Endogenous testosterone attenuates neointima formation after moderate coronary balloon injury in male swine

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Aims Previous studies from our laboratory have demonstrated that testosterone increases coronary smooth muscle protein kinase C delta (PKCδ) both in vivo and in vitro and inhibits coronary smooth muscle proliferation by inducing G0/G1 cell cycle arrest in a PKCδ-dependent manner. The purpose of the present study was to determine whether endogenous testosterone limits coronary neointima (NI) formation in a porcine model of post-angioplasty restenosis.

Methods and results Sexually mature, male Yucatan miniature swine were either left intact (IM), castrated (CM), or castrated with testosterone replacement (CMT; Androgel, 10 mg/day). Angioplasty was performed in both the left anterior descending and left circumflex coronary arteries with balloon catheter overinflation to induce either moderate (1.25–1.3x diameter; 3 × 30 s) or severe (1.4x diameter; 3 × 30 s) injury, and animals were allowed to recover for either 10 or 28 days. Injured coronary sections were dissected, fixed, stained (Verheoff-Van Gieson, Ki67, PKCδ, p27), and analysed. Vessels without internal elastic laminal rupture were excluded. Following moderate injury, intimal area, intima-to-media ratio (I/M), and I/M normalized to rupture index (RI) were increased in CM compared with IM and CMT. RI, medial area, and intimal/medial thickness (IMT) were not different between groups. NI formation was inversely related to serum testosterone concentration. Conversely, following severe injury, there were no significant differences between the groups. Testosterone inhibited proliferation and stimulated PKCδ and p27kip1 expression during NI formation (10 days post-injury).

Conclusion These findings demonstrate that endogenous testosterone limits coronary NI formation in male swine and provides support for a protective role for testosterone in coronary vasculoproliferative diseases, such as restenosis and atherosclerosis.

KEYWORDS Coronary; Vascular smooth muscle; Testosterone; Angioplasty; Restenosis; Porcine

1. Introduction

The role of endogenous testosterone in men’s health is controversial, especially with regard to cardiovascular disease. It has been noted for decades that men, 30–50 years of age, have an increased incidence of coronary artery disease (CAD) compared with women of similar age.¹⁻³ This sex difference in the prevalence of CAD led to the widespread belief that testosterone increases the risk of heart disease in men. However, recent clinical studies have failed to support a detrimental effect of testosterone on the incidence or severity of CAD⁴⁻⁶ or carotid atherosclerosis⁷ in men. On the contrary, a growing body of epidemiological and clinical trial data indicates that low testosterone levels in men are associated with a higher risk of cardiovascular disease.⁸ For example, both low testosterone levels and free androgen index have been reported in men with CAD,⁴⁻⁶,⁹,¹⁰ aortic atherosclerosis,¹¹ and carotid atherosclerosis.¹² The prospective EPIC Norfolk study followed 11 606 men for 6–10 years and found an inverse relationship between endogenous testosterone levels and overall mortality and cardiovascular disease.⁴ Men in the lowest quartile of testosterone had an approximately two-fold greater CAD risk vs. men in the highest quartile.⁴ Similarly, low testosterone levels are associated with increased risk factors for cardiovascular disease, especially obesity, hypertension, hyperglycaemia, and hypercholesterolaemia.⁶,⁷,¹² Conversely, higher levels of...
endogenous testosterone are associated with a favourable CV risk profile, including elevated HDL cholesterol, and reduced blood pressure, triglycerides, and glucose. These findings suggest that testosterone may limit the progression of CAD indirectly through beneficial modification of risk factors, independent of direct androgenic actions on the vascular wall. However, covariate-adjusted analysis indicates an independent, beneficial effect of testosterone on CAD disease in men. Furthermore, testosterone produced significant reductions of neointimal (NI) plaque development in aortas of male rabbits \textit{in vitro} \cite{21} and \textit{in vivo} \cite{15} implicating a direct effect of testosterone on the coronary vascular wall.

