Testosterone: a vascular hormone in health and disease

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Abstract

Coronary heart disease is a leading cause of premature death in men. Epidemiological studies have shown a high prevalence of low serum testosterone levels in men with cardiovascular disease (CVD). Furthermore, a low testosterone level is associated in some but not in all observational studies with an increase in cardiovascular events and mortality. Testosterone has beneficial effects on several cardiovascular risk factors, which include cholesterol, endothelial dysfunction and inflammation: key mediators of atherosclerosis. A bidirectional relationship between low endogenous testosterone levels and concurrent illness complicates attempts to validate causality in this association and potential mechanistic actions are complex. Testosterone is a vasoactive hormone that predominantly has vasodilatory actions on several vascular beds, although some studies have reported conflicting effects. In clinical studies, acute and chronic testosterone administration increases coronary artery diameter and flow, improves cardiac ischaemia and symptoms in men with chronic stable angina and reduces peripheral vascular resistance in chronic heart failure. Although the mechanism of the action of testosterone on vascular tone in vivo is not understood, laboratory research has found that testosterone is an L-calcium channel blocker and induces potassium channel activation in vascular smooth muscle cells. Animal studies have consistently demonstrated that testosterone is atheroprotective, whereas testosterone deficiency promotes the early stages of atherogenesis. The translational effects of testosterone between in vitro animal and human studies, some of which have conflicting effects, will be discussed in this review. We review the evidence for a role of testosterone in vascular health, its therapeutic potential and safety in hypogonadal men with CVD, and some of the possible underlying mechanisms.

Key Words
- Atherosclerosis
- Testosterone
- Inflammation
- Vasoreactivity

Introduction

For many decades, testosterone has been perceived by the medical community to play a role in the development of prostate cancer and cardiovascular disease (CVD), with its potential as a cardiovascular therapeutic agent being generally overlooked. This assumption was largely based upon the observed clinical benefit of androgen deprivation therapy (ADT) in prostate carcinoma patients and the case reports of sudden cardiovascular death among male athletes abusing anabolic steroids (Cohen & Hickman 1987, Thompson et al. 1989, Sullivan et al. 1998, Denmeade & Isaacs 2002). However, there is no compelling evidence that testosterone replacement to levels within the normal healthy range contributes adversely to the pathogenesis of CVD (Carson & Rosano 2011) or
prostate cancer (Morgentaler & Schulman 2009), and conversely, recent evidence indicates that testosterone may be beneficial in the management of the commonly associated conditions (e.g. obesity, metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM)) that are well known to be associated with an increased incidence of CVD.

Atherosclerosis is a complex disease of the arteries characterised by endothelial dysfunction, vascular inflammation and the build-up of lipids within the intima of the vessel wall. Many cardiometabolic risk factors facilitate the development of atherosclerosis, although the precise underlying mechanisms remain disputed. The formation of an atherosclerotic plaque as the disease progresses can lead to stenosis of the artery, reduced blood flow and increased blood pressure as vascular function is further disrupted. Therefore, a negative cycle of metabolic, vascular and inflammatory dysregulation in the vessel wall evolves. The maintenance of vascular tone through a correct response to vasoconstrictive and vasodilatory agents is especially important in the context of atherosclerosis in the coronary circulation. Reduced vasodilatory responses and enhanced vasoconstriction may further restrict haemodynamic flow through atherosclerotic vessels with stenosis, exacerbating clinical symptoms and perpetuating vascular dysfunction, which can also lead to vasospasm and, for example, worsening angina symptoms.

The ageing blood vessel is less able to protect itself from injury induced by diseases such as dyslipidaemia, T2DM and obesity, with a loss of compliance in the aorta and the principal arterial conduits (Franklin et al. 1997). In turn, this loss of compliance is a powerful determinant of cardiovascular risk and the development of atherosclerosis (Blacher et al. 1999). It is also known that ageing is associated with the development of low-grade systemic inflammation (or ‘inflammageing’), characterised by raised serum C-reactive protein (CRP) and pro-inflammatory cytokine levels (Bartlett et al. 2012). Importantly, this inflammageing is implicated in the pathogenesis of atherosclerosis as well as T2DM and is associated with increased mortality. Although ageing is associated with an increased incidence of CVD in males and females, 68% of premature cardiovascular mortality is accounted for by men (http://www.bhf.org.uk/research/heart-statistics.aspx, 21/03/2012). Therefore, as testosterone levels progressively decline in ageing men, and diminished vasoreactivity and vascular inflammation are associated with ageing per se (English et al. 2000a), a vascular role for testosterone in cardiovascular protection has been hypothesised.

The majority of epidemiological studies have found that there is a high prevalence of low testosterone levels in men with coronary heart disease (CHD), and this association exists regardless of the age of the patient (Jones 2010a). In fact, an increase in all-cause and cardiovascular mortality has been correlated with low testosterone levels in population studies (Khaw et al. 2007, Laughlin et al. 2008, Vikas et al. 2009, Ponikowska et al. 2010) and also within a population of men with proven CHD (Malkin et al. 2010). Some evidence now even suggests that low testosterone levels should be considered an independent cardiovascular risk factor (Jones & Saad 2009, Jones 2010a,b). The causality of the relationship between low testosterone levels and vascular disease is unclear. Indeed, Araujo et al. (2011) suggest that cardiovascular mortality in such patients is driven by the underlying health status and that a low testosterone level is simply a marker of poor general health. It is likely, however, that a bidirectional effect between decreased testosterone concentrations and disease pathology exists as concomitant cardiovascular risk factors (including inflammation, obesity and insulin resistance) are known to reduce testosterone levels and that testosterone confers beneficial effects on these cardiovascular risk factors (see Kelly & Jones 2013). A recent prospective population-based study of elderly Swedish men has demonstrated that high endogenous testosterone levels predict reduced fatal and non-fatal cardiovascular events over a 5-year follow-up period (Ohlsson et al. 2011). Testosterone levels in the highest quartile were associated with reduced cardiovascular events compared with those in the three combined lower quartiles, which remained after adjustment for traditional risk factors. Although this further indicates a protective influence of testosterone in CVD, a high testosterone level in elderly men may be a sign of good general health and thereby associated with a reduced risk of cardiovascular events.

