.

Following graduation, I plan to attend Baylor College of Medicine in Houston, Texas. I have been accepted for entrance in August 2001 and expect to graduate with a Doctor of Medicine with the Class of 2005.

TEXAS A&M UNIVERSITY
University Laboratory Animal Care Committee

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Dr. Duncan S. MacKenzie, Chair
Melissa Taylor, Staff Assistant

214, Jack K. Williams Administration Building

July 18, 2000

MEMORANDUM

TO: Dr. John N. Stallone
VTPP
MS 4466



FROM: Dr. Duncan S. MacKenzie, Chair
University Laboratory Animal Care Committee

SUBJECT: Approval of Amendment to Animal Use Protocol #1999-102
Title: "Sexual Dimorphism in Constrictor Prostanoid-Potentiated Contraction:
Roles of Endothelium and Estrogen"

This is to inform you that the University Laboratory Animal Care Committee has approved the attached amendment. Your copy should be retained for future reference.

The Committee thanks you for your efforts to keep the ULACC informed of any changes to your protocol. If we can be of any further assistance, please contact me or Melissa Taylor, Staff Assistant, at 845-1828.

Best of success in your research endeavors.

DSM/jjb

cc: Housing Facility: John Park, LARR, MS 4473
Campus Veterinarian: Dr. Richard Ermel, Director-LARR
ULACC file