Accumulation of smooth muscle cells in the intima is a hallmark of coronary atherosclerosis and post-angioplasty restenosis. \cite{15, 16} Since smooth muscle proliferation and apoptosis coincide in arteriosclerotic lesions, the balance between these two processes determine SMC accumulation during vascular remodelling and lesion development.\cite{17-19} Smooth muscle cell proliferation is tightly regulated by the complex interaction of numerous cell-cycle regulatory proteins at specific check points of cell growth.\cite{20} These cell cycle regulatory proteins are influenced by kinase-signalling pathways, including PKC.\cite{21} Overexpression of PKC\(\alpha\) inhibits growth rates and proliferation of rat aortic smooth muscle cells.\cite{21, 22} We have previously shown that testosterone increases PKC\(\alpha\) in porcine coronary smooth muscle \textit{in vivo} and that both testosterone and dihydrotestosterone (DHT) increase PKC\(\alpha\) expression and activity in coronary smooth muscle \textit{in vitro}.\cite{23, 24} Testosterone induced a PKC\(\alpha\)-dependent G1/S phase cell cycle arrest and stimulated apoptosis in coronary smooth muscle, providing a potential mechanistic basis for epidemiological observations regarding effects of endogenous testosterone on coronary vasculoproliferative diseases. The porcine coronary overstretch injury model, widely recognized as the most appropriate model for studying post-angioplasty restenosis, produces a medial injury and development of a smooth muscle-rich NI nearly identical to what is seen in humans.\cite{25-27} Thus, the purpose of the present study was to determine if endogenous testosterone alters coronary smooth muscle hyperplasia \textit{in vivo} using a porcine model of post-angioplasty restenosis.

## 2. Materials and methods

### 2.1 Animals

Sexually mature male Yucatan swine were obtained from the breeder (Sinclair Research Farm, Columbia, MO, USA) and housed at the College of Veterinary Medicine. Animal protocols were approved by the University of Missouri Animal Care and Use Committee in accordance with the ‘Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training’ published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.2 Castration and hormone replacement

Castration and hormone replacement was performed as described previously.\cite{24, 28} Males were castrated (CM) and subsequently randomised to receive testosterone replacement (CMT; Androgel, Solvay Pharmaceuticals, 10 mg/day) or vehicle. Testosterone replacement occurred at the time of castration to avoid disruption of hormonal influence.

### 2.3 Coronary balloon angioplasty

Animals underwent coronary intervention as described previously\cite{29} 5-6 weeks after castration. A 6F HS or LCB SH guide catheter (Boston Scientific) was introduced through a 7F sheath placed in the right femoral artery, and positioned at the left main coronary. Angiograms of both the left circumflex (LCX; RAO 40) and left anterior descending (LAD; LAO 30, cranial 30) arteries were obtained. Coronary artery diameters were measured using quantitative angiography software (Infiniti). Intravascular ultrasound (IVUS) pullbacks were obtained in both the LCX and LAD prior to angioplasty and at the time of sacrifice using a 20 MHz catheter at a pullback rate of 1 mm/s (Volcano Therapeutics). Coronary injury was induced by performing either a moderate (1.25-1.3x) or severe (1.4x) balloon (Maverick, Boston Scientific) overinflation three times for 30 s each, waiting 1 min between inflations. Sites of injury were selected based on homogeneity of diameter and proximity to anatomical landmarks (generally between the first and second diagonal in the LAD and between the first and second obtuse marginal branches in the LCX). Balloon lengths (15 or 20 mm) were chosen based on target vessel uniformity. Angiograms were obtained with the balloon catheter in place prior to inflation to allow identification during dissection. Following injury, femoral access sites were closed and swine were allowed to recover for either 10 or 28 days.

### 2.4 Histology and morphology

At the time of sacrifice, animals were sedated and anaesthetized as prior. The hearts were removed and placed in Krebs bicarbonate solution (4°C) during coronary dissection. Injured segments were identified by anatomical landmarks from angiograms obtained at the time of coronary injury, dissected, and fixed in 10% paraformaldehyde. Spatially calibrated digital images of Verhoff Van Gieson (VVG)-stained sections of coronary arteries were obtained. Analysis of vessel morphometry was performed independently by at least two blinded investigators using Image J software (Scion Image). Vessel area was measured as the area defined by the external elastic lamina (EEL) and NI area calculated [vessel area \(\times\) (lumen area + medial area)]. In the swine model of coronary restenosis, the extent of NI formation is directly proportional to the extent of IEL disruption (i.e. IEL rupture); thus, it is necessary to normalize the NI formation to the degree of IEL damage when comparing between intervention groups. Rupture length (RL) was measured as the length of discontinuity of the injured IEL, and the extent of injury was normalized to the rupture index (RI = RL/IEL).\cite{30} The NI was normalized as [intimal area/medial area]/RI. The intima-to-media thickness ratio (IMT) was calculated by dividing the measurement of the thickest portion of the NI from the lumen to the EEL boundary by the uninjured media opposite the injury. Coronary remodelling index (CRI) was calculated as the ratio of vessel volume\(_{norm}\)/vessel volume\(_{norm}\). Vessel volume [V\(V\); defined by the EEL] was determined from three-dimensional reconstruction of the pre- and post-IVUS images over the injured area (Qvus, Medis).