ADT for the treatment of prostate cancer increases the risk of CHD, diabetes and cardiovascular death (Keating et al. 2006, Levine et al. 2010, Jones 2011). This supports a key role of testosterone in atheroprotection, noted by a science advisory from the American Heart Association (Levine et al. 2010). In a recent meta-analysis of over 4000 prostate cancer patients from eight randomised trials with ADT, Nguyen et al. (2011) have found no increased risk of cardiovascular death. The patients in these trials, however, were not stratified by pre-existing cardiovascular comorbidity and received a relatively short-duration ADT.
(<3 years) and studies were not specifically designed to reveal a relationship between ADT and cardiovascular morbidity and therefore cannot exclude the possibility of long-term detrimental effects and in-patient subgroups. Achieving a normal physiological testosterone concentration through the administration of testosterone replacement therapy (TRT) has been shown to improve risk factors for atherosclerosis including reducing central adiposity and insulin resistance and improving lipid profiles (in particular, lowering cholesterol), clotting and inflammatory profiles and vascular function (reviewed in Jones & Saad (2009) and Jones (2010a)). The exact mechanism by which testosterone produces these effects is largely unknown. Some studies have reported confounding results, and there is a shortage of long-term placebo-controlled trials. Indeed, the only way to conclusively substantiate a causal relationship between testosterone and cardiovascular morbidity and mortality is to undertake 5-year clinical outcome trials, and a need for such studies is apparent.

Numerous animal studies support an anti-atherogenic action of testosterone in males, whereby castration accelerates aortic plaque build-up in models of atherosclerosis (Bruck et al. 1997, Alexandersen et al. 1999, Nathan et al. 2001, Nettleship et al. 2007a, Bourghardt et al. 2010). Testosterone replacement in these animals significantly diminished plaque formation, indicating a direct role for testosterone in the aetiology of atherogenesis. Despite these indications, testosterone is not routinely monitored in men at cardiovascular risk and the use of testosterone as a cardiovascular therapeutic agent in hypogonadal men remains disputed due to the relatively limited understanding of the underlying mechanisms of action and a lack of large randomised placebo-controlled clinical trials focussing on CVD-related morbidity and mortality.

Another reason for the action of testosterone on cardiovascular function being difficult to define is that androgens may potentially affect cellular function by both genomic and non-genomic mechanisms. Testosterone may signal through its cytoplasmic cognate (androgen receptor (AR)) directly or via 5α-reductase conversion to its more potent metabolite, dihydrotestosterone (DHT). Alternatively, testosterone is converted to 17β-oestradiol (E2) by aromatase and subsequently activates the oestrogen receptor (ER), diversifying the potential signalling pathways. Indeed, sex hormone receptors and testosterone-convert ing enzymes are expressed in the arterial wall and in cultured vascular cells (Wu & von Eckardstein 2003). In addition to these genomic mechanisms, testosterone may act via non-classical routes during the cell membrane channel activation and/or in the presence of G-protein-coupled, agonist-sequestrable receptors that initiate rapid intracellular signalling cascades and transcription-independent signalling (Heinlein & Chang 2002). The influence of these heterogeneous signalling pathways on cardiovascular function remains to be clarified.

This review focusses on some of the clinical, experimental and mechanistic evidence implicating testosterone as a vascular hormone in cardiovascular disorders.

Testosterone and vascular function

A negative correlation between testosterone and hypertension has been demonstrated in men (Barrett-Connor & Khaw 1988, Khaw & Barrett-Connor 1988, Hughes et al. 1989, Phillips et al. 1993, Simon et al. 1997, Svartberg et al. 2004). In a subpopulation of 206 men from the Baltimore Longitudinal Study of Ageing, serum testosterone levels were proved to be an independent negative predictor for developing arterial stiffness, assessed from the peak systolic and end diastolic diameters of the common carotid artery and simultaneous brachial artery blood pressure (Hougaku et al. 2006). This association remained after adjusting for other risk factors including age, pulse pressure, fasting plasma glucose, BMI and total cholesterol. Similarly, free serum testosterone was negatively correlated with the measures of vascular stiffness in a retrospective, cross-sectional study of older men (Dockery et al. 2003a). This is further supported by the evidence that patients with prostate cancer undergoing ADT develop arterial stiffness within 3 months, as measured by the pulse-wave velocity (Smith et al. 2001, Dockery et al. 2003b). In addition and potentially offering some explanation to epidemiological findings, low levels of testosterone were related to increased oxidative stress and a reduced antioxidant capacity in men, potentially as a mechanism of disturbed flow due to vascular damage and loss of reactivity (Mancini et al. 2008). Low testosterone levels in men are associated with erectile dysfunction (ED), which in part may be a result of impaired penile blood flow due to diminished vasoreactivity (Yassin & Saad 2008, Corona et al. 2009). Indeed, ED is predominantly a vascular condition. It is well known that impaired erectile function and CVD are closely related in that ED can be the first clinical manifestation of atherosclerosis often preceding a cardiovascular event by 3–5 years (Jackson 2012). These data may therefore suggest that sub-normal levels of testosterone impact negatively on vascular dynamics and the reactivity of blood vessels.

A number of patient-based studies have investigated the influence of testosterone therapy on vascular reactivity...
in men. As early as the 1940s, the therapeutic use of testosterone was reported to improve angina pectoris in men with coronary artery disease (CAD; Hamm 1942, Lesser 1942, Walker 1942, Stigher & Tulgan 1943) and show a benefit in intermittent claudication, the clinical manifestation of lower leg peripheral artery disease (Edwards et al. 1939, Walker 1942). Since these early investigations, the majority of clinical studies have demonstrated that testosterone therapy, whether administered chronically or acutely, results in improvements in coronary blood flow and cardiac ischaemia in men with CAD, an effect that is considered to arise from the beneficial modulation of vascular tone (see Table 1). Indeed, the vascular system is a target for direct androgen action and the beneficial effects of TRT on symptoms of angina, blood pressure and ED are also observed and may be due to the vasodilatory actions of testosterone and the restoration of normal vascular function (Khaw & Barrett-Connor 1988, Corona et al. 2008).

In 1977 in the first randomised controlled trial, weekly injections of testosterone cypionate were shown to decrease the sum of ST-segment changes (representing phase 2 of the cardiac action potential with the elevation or depression of the ST segment being indicative of myocardial ischaemia or injury and CAD) in the electrocardiogram after 4 and 8 weeks of therapy (Jaffe 1977). Our group performed the first randomised, double-blind placebo-controlled trial using testosterone therapy add-on to usual therapy to maintain serum testosterone levels within the normal range (mean increase in total testosterone was 5.2 nmol/l at 3 months; English et al. 2000b). This study treated men with chronic stable angina independent of the testosterone status for 3 months. It reported that testosterone treatment significantly increased time to 1 mm ST-segment depression compared with placebo over time (Fig. 1A) and that men with lower testosterone levels had a greater response (Fig. 1B). A further randomised placebo-controlled cross-over study demonstrated an improved ischaemic threshold in men with overt hypogonadism (mean total testosterone was 4.2 nmol/l) and chronic stable angina after 1 month of bi-weekly i.m. depot of mixed testosterone esters (Malkin et al. 2004b).

### Table 1  Effects of testosterone on coronary blood flow and cardiac ischaemia

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Patients (age in years)</th>
<th>Testosterone administration</th>
<th>Outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>13 men with CAD (61 ± 11)</td>
<td>Intracoronary infusion (10⁻¹⁰⁻¹⁰⁻⁷ mol/l)</td>
<td>Increased coronary artery dilatation following Ach-induced contraction and increased coronary blood flow</td>
<td>Webb et al. (1999a)</td>
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<tr>
<td></td>
<td>14 men with CAD (57 ± 2)</td>
<td>I.v. (2300 μg/ml)</td>
<td>Increased time to 1 mm ST-segment depression</td>
<td>Webb et al. (1999b)</td>
</tr>
<tr>
<td></td>
<td>14 men with CAD (58 ± 4)</td>
<td>I.v. (2.5 mg)</td>
<td>Increased time to 1 mm ST-segment depression post-exercise; increased total exercise time – improved exercise-induced myocardial ischaemia</td>
<td>Rosano et al. (1999)</td>
</tr>
<tr>
<td>Chronic</td>
<td>50 men with abnormal post-exercise ECG (ECG) (58)</td>
<td>I.m. testosterone cypionate (4 and 8 weeks) (200 mg weekly)</td>
<td>Decreased post-exercise ST-segment depression</td>
<td>Jaffe (1977)</td>
</tr>
<tr>
<td></td>
<td>62 men with CHD</td>
<td>Oral testosterone undecanoate (10 weeks) (120 mg/day for two weeks then 40mg/day thereafter)</td>
<td>Improved angina pectoris and myocardial ischaemia in ECG and Holter</td>
<td>Wu &amp; Weng (1993)</td>
</tr>
<tr>
<td></td>
<td>46 men with chronic stable angina and &gt;70% stenosis of at least one coronary artery (62 ± 2)</td>
<td>Transdermal (4 and 12 weeks) (5 mg patches/day)</td>
<td>Increased time to post-exercise 1 mm ST-segment depression</td>
<td>English et al. (2000b)</td>
</tr>
<tr>
<td></td>
<td>10 men with chronic stable angina and &gt;70% stenosis of at least one coronary artery or previous MI with angina (60.8 ± 4.6)</td>
<td>I.m. injection mixed testosterone esters (12 weeks) (100 mg/2 weeks)</td>
<td>Increased time to 1 mm ST-segment depression post-exercise; reduced exercise-induced myocardial ischaemia</td>
<td>Malkin et al. (2004b)</td>
</tr>
<tr>
<td></td>
<td>13 men with chronic stable angina (64.8 ± 7.0)</td>
<td>I.m. testosterone undecanoate (52 weeks) (1000 mg/2 weeks)</td>
<td>Increased time to 1 mm ST-segment depression post-exercise and increased maximal exercise time; reduced exercise-induced myocardial ischaemia</td>
<td>Mathur et al. (2009)</td>
</tr>
</tbody>
</table>
Testosterone therapy improves angina threshold in men with chronic stable angina. English et al. (2000b) demonstrated that time to 1 mm ST-segment depression following exercise is greatly increased in the testosterone-treated group compared with the placebo group at baseline and at weeks 6 and 14 (A). In addition, a significant correlation is observed between baseline bioavailable testosterone levels and change in response to exercise-induced cardiac ischaemia (B).

In this study, time to electrocardiogram 1 mm ST-segment depression by Bruce protocol exercise testing (a standard measure of exercise-induced ischaemia) was greatly increased (74 s) in the treated group compared with the placebo group. Using the same method of assessment, Mathur et al. (2009) also demonstrated that 12 months of testosterone undecanoate treatment reduced exercise-induced myocardial ischaemia and increased maximal exercise time in hypogonadal men with chronic stable angina. This study is important in showing that there was no decrease in the response (i.e. no tachyphylaxis) of testosterone and that patient benefit persists in the long term.

The acute effects of testosterone treatment on cardiac ischaemia have also been demonstrated with i.v. infusion of high concentrations of testosterone into the coronary blood flow in men with CAD increasing time to exercise-induced cardiac ischaemia (Rosano et al. 1999, Webb et al. 1999a). Direct infusion of physiological concentrations of testosterone into the right coronary artery of men with CAD rapidly increased the coronary artery diameter and coronary blood flow after pre-constriction with acetylcholine (ACh), consistent with a rapid and direct vasodilatory action (Webb et al. 1999b).

A frequently used method for assessing vascular reactivity is the monitoring of changes in brachial artery diameter by a non-invasive ultrasound assessment as it is considered an accurate and reproducible measurement of endothelium-dependent response to shear stress (Celermajer et al, 1992, Takase et al, 1998). This measure is a marker for increased cardiovascular risk (Kuvin et al, 2001) and correlates with endothelium-dependent coronary and peripheral artery responses (Anderson et al, 1995, Sorensen et al, 1995). Employing this methodology, Ong et al. (2000) demonstrated that high-dose testosterone administered intravenously to men with CAD was associated with a significant increase in brachial artery vasodilation compared with the placebo treatment. Physiological testosterone treatment, however, was reported to have no effect on vascular reactivity. CAD patients in this study had testosterone levels in the low normal range (11.1 ± 6.1 nmol/l), which may influence the efficacy of response to physiological testosterone as reported by English et al. (2000b). Pugh et al. (2003) found that the acute administration of testosterone (60 mg buccal testosterone) to men with moderate chronic cardiac failure in a placebo-controlled trial reduced peripheral vascular resistance and improved the cardiac index, a measure of cardiac output during invasive monitoring. No effect was observed on either pulmonary capillary wedge or pulmonary artery pressure. Again those patients with lower baseline testosterone levels derived the greater benefit from the treatment (see Fig. 2). The effect was maximal after 180 min, which coincided with the maximal testosterone level achieved. The lack of an effect at physiological doses in Ong’s study may have also been due to the acute nature (within minutes) of hormone administration, and the authors suggest that chronic exposure to a physiological concentration of testosterone may have a beneficial effect on vascular reactivity in the long term. Indeed, 12-week oral testosterone treatment, which produced plasma free testosterone levels within the physiological range, has been shown to result in a marked increase in both flow- and nitroglycerin-mediated brachial artery vasodilation in men with CAD (Kang et al, 2002).