### 2.5 Immunohistochemistry

Immunohistochemistry was performed as described previously.\cite{31} Sections were incubated with avidin-biotin two-step blocking solution (Vector SP-2001) to inhibit background staining and in 3% hydrogen peroxide to inhibit endogenous peroxidase. Primary antibodies, K667 (1:200, Zymed), p27 (1:400; Santa Cruz), and PKC\(\alpha\) (1:400; Santa Cruz) were incubated overnight at 4°C. Ki-67 is a nuclear protein expressed in G1-, S-, and G2 phases of the cell cycle but is absent in the G0-phase and thus indicative of proliferation.\cite{32} After appropriate washing steps, sections were incubated with biotinylated secondary antibody in phosphate-buffered saline containing 15 mM sodium azide and peroxidase-labelled streptavidin (Dako LSAB\(+\) kit, peroxidase, K0690). Diaminobenzidine (DAB, Dako) was applied for 5 min to

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visualization of the reaction product. Ki67 and p27kip1 sections were counterstained with haematoxylin. Sections were photographed with an Olympus BX40 photomicroscope and Spot Insight Colour camera (Diagnostic Instruments). The relative area and mean density of positive staining for PKCα were determined for each area of interest. Quantification of p27kip1 and Ki67 (proliferation index) was performed as a percent of total nuclei that was stained utilizing ImagePro Plus (Media Cybernetics).

2.6 Hormone assays
Blood samples (5 mL) were collected at the time of surgery prior to castration and at the time of sacrifice. Samples were collected into plain tubes, centrifuged at 1000 rpm for 5 min and the serum decanted and frozen at −80°C until analysis. Testosterone was determined by radioimmunoassay (RIA) with a commercially available kit (Diagnostic Products, Los Angeles, CA, USA). Essentially, a solid-phase 125I RIA was utilized with a sensitivity of 4 ng/dL and an inter-assay and intra-assay coefficient of variation of 8% and 6%, respectively. Cross-reactivity studies to the antibody established that the RIA was specific for testosterone with only 19-nortestosterone cross-reactively. Parallelism studies were also carried out with pooled boar serum, both at the high and low ends of the standard curve, resulting in good linearity.

2.7 Statistics
Data are expressed as mean ± SEM. Group comparisons were made by analysis of variance using SPSS 15 software (SPSS, Inc., Chicago, IL) and post hoc analyses applied when significantly main or interaction effects were determined. A P-value ≤ 0.05 was set as the criterion for significance in all comparisons.

3. Results
3.1 Serum testosterone levels
Animals were of similar age (7–8 months) at the time of sacrifice. Body weights were similar in intact, orchietomized, and hormone-replaced males at the time of sacrifice (28.6 ± 1.34, 31.5 ± 1.4 and 31.9 ± 0.8 kg, respectively). Heart weights (IM, 147.8 ± 8.3; CM, 166.4 ± 6.3; and CMT, 167.9 ± 7.6 g, respectively) and heart weight to body weight ratios (IM, 5.2 ± 0.2; CM, 4.7 ± 0.0.5; and CMT, 5.26 ± 0.2 g, respectively) were also similar among groups. Total serum testosterone levels in intact male swine are similar to those found in human males (200–1300 ng/dL, 7–44 nM).33,34 Castration reduced circulating testosterone levels >90% (Figure 1A). Testosterone replacement in orchietomized males maintained serum testosterone concentration similar to, but significantly higher than, intact males. Prostate to body weight ratios were decreased by castration, an effect partially reversed by testosterone replacement (Figure 1B), indicating a direct association between circulating and bioavailability of testosterone in each of the groups.