In contrast to the observed vasodilatory effects of testosterone in men with CAD, prostate carcinoma patients undergoing therapeutic or surgical castration did not demonstrate nitrate-mediated brachial artery vasodilatation (Herman et al, 1997). In fact, increased flow-mediated dilatation was observed in the androgen...
The vascular response to testosterone is enhanced in patients with the lowest baseline levels. Pugh et al. (2003) indicated that response to testosterone treatment in cardiac index (A) and systemic vascular resistance (B) is significantly greater in subjects with lower endogenous androgens at baseline. The results are presented as patients with bio-available testosterone levels either above or below the median concentration of 4.57 nmol/l (normal range 2.5–11.9 nmol/l). Reduced state groups compared with the controls who were healthy men or patients with non-prostate cancers. Similarly, hypogonadal men with diabetes exhibit decreased flow- and nitrate-mediated brachial artery vasodilation or no change following testosterone treatment or transdermal DHT administration (Ly et al. 2001, Kenny et al. 2002, Zitzmann et al. 2002). In addition, the number of CAG repeats in exon one of the AR, thereby reducing sensitivity to testosterone, was associated with endothelium-dependent and -independent brachial artery vasodilation in healthy men, suggesting an inhibitory effect of AR-mediated testosterone action on vasoreactivity (Zitzmann et al. 2001). Zitzmann et al. (2002) reported that elevated flow-mediated brachial artery dilation in hypogonadal patients was restored to control levels following 3 months of testosterone replacement. These findings were supported by a similar study demonstrating reduced vasodilation after receiving 6 monthly testosterone depot treatment compared with pre-treatment (Sader et al. 2003). However, as these studies of hypogonadal men included only relatively healthy individuals, a positive influence of testosterone treatment may only be observed when vascular reactivity is sufficiently impaired as observed in CAD patients (Jones et al. 2004b). The reduction in vasodilation following testosterone treatment in hypogonadal men may merely represent a restoration of vascular responsiveness rather than a loss of sensitivity to vasodilatory stimuli (Jones et al. 2004b).

Studies mainly in Brattleboro hypertensive rats have suggested that androgens increase tubular sodium and water reabsorption and activate the renin–angiotensin system (Reckelhoff et al. 2005). Testosterone increases extracellular water levels and decreases aldosterone levels, but it has no effect on plasma renin or atrial natriuretic peptide levels, although neither systolic blood pressure nor diastolic blood pressure changed in this study (Johannsson et al. 2005). This has been supported by a recent study that also showed that testosterone reduces aldosterone levels over a 6-month treatment period (Goncharov et al. 2012). The mechanism of this effect was suggested to be due to the activation of the renin–angiotensin system or by the increased expression of sodium channels (Johannsson et al. 2005). Animal studies revealed that testosterone replacement in young orchiectomised spontaneously hypertensive rats exacerbates systolic hypertension and reduces pressure natriuresis (Reckelhoff et al. 1998). Clinical studies, however, have revealed either small reductions of 2–3 mm in diastolic pressure or no significant effects when testosterone is replaced within normal physiological limits in humans (Wang et al. 2004, Jones & Saad 2009).

**Isolated vessels**

Malkin et al. (2006a) demonstrated that testosterone treatment in the high physiological range for 3 months increases the vasoconstrictor response to noradrenaline and reduces dilatation to ACh and sodium nitroprusside in ex vivo subcutaneous resistance arteries (isolated from gluteal biopsies) of androgen-deficient men compared with pre-treatment. In the same study, a cross-sectional analysis of men with and without heart failure revealed that testosterone caused the vasodilatation of pre-constricted subcutaneous resistance vessels in all men, although it was noted that the normal dilating effect of testosterone was augmented in patients with androgen deficiency. Following testosterone treatment, the increased vasodilatory action of testosterone on
isolated vessels from androgen-deficient men was reduced (Malkin et al. 2006a). Therefore, testosterone alters the responsiveness of subcutaneous resistance arteries to testosterone and also modifies vessel response to other vasoactive agents.

Isolated pulmonary arteries pre-constricted with U46619, a potent thromboxane mimic, demonstrate vasodilatation upon testosterone exposure in vessels from both male and female human lobectomy samples (Smith et al. 2008). Rowell et al. (2009) extended these investigations to human pulmonary vasculature and demonstrated significant vasodilatation in response to testosterone in isolated pulmonary arteries pre-constricted with potassium chloride. Vasodilatation in response to testosterone at physiological concentrations was only observed in arteries from male subjects and the maximal response at supraphysiological doses was also significantly greater in vessels from the male subjects. Similarly, radial artery isolated from patients undergoing bypass surgery was shown to vasodilate in response to testosterone after pre-contraction with potassium chloride or phenylephrine (Seyrek et al. 2007). The same group had previously found that testosterone relaxes isolated human internal mammary artery pre-contracted with potassium chloride or prostaglandins (Yildiz et al. 2005).

The majority of animal studies support a vasodilatory action of testosterone in several different species (see Table 2). Conversely, alterations in pressor responses to pre-constricted isolated vessels following androgen treatment have also been reported in a few animal studies (Schrör et al. 1994, Farhat et al. 1995, Ceballos et al. 1999, Quan et al. 1999, Teoh et al. 2000a). Ceballos et al. (1999) exposed isolated coronary arteries from male rats to acute physiological concentrations of testosterone and demonstrated an attenuation of the vasodilatation induced by adenosine. Similarly, Teoh et al. (2000b) reported that short-term exposure to physiological levels of testosterone potentiated vasocontractile responses elicited by endothelin-1, 5-hydroxytryptamine, U46619 and potassium chloride in porcine coronary artery rings. However, the gender origin of these isolated vessels was not specified in this study, and consequently it is unknown whether alterations of contractile vasoreactivity are sex dependent. Indeed, Farhat et al. (1995) highlighted further gender differences in contractile responses to both prostaglandin F2α (PGF2α) and potassium chloride in isolated porcine

Table 2 Vasoreactive effects of testosterone on isolated vessels

<table>
<thead>
<tr>
<th>Action</th>
<th>Vessel</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coronary artery</td>
<td>Rat</td>
<td>English et al. (2000a, 2001, 2002), Pugh et al. (2002) and Jones et al. (2004a)</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Rabbit</td>
<td>Yue et al. (1995)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>Creus &amp; Khalil (1999a), Murphy &amp; Khalil (1999), Teoh et al. (2000a,b) and Deenadayalu et al. (2001)</td>
<td></td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>Dog</td>
<td>Chou et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>Pulmonary vein</td>
<td>Sheep</td>
<td>Yildirm &amp; Erol (2011)</td>
<td></td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>Human</td>
<td>Smith et al. (2008) and Rowell et al. (2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Jones et al. (2003a,b,c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>English et al. (2001) and Jones et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>Human</td>
<td>Rowell et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>Radial artery</td>
<td>Human</td>
<td>Jones et al. (2004c)</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous resistance artery</td>
<td>Human</td>
<td>Tep-areenan et al. (2002) and Toot et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>Renal afferent arteriole</td>
<td>Mouse</td>
<td>Malkin et al. (2004a)</td>
<td></td>
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<tr>
<td>Umbilical artery</td>
<td>Human</td>
<td>Perusquía et al. (2007) and Cairrño et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Prostatic small artery</td>
<td>Pig</td>
<td>Navarro-Dorado et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Mammary artery</td>
<td>Human</td>
<td>Yildiz et al. (2005)</td>
<td></td>
</tr>
<tr>
<td>Vasoconstriction</td>
<td>Coronary artery</td>
<td>Rat</td>
<td>Ceballos et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>Farhat et al. (1995), Quan et al. (1999), and Teoh et al. (2000b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Guinea pig</td>
<td>Schrör et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>Rat</td>
<td>Toot et al. (2012)</td>
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coronary arteries following testosterone treatment. Two weeks of s.c. testosterone treatment was associated with an elevation in the vasoconstrictor response in vessels from female animals, but only to potassium chloride in vessels from males. In addition, the testosterone supplementation, while associated with a significant increase in the circulating testosterone levels in female animals, produced a significant decline in circulating levels in male animals, suggesting that the potentially deleterious changes in vascular reactivity may actually have resulted from a reduced circulating testosterone profile in the male animals.

Supraphysiological testosterone levels, which could be considered pharmacological concentrations, are usually required in vitro for the assessment of the effects of hormones on vascular tone. Although this does lead to questions in terms of the physiological relevance of the actions, it is well known that well-established physiological agents such as noradrenaline and Ach that affect vascular tone need to be administered in high (μM) dosages (Malkin et al. 2006a). In addition, the effects of vasodilation are studied on potassium chloride-induced vasoconstriction and this also may be a factor as to why high concentrations of ligands are required.

Vascular mechanisms of testosterone

The underlying cellular and molecular mechanisms by which testosterone modulates vascular reactivity are not fully understood. In most cases, experimental animal studies indicating that testosterone induces vascular relaxation suggest that this takes place via rapid nongenomic mechanisms and that these actions are endothelium independent in a variety of vascular beds including coronary, mesenteric, iliac, renal and femoral arteries (Yue et al. 1995, Chou et al. 1996, Perusquía et al. 1996, Crews & Khalil 1999a, Murphy & Khalil 1999, English et al. 2000a, 2001, 2002, Deenadayalu et al. 2001, Ding & Stallone 2001, Jones et al. 2003a). In contrast, other studies have shown that endothelial denudation significantly inhibits testosterone-mediated vasorelaxation in rat mesenteric artery (Tep-areenan et al. 2002) and isolated human pulmonary artery, with decreased sensitivity to testosterone-induced vasodilation (Rowell et al. 2009). These discrepancies in the role of the endothelium in testosterone-induced vasorelaxation have been considered due to differences in individual study designs with testosterone concentration, acute or chronic exposure, vascular bed investigated and precontractile agent all influencing the outcome. It is also likely, however, that the actions of testosterone on vasoreactivity are partially both endothelium dependent and independent. Indeed, the endothelium-dependent and -independent vasodilatory effects of testosterone were described in studies conducted on canine coronary conductance and resistance arteries in vivo (Chou et al. 1996).

Ion channel modulation

The key mechanism underlying the potential vasodilatory actions of testosterone has been proposed by several studies to involve an effect on smooth muscle cell ion channel function, influencing either potassium (K⁺) channel opening and/or calcium (Ca²⁺) channel inactivation (Jones et al. 2003b) (Fig. 3). The vasorelaxant effects of testosterone on human umbilical arteries, pre- contracted with serotonin or histamine or potassium chloride, were partially mediated by the activation of both voltage-sensitive K⁺ channels and large-conductance, Ca²⁺-activated K⁺ channels (Cairrão et al. 2008). This supports earlier animal studies that suggest that testosterone-induced vasorelaxation is mediated predominantly via K⁺ channels in rat mesenteric arterial beds (Tep-areenan et al. 2002), rabbit coronary arteries (Yue et al. 1995) and rat aorta (Ding & Stallone 2001). In a particularly well-conducted study using patch-clamp recordings, Deenadayalu et al. (2001) identified a specific large-conductance, Ca²⁺- and voltage-activated K⁺ channel as the primary effector mediating testosterone-induced relaxation of porcine coronary arteries. This mechanism is supported in the isolated human internal mammary artery, whereby testosterone-induced vasorelaxations are reduced by the voltage- sensitive K⁺ channel inhibitor tetraethylammonium (TEA), with the authors suggesting a Ca²⁺-activated K⁺ channel action (Yildiz et al. 2005). The relaxation at low concentrations of testosterone, however, was not altered by other K⁺ channel inhibitors (including ATP-sensitive K⁺ channel inhibitor glibenclamide (GLI) and voltage-sensitive K⁺ channel inhibitor 4-aminopyridine (4-AP)). Conversely, the relaxation of isolated human radial artery in response to testosterone was not blocked by TEA, 4-AP or barium chloride and vasodilatation was most effectively reduced with the addition of GLI, suggesting an ATP-sensitive K⁺ channel opening action of testosterone (Seyrek et al. 2007). The permissive role of testosterone in vasorelaxation in the erectile tissue is also considered to occur via the induction of acute non-genomic K⁺ channel activation of vascular smooth muscle cells (VSMCs; Ding & Stallone 2001).