3.2 Effect of testosterone on neointimal response to moderate injury
Moderate injury (1.25–1.3x overinflation) was performed on 43 vessels in 24 pigs (eight animals per group). One IM pig died of unknown causes post-surgery, and was excluded from analysis. In the remaining 23 animals, 41 vessels underwent injury with 22 (54%) demonstrating a ruptured IEL and included in analysis. SubstantialNI formation in porcine coronary arteries following balloon injury requires disruption of the IEL, therefore vessels without IEL injury were excluded from analysis. There was no difference in response between LAD and LCX, therefore these arteries were pooled within each group. Vessel, lumen, and medial areas were not different between groups (Table 1). Consistent with the findings of others using the coronary balloon injury model,25–27 balloon injury produced a smooth muscle-rich NI as demonstrated by robust expression of α-smooth muscle actin (α-SMA) in the NI (Figure 2B). RL and RI were similar among groups, indicating a consistent injury stimulus between groups (Table 1). Figure 3 provides representative VVG-stained sections of injured coronaries 28 days post-angioplasty from IM, CM, and CMT animals (left panels) and corresponding group data for intimal formation (right panel). IEL rupture resulted in significant NI formation in all groups. However, the intimal area, intima-to-medial ratio (I/M), and normalized intima-to-medial ratio (IM/RI) were greater in CM when compared with both IM and CMT groups. A direct, inverse relationship between serum testosterone concentration and NI formation is demonstrated in Figure 4. Balloon angioplasty resulted in significant positive remodelling as demonstrated by an increase in vessel volume (Vv) 28 days post-injury (Figure 5). Testosterone status had no effect on pre- or post-injury vessel volume or CRI, indicating no relationship between endogenous testosterone levels and vessel remodelling.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Serum testosterone and prostate status. (A) Serum testosterone (T) values from intact male (IM, n = 11), castrated male (CM, n = 11), and castrated male with testosterone (CMT, n = 11) at the time of sacrifice. (B) Corresponding prostate to body weight ratios (PW/BW). Values are mean ± SEM. *P < 0.05 vs. all.
3.3 Effect of testosterone on neointimal response to severe injury

Severe injury (1.4x overinflation) was performed on 22 vessels in 12 pigs (four animals per group) with 20 (91%) demonstrating a ruptured IEL and subsequently included in the analysis. Increased severity of the injury with 1.4x overinflation is demonstrated by a greater RI, intimal area, and IM/RI compared with moderate injury (Table 1). Figure 6 provides representative VVG-stained sections of severely injured coronaries 28 days post-angioplasty from IM, CM, and CMT animals (left panels) and corresponding group data for intimal formation (right panel). Contrary to moderate injury, testosterone status had no effect on intimal area, I/M, or IM/RI with severe injury.

3.4 Effect of testosterone on neointimal development

We have previously shown that testosterone inhibits coronary smooth muscle cell proliferation and increases expression of the cyclin-dependent kinase inhibitor, p27kip1, in a PKCδ-dependent manner.35 Therefore, we determined the levels of PKCδ, p27kip1, and Ki67 in coronary arteries 10 days following moderate injury additionally in four, four, and six animals (IM, CM, and CMT, respectively). We selected this time point based on previous studies using coronary balloon overstretch injury, which demonstrated this time frame to be associated with peak NI proliferation following balloon angioplasty in porcine coronary arteries.36 Testosterone significantly reduced the NI proliferation index, as indicated by less Ki67 positive nuclei in IM and CMT compared with CM (Figure 7). In uninjured coronary artery media, p27kip1 expression was high (Figure 8A) and similar between groups (data not shown). Consistent with an inhibition of NI proliferation, p27kip1 expression was

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**Table 1** Coronary artery morphometry 28 days post-angioplasty

<table>
<thead>
<tr>
<th>Injury</th>
<th>Vessel area (mm²)</th>
<th>Lumen area (mm²)</th>
<th>Medial area (mm²)</th>
<th>Intimal area (mm²)</th>
<th>Rupture length (mm)</th>
<th>Rupture index</th>
<th>I/M ratio</th>
<th>IM/RI</th>
<th>IMT/RI</th>
<th>IMT ratio</th>
<th>I/M ratio</th>
<th>IM/RI</th>
<th>CMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate</td>
<td>6.08 ± 1.14 (7)</td>
<td>3.66 ± 1.22 (7)</td>
<td>1.53 ± 0.21 (7)</td>
<td>0.89 ± 0.25 (7)</td>
<td>1.25 ± 0.18 (7)</td>
<td>0.19 ± 0.20 (7)</td>
<td>0.59 ± 0.15 (7)</td>
<td>3.23 ± 0.54 (7)</td>
<td>0.93 ± 0.96 (7)</td>
<td>22.33 ± 3.03 (7)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Severe</td>
<td>6.08 ± 0.97 (6)</td>
<td>3.71 ± 0.88 (6)</td>
<td>1.81 ± 0.27 (6)</td>
<td>0.48 ± 0.08 (6)</td>
<td>2.83 ± 0.54 (6)</td>
<td>2.35 ± 0.20 (7)</td>
<td>0.59 ± 0.15 (7)</td>
<td>3.23 ± 0.54 (7)</td>
<td>0.93 ± 0.96 (7)</td>
<td>22.33 ± 3.03 (7)</td>
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</table>