Several studies have suggested that testosterone primarily inhibits the calcium-dependent elements of
vasoconstriction by the inactivation of L-type voltage-operated Ca\(^{2+}\) channels to induce the documented vasodilatory effects (Fig. 3). Testosterone abolished the Ca\(^{2+}\)-dependent contraction induced by potassium chloride or PGF2\(\alpha\) in male rat coronary arteries (English et al. 2002; Jones et al. 2002) and mouse thoracic aorta (Jones et al. 2003a). Scragg et al. (2004) showed that the vasodilatation response to testosterone occurs selectively via L-type Ca\(^{2+}\) channel inhibition in HEK293 embryonic kidney cells transfected with the \(\alpha1\)-C subunit that forms the pore of the L-Ca\(^{2+}\) channel and in the A7r5 rat VSMC line that stably expresses the L-Ca\(^{2+}\) channel (Fig. 4a). Indeed, it was further demonstrated in electrophysiological patch-clamp studies that testosterone at increasing physiological concentrations can dose dependently inactivate L-type voltage-operated Ca\(^{2+}\) channels, preventing Ca\(^{2+}\) influx in rat A7r5 VSMCs (Hall et al. 2006; Fig. 4b). When combined with 10 nM testosterone, the treatment of cells with the well-known L-type voltage-gated Ca\(^{2+}\) channel blocker nifedipine (a commonly used anti-anginal and anti-hypertensive agent) at its maximally effective dose produced no further suppression of Ca\(^{2+}\) influx, suggesting that testosterone acts at the same site as nifedipine. The addition of pimozide to block T-type voltage-gated channels further reduced the testosterone-induced inhibition of Ca\(^{2+}\) influx, indicating actions independent of this channel. In another study, Scragg et al. (2007) extended the earlier implications of Hall et al.
by demonstrating that this rapid action of testosterone acts at the nifedipine-binding site. HEK293 cells transfected with a point mutation in the α₁-C subunit of the L-type Ca²⁺ channel almost completely abolished nifedipine sensitivity and rendered the same channel insensitive to testosterone (Scragg et al., 2007; Fig. 4c).

These data thereby support a beneficial role for testosterone as an endogenous L-Ca²⁺ channel blocker.

An alternative mechanism for the action of testosterone on store-operated Ca²⁺ channels (SOCCs) has additionally been postulated (Fig. 3). Jones et al. (2003c) clearly demonstrated that the rapid transient increase in intracellular Ca²⁺ levels responsible for constriction in response to PGF2α acts via SOCCs in A7r5 cells. The response was resistant to voltage-operated Ca²⁺ channel blockade, but it was reduced by pre-incubation with SK&F 96365, a potent SOCC blocker. Testosterone also inhibited the Ca²⁺ influx response to PGF2α with a similar efficacy as SK&F 96365, suggesting an antagonistic effect of testosterone on SOCCs in these cells (Jones et al., 2003c). In a previous study, Jones et al. (2002) used thapsigargin as an indirect activator of SOCCs to investigate the vasodilatory action of testosterone in pulmonary arteries. Thapsigargin inhibits the active uptake of Ca²⁺ into intracellular stores but leaves the passive release unaffected, thus emptying these stores over time and triggering SOCC opening and Ca²⁺ influx. Testosterone, however, was unable to induce vasodilation in thapsigargin pre-constricted vessels, suggesting that there was no effect on SOCCs when intracellular Ca²⁺ stores were depleted in pulmonary arteries. These differences may be due to the mode of SOCC activation or simply represent}

Figure 4
The vasodilatory actions of testosterone occur via L-type Ca²⁺ channel modulation. Scragg et al. (2004) demonstrated an inhibitory effect of testosterone over time on Ca²⁺ channel currents recorded in HEK293 cells stably transfected with the α₁-C subunit of a cloned human cardiovascular L-type Ca²⁺ channel and rat aortic smooth muscle cell line A7r5 expressing native L-type Ca²⁺ channels (A). Hall et al. (2006) reported a dose-dependent reduction in K⁺-evoked Ca²⁺ increase in A7r5 cells. Nifedipine had no further effects on testosterone response, while pimozide further decreased the response, suggesting an L-type rather than a T-type Ca²⁺ channel action of testosterone (B). Scragg et al. (2007) further demonstrated that HEK293 cells transfected with the α₁-C subunit of the L-type Ca²⁺ channel containing a point mutation (T1007Y) prevent the inhibitory effects of both nifedipine and testosterone on Ca²⁺ currents compared with cells transfected with the wild-type subunits (C). The authors therefore demonstrate that testosterone acts at the nifedipine-binding site to elicit L-type Ca²⁺ channel inhibition and subsequent vasodilatation.

(2006)
alternative mechanisms of action of testosterone in systemic and pulmonary vascular smooth muscle.

**Nitric oxide**  In addition to the well-documented ion channel modulation, testosterone can also influence endothelial function through the modulation of nitric oxide (NO) release (Miller & Mulvagh 2007; Fig. 3). Testosterone is metabolised directly to E2 by aromatase, which is well known to stimulate NO release. NO is a potent vasodilator that can be synthesised by NO synthase (NOS) and is released by the vascular endothelium, among other tissues, and NO-induced vasodilation is often therapeutically targeted in hypertension and angina (Miller & Megson 2007). In vitro, cultured human endothelial cells increase NO synthesis in response to physiological concentrations of testosterone via non-genomic activation of intracellular signalling pathways and Ca2+ influx (Goglia et al. 2010, Yu et al. 2010, Campelo et al. 2012). Moreover, increased endothelial NOS (eNOS) expression and phosphorylation were observed in testosterone- and DHT-treated human umbilical vein endothelial cells (HUVECs) stimulated with hydrogen peroxide to induce senescence (Ota et al. 2012). In addition, testosterone significantly increased NO production via comparable mechanisms in rat aortic strips (Campelo et al. 2012), and testosterone stimulates NO synthesis, which consequently increases cyclic guanosine monophosphate (cGMP) formation to induce vasorelaxation in VSMCs (Deenadayalu et al. 2001, White et al. 2007).

A large amount of research has focussed on the vasoreactive mechanisms of testosterone in the penile tissue with regard to sexual function and ED. In patients with ED, there is a positive correlation between free testosterone levels and the compliance of cavernous arteries of the penis, suggesting an involvement of circulating androgens in the regulation of intrapenile vasodilatation (Aversa et al. 2000). Similar to alternative vascular beds, one of the major actions of testosterone is on NO and its signalling pathways. The expression of NOS inside the penis is partially dependent on the presence of adequate androgen levels (Penson et al. 1996, Mills & Lewis 1999, Mills et al. 1999a, Aversa et al. 2000). Androgen deprivation leads to a reduction in neuronal NOS expression associated with a decrease of intracavernosal pressure in penile arteries during erection, an effect that is promptly reversed by androgen replacement therapy (Lugg et al. 1995, Penson et al. 1996, Mills et al. 1999b). In addition to direct effects on NOS expression, testosterone may also affect phosphodiesterase type 5 (PDE5 (PDE5A)) gene expression, an enzyme controlling the degradation of cGMP, which acts as a vasodilatory second messenger (Morelli et al. 2004). PDE5 inhibitors are currently the frontline treatment option for ED (Lee 2011); however, the efficacy of their use may be at least in part dependent upon the androgen status. Indeed, the response to PDE5 inhibitors for ED therapy was blunted in patients with subclinical hypogonadism (Guay et al. 2001, Kalinchenko et al. 2003) and PDE5 inhibitors are ineffective in improving erectile function in androgen-deficient animals (Traish et al. 1999). However, combined therapy with testosterone replacement and sildenafil in hypogonadal men converts 60% of sildenafil non-responders to responders (Greenstein et al. 2005, Shabsigh et al. 2004).

**Vessel structure**  Testosterone is also thought to modulate VSMCs through the upregulation of proliferation genes and through possible inhibitory interactions with intracellular signalling pathways induced by pro-inflammatory cytokines, decreasing inflammation-induced apoptosis and promoting proliferation (Williams et al. 2002, Nakamura et al. 2006). Although some studies have linked abnormal VSMC proliferation to the pathogenesis of both atherosclerosis and re-stenosis (for review, refer to Rivard & André (2000)), smooth muscle cells secrete and deposit extracellular matrix proteins (collagen, elastin, etc.) often considered protective against atherosclerotic plaque destabilisation (Johnson 2007). However, smooth muscle cells also release numerous proteases (matrix metalloproteinases (MMPs)) that are capable of digesting matrix proteins, making the role of VSMC proliferation in atherosclerotic plaque progression a complex issue that is not fully understood. Likewise, VSMC apoptosis can induce features of plaque vulnerability in atherosclerosis, yet the accumulation of VSMCs is also viewed as responsible for lesion formation (Clarke et al. 2006). Thus, the significance of the action of testosterone on VSMC apoptosis and proliferation in atherosclerosis is difficult to delineate and may be dependent upon the stage of plaque development.

Whether as a result of smooth muscle cell proliferation or due to the build-up of lipid and inflammation in atherosclerosis, thickening of the intima-media leads to detrimental alterations in local haemodynamics, shear stress, blood pressure and ultimately vascular function. Intima-media thickness (IMT, a measure of the thickness of the innermost two layers of the arterial wall) is associated with atherosclerosis and is viewed as a surrogate endpoint for evaluating the regression and/or progression of atherosclerotic CVD. Several human studies have shown that carotid IMT (CIMT) and aortic calcification negatively...
correlate with serum testosterone (Hak et al. 2002, van den Beld et al. 2003, Fukui et al. 2003, Muller et al. 2004, Demirbag et al. 2005, Mäkinen et al. 2005, Svartberg et al. 2006). Low levels of testosterone in men were inversely related to the mean progression of IMT in the common carotid artery even after adjusting for age, and this relationship was found to be independent of other cardiovascular risk factors (Muller et al. 2004). We, and others, have discovered that long-term testosterone treatment reduced CIMT in men with low testosterone levels and angina (Rosano et al. 1999, Mathur et al. 2009, Zitzmann et al. 2009).

Men with CVD in the majority of epidemiological studies have been demonstrated to have a high prevalence of low testosterone levels (Jones & Saad 2009) and Jones (2010a,b), for review). There is also an inverse relationship between the degree of coronary atherosclerosis and testosterone (Phillips et al. 1993, Nettleship et al. 2007b). A case–control study of men undergoing coronary angiography found that those with evidence of coronary atheroma had lower levels of bioavailable testosterone compared with men with normal coronary arteries (English et al. 2000c). The key question is whether or not the low testosterone state contributes to the pathogenesis of atherosclerosis or whether or not it is merely a biomarker of ill-health.

**Potential signalling mechanisms of testosterone in vascular reactivity**

The mechanisms by which testosterone acts on the vasculature to elicit effects on tone, compliance and function are a matter of some debate. The rapid onset of acute vasodilatory actions of testosterone (within minutes) and evidence from ion channel investigations suggest that at least some of these actions are nongenomic, function independently of (AR) nuclear translocation and do not require protein synthesis (Jones et al. 2003b). Indeed, as has been discussed previously, the direct actions of testosterone on ion channel function bypass the necessity for classical nuclear AR activity. This is supported by the fact that vasodilatory effects have been shown with testosterone bound to BSA, which prevents transmembrane diffusion and/or endocytosis and, therefore, access to the cytoplasmic AR in smooth muscle cells (Ding & Stallone 2001). Furthermore, polar testosterone analogues that are unable to permeate the cell membrane have been reported to produce a greater vasodilatory response than non-polar, permeable analogues (Ding & Stallone 2001), and similarly non-genomic testosterone analogues elicit increased vasodilatation compared with the genome-acting analogues (Yue et al. 1995, Ding & Stallone 2001). These data may allow for the presence of cell surface ARs, and binding sites in cell membranes that display features compatible with rapid steroid signalling have been characterised (Gerdes et al. 2000). However, the acute vasodilatory actions of testosterone in rat coronary arteries and thoracic aorta of mice were not abolished by the AR blocker flutamide, suggesting an AR-independent mechanism (Channer & Jones 2003, Jones et al. 2004a).