IM, intact male; CM, castrated male; RI, rupture index; I/M ratio, intima-to-media ratio; IMT, intimal/medial thickness.

*p < 0.05 vs. IM and CMT.

†P < 0.05 vs. IM and CM.

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Figure 2: Coronary balloon injury produces a smooth muscle-rich neointima (NI). Representative photomicrographs of coronary arterial sections stained for (A) collagen/elastin (Verheoef-Van Gieson, VVG) and (B) α-smooth muscle actin (α-SMA) 28 days following moderate balloon injury in a castrated male. Arrowheads indicate ruptured ends of internal elastic lamina (IEL) induced by balloon overinflation. The NI (n) formed expresses high levels of α-SMA, similar to the uninjured media (m), consistent with the predominant role of smooth muscle in NI formation. α-SMA expression was similar among groups (data not shown).
greater in the NI of injured coronary arteries in IM and CMT compared with CM (Figure 8B and E). Our previous data demonstrate that testosterone stimulates PKCδ expression and activity and that testosterone-induced changes in proliferation and p27kip1 levels are PKCδ-dependent.24,35 Accordingly, we found that PKCδ expression was greater in the NI of IM and CMT compared with CM (Figure 9B and E). Together, these data are consistent with testosterone inhibition of NI development post-angioplasty by a PKCδ-dependent inhibition of smooth muscle proliferation, mediated, in part, via sustained p27kip1 expression.

4. Discussion
The present study provides the first evidence that endogenous testosterone limits coronary NI response in the porcine model of post-angioplasty restenosis. Specifically, NI formation was inversely related to serum testosterone levels with moderate, but not severe injury. Furthermore, this inhibition of NI formation was associated with an increased PKCδ and p27kip1 and reduced proliferation, similar to that previously shown in vitro.24,35 We chose the porcine post-angioplasty restenosis model for two reasons. First, the NI formed is primarily a smooth muscle hyperplastic response,26 and as such, provides an in vivo model for coronary smooth muscle proliferation and migration, common to vasculoproliferative diseases, such as atherosclerosis and restenosis. Secondly, the swine model is the pre-eminent non-primate model for human post-angioplasty restenosis.26,27 Not only do swine have similar coronary vessel anatomy, medial thickness, and endothelial to smooth muscle ratios,17 but the NI that develops in response to injury is nearly identical to what is seen in humans.27 Therefore, the results obtained using the porcine model of post-angioplasty restenosis provide the best translational potential for human CAD and restenosis treatments.

Accumulation of smooth muscle in the intima is a hallmark of coronary atherosclerosis and post-angioplasty restenosis.15,16 Since smooth muscle proliferation and apoptosis coincide during NI formation, the balance between these two processes determine smooth muscle accumulation during vascular remodelling and net lesion development.17–19 The attenuation

Figure 3  Effect of testosterone on neointimal response to moderate injury. (Left panel) Representative photomicrographs of Verheoff-Van Gieson (VVG)-stained coronary arteries 28 days following moderate balloon injury in intact male (IM), castrated male (CM), and CM with testosterone (CMT). n, neointima; m, media; breaks in IEL are indicated by arrowheads. (Right panel) Group data for intimal area, intima-to-media ratio (I/M), and intima-to-media ratio normalized to rupture index (IM/RI). All data are means ± SEM. n Values as in Table 1. *P < 0.05 vs. IM and CMT.
Testosterone and coronary neointima formation

We have previously shown that endogenous testosterone increases PKCδ protein levels in coronary smooth muscle of swine and that both testosterone and DHT increase PKCδ expression and activity in coronary smooth muscle in vitro, consistent with the present study where testosterone increased PKCδ levels in the NI. Thus, both in vitro and in vivo data support beneficial effects of endogenous testosterone on coronary vasculoproliferative disease.

The observed attenuation of NI formation in the present study is consistent with that found in a rabbit model of atherosclerosis, but is in contrast with Chen et al., who found no effect of castration, with or without testosterone replacement, on carotid NI formation in the rat. Numerous factors could contribute to this apparent contradiction, including differences in species, vessel, and type of injury. It is well-established that fundamental differences exist in the underlying mechanisms of NI formation between species. Similarly, the rat carotid exhibits only a minor inflammatory response to injury, whereas human, swine, and non-human primates demonstrate a robust inflammatory response, which is positively related to NI formation. Another key difference between rodent and large mammal models is the relative influence of the endothelium on vascular wall remodelling. In rodents, endothelial denudation or injury with a compliant balloon or wire produces substantial NI formation. This apparent dominant influence of the endothelium on vascular wall remodelling in rodent models is consistent with the much higher endothelial to smooth muscle ratio in rodent arteries compared with large mammals. Conversely, substantial NI formation in the pig, non-human primates, and humans following injury requires disruption of the internal elastic lamina. The porcine overstretch injury model induces a medial tear, allowing for the development of substantial lesions and increased smooth muscle cell proliferation, which is identical to that seen in humans. Touchard and Schwartz compared several animal models of restenosis concluding that the porcine overstretch injury model was superior at mimicking lesion development in human coronaries, and therefore was the most appropriate model for studying post-angioplasty restenosis in human patients. Thus, the findings of the present study in swine provide a unique insight regarding the potential coronary response in humans.

Differences in vascular smooth muscle responses to injury in rodent and pig models may also arise because of innate phenotypic differences in vascular smooth muscles. A major influence on the smooth muscle response may be the developmental origin of the smooth muscle cells comprising different vascular beds, e.g. carotid vs. coronary. Coronary smooth muscle cells uniquely arise from the proepicardium during development and are thus separate and distinct from the systemic vasculature. Differences in developmental origin have been shown to produce persistent differences in phenotype, including differing proliferative responses to mitogenic stimulation. Vessel-specific responses to vascular injury have also been noted in coronary and carotid arteries.

Species differences also exist in the innate phenotype and response to stimulation of vascular smooth muscles. For example, the media of the rat has been proposed to consist of two distinct, and non-interchangeable phenotype subpopulations, i.e. atheroprone and atheroresistant.

Figure 4 Relationship between serum testosterone and neointima formation. Group data for normalized intima-to-media ratios (IM/RI) plotted as a function of serum testosterone. In the moderate injury groups, linear regression demonstrated a significant inverse relationship between serum testosterone and IM/RI (r² = 0.99; P < 0.05). IM, intact male; CM, castrated male; CMT, castrated male with testosterone replacement. Data are plotted as mean ± SEM.

Figure 5 Coronary remodelling. Vessel volume (Vv; left panel; defined by the external elastic lamina) and coronary remodelling index (right panel; CRI = Vv-post/Vv-pre) was determined within the site of injury from intravascular ultrasound pullbacks obtained immediately before (pre-) and 28 days following (post-) moderate injury in intact male (IM), castrated male (CM), and CM with testosterone (CMT). Balloon injury resulted in positive remodelling as indicated by the increase in vessel area post-injury and a CRI > 1.0. Testosterone status had no effect on coronary artery remodelling. Data are plotted as mean ± SEM; *P < 0.05 vs. pre.

of NI development by endogenous testosterone in the present study in vivo is consistent with our previous observation that testosterone inhibits coronary smooth muscle proliferation and stimulates apoptosis. In vitro, testosterone blocked coronary smooth muscle cycle progression at the G1-to-S phase transition, attenuated Rb phosphorylation, and upregulated the CDKIs p21cip1 and p27kip1. The present study found similar inhibition of proliferation and increased p27kip1 expression in the developing NI by testosterone in vivo. Interestingly, this mechanistic profile is similar to cytostatic drugs, such as rapamycin, which have emerged clinically as a means to produce cell cycle arrest, inhibit smooth muscle proliferation, and limit restenosis. Furthermore, testosterone-induced G1/G0 arrest, downregulation of cyclin D1 and E, and upregulation of p21cip1 were PKCδ-dependent.
NI formation in the rat is proposed to occur exclusively through expansion of the atheroprone subpopulation. Conversely, the pig and human coronary media possess both smooth muscle cell subpopulations, but these are interchangeable, i.e. the atheroresistant can undergo phenotypic modulation to become atheroprone and contribute to NI formation. These potential differences reinforce the need for utilizing coronary arteries in large mammal models for human comparison. It is currently unknown whether differential responses to testosterone in the subpopulations contribute to species and/or vessel differences.