Likewise, additional receptor antagonist studies have further indicated that neither intracellular nor membrane-associated ARs are required for the rapid vasodilator effect (Yue et al. 1995, Tep-areenan et al. 2002). Research carried out in our laboratory has demonstrated that testosterone-mediated vasodilatation is maintained in vessels isolated from testicular feminised (Tfm) mice, which lack a functional AR, supporting AR-independent mechanisms and potentially direct actions on ion channel function in acute responses (Jones et al. 2003a).

While acute responses appear to be AR independent, long-term AR-mediated effects on the vasculature have also been described, primarily in the context of vascular tone regulation via the modulation of gene transcription. Indeed, the AR is expressed ubiquitously in the cells of the vasculature. Testosterone and DHT increased the expression of eNOS in HUVECs, an effect that was significantly reduced by AR blockade (Goglia et al. 2010). Furthermore, Ar knockout (ARKO) mice displayed significantly reduced aortic eNOS expression and phosphorylation compared with the wild-type mice, indicating that AR activation may be required for the preservation of NO bioavailability and the regulation of vascular tone (Ikeda et al. 2009). In addition to slower acting gene regulation, rapid AR activation of intracellular signalling pathways has also been suggested. Yu et al. (2010) described how the rapid phosphorylation of eNOS or NO production was abolished by pre-treatment with an AR antagonist, nilutamide, or by transfection with AR siRNA and that DHT also stimulated eNOS phosphorylation in human aortic endothelial cells (HAECs). The authors suggest that the activation of PI3-kinase/Akt intracellular signalling pathway and the direct interaction of the AR with p85α (the regulatory subunit of PI3-kinase) were responsible for the observed effects (Yu et al. 2010). Furthermore, the lack of a functional AR in Tfm mice is associated with reduced endothelium-dependent vasodilatation even when the low endogenous levels of testosterone (characteristic of Tfm mice) are restored to the wild-type levels, indicating...
that AR genomic pathways also influence vascular tone (Jones et al. 2003a).

The influence of ERs on the vascular action of testosterone, via conversion to E2 by aromatase, has also been proposed as a potential mechanism for the observed AR-independent effects. Indeed, E2 is recognised to elicit marked vasodilatation in a variety of vascular beds (Chester et al. 1995, Browne et al. 1999, Teoh et al. 2000a,b, English et al. 2001, Salom et al. 2001), and aromatase is expressed in the cells of the vasculature (Harada et al. 1999, Mukherjee et al. 2002). Additionally, oestrogens have been shown to activate eNOS and stimulate NO production in an ERα-dependent manner (Chen et al. 1999, Simoncini et al. 2000a). Several studies, however, have demonstrated that the vasodilatory actions of testosterone are not reduced by aromatase inhibition (Yue et al. 1995, Tep-areenan et al. 2002) or ER antagonism (Chou et al. 1996) and non-aromatisable DHT elicited similar vasodilation to testosterone treatment in arterial smooth muscle (Deenadayalu et al. 2001, Hall et al. 2006). Furthermore, the vasodilatory effects triggered by E2 were reported to be significantly lower than those observed with testosterone treatment in the pulmonary vasculature (English et al. 2001), leading to the assumption that aromatase-mediated conversion of testosterone into E2 and subsequent activation of the ER are unlikely to be involved in the response in this vascular bed.

**Testosterone and vascular inflammation**

It is now accepted that atherosclerosis is a chronic inflammatory pathology. Individuals with hyperlipidaemia and signs of systemic inflammation develop atherosclerosis, with specific defects in lipid processing and immune activity consequentially occurring at the vessel wall. The initiating factors leading to specific local vascular changes may be numerous and often remain elusive in cardiovascular research; however, it is known that the activation of endothelial cells promotes the adhesion of leukocytes to the blood vessel wall as an early atherogenic event. This activation also leads to increased vascular permeability for not only inflammatory leukocytes, but also the circulating lipid components, such as LDL. LDL in the intima is oxidised to form pro-inflammatory cell-activating oxLDLs, which further promote the early stages of atherogenesis. The build-up of lipid and inflammation in the vessel wall causes changes in local fluid dynamics with the thickening of the intima consequentially leading to the loss of normal vascular reactivity with the associated detrimental outcomes discussed previously.

The anti-inflammatory mechanisms of androgens have been long recognised, yet evidence from previous studies that have investigated sex hormones and inflammatory markers has not been consistent (Jones & Saad 2009). Observational evidence suggests that several pro-inflammatory cytokines (including interleukin 1β (IL1β), IL6, tumour necrosis factor α (TNFα), and highly sensitive CRP) and serum testosterone levels are inversely associated in patients with CAD, T2DM and/or hypogonadism (Yang et al. 2005, Maggio et al. 2006, Kapoor et al. 2007, Nettleship et al. 2007b). Moreover, Nettleship et al. (2007b) demonstrated an association between circulating levels of IL1β and atherosclerotic burden and found that patients with the highest IL1β concentrations had lower endogenous testosterone levels. Additionally, TRT has been reported to significantly reduce TNFα and elevate the circulating anti-inflammatory IL10 in hypogonadal men with CVD (Malkin et al. 2004a,b). The Moscow Study demonstrated that 30 weeks of testosterone treatment to normalise levels in hypogonadal men with the MetS resulted in a significant reduction in the circulating CRP, IL1β and TNFα, with a trend towards lower IL6 compared with placebo (Kalinchenko et al. 2010). Following 1 year of treatment with parenteral testosterone undecanoate, CRP decreased significantly in hypogonadal elderly men (Haider et al. 2009). Khosla et al. (2002) reported that TNFα, IL6 and IL6 soluble receptor were elevated in elderly men where hypogonadism was induced by GnRH antagonists. Testosterone replacement in these men abolished this heightened inflammatory response