The mitigating effect of endogenous testosterone on NI formation observed in the present study was limited to moderate injury. Increasing the balloon to artery diameter from 1.25–1.3 (moderate) to 1.4 (severe) resulted in an approximately two-fold increase in NI development and a loss of the influence of testosterone, suggesting that the inhibitory effect of testosterone was overwhelmed. This apparent loss of the salutary effect of testosterone with increasing severity of arterial injury is similar to other interventions, such as nitric oxide. One possible implication is that higher concentrations of testosterone may be necessary to limit NI development following severe coronary injury. However, given the potentially detrimental off-target effects of supraphysiological testosterone supplementation, e.g. prostate hypertrophy, targeted, local delivery of testosterone, as done for other drugs may present a rationale therapeutic approach.

In conclusion, both in vitro and in vivo data derived from the swine model support beneficial effects of endogenous testosterone on coronary post-angioplasty restenosis in males. Although both post-angioplasty restenosis and atherosclerosis share common aetiologies with regard to smooth muscle proliferation, migration, and phenotype modulation, caution must be exercised when extrapolating these findings to the observed beneficial effects of testosterone on coronary heart disease and cardiovascular events in humans. The effect of testosterone in the long-term progression of complex atherosclerotic lesions is likely complex and context-dependent. For example, the anti-proliferative effect of testosterone could beneficially reduce lesion burden of a stable plaque, but conversely may increase risk of plaque rupture of a vulnerable plaque by inhibiting cap stabilization by smooth muscle. Therefore,

Figure 6  Lack of an effect of testosterone on neointimal response to severe injury. (Left panel) Representative photomicrographs of Verhoff Van Geissen (VVG)-stained coronary arteries 28 days following severe balloon injury in intact male (IM), castrated male (CM), and CM with testosterone (CMT). n, neointima; m, media. (Right panel) Group data for intima area, intima-to-media ratio (I/M), and intima-to-media ratio normalized to rupture index (IM/RI). All data are means ± SEM. n Values as in Table 1.
while this study supports a beneficial role of testosterone in limiting vasculoproliferative disease, more study will be necessary to completely resolve the role of testosterone on coronary pathophysiology.

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References


Figure 7 Testosterone inhibits neointimal proliferation. Cell proliferation was assessed by Ki67-positive nuclei in both the uninjured media (black bars) and neointima (gray bars) 10 days following moderate balloon injury in intact male (IM), castrated male (CM), and CM with testosterone (CMT). Data are expressed as Ki67-positive nuclei relative to total nuclei. Bars represent means ± SEM; n = 7, 5, and 9 for IM, CM, and CMT, respectively. *P < 0.05 vs. IM and CMT.

Figure 8 Testosterone increases neointimal (NI) p27kip1. Representative photomicrographs demonstrating p27kip1 expression in an uninjured control artery (left anterior descending) from an intact male (IM) (A), and NI from IM (C), castrated male (CM) (D), and CM with testosterone (CMT) (E) 10 days following balloon angioplasty; I, lumen; m, media; a, adventitia; n, neointima. Representative non-immune control for an IM is shown in (B). Bars represent means ± SEM; n = 7, 5, and 9 for IM, CM, and CMT, respectively. *P < 0.05 vs. IM and CMT.

Figure 9 Testosterone increases protein kinase C (PKC)-δ in the neointima (NI). Representative photomicrographs demonstrating PKCδ expression injured left anterior descending from an intact male (IM) (A), and NI from IM (C, same as boxed area in A), CM (D), and CMT (E) 10 days following balloon angioplasty; I, lumen; m, media; a, adventitia; n, neointima. Representative non-immune control for an IM is shown in (B). PKCδ staining density (black bars) and % positive area (grey bars) within the NI were lower in CM compared with both IM and CMT (F). Bars represent means ± SEM; n = 8, 6, and 11 for IM, CM, and CMT, respectively. *P < 0.05 vs. IM and CMT.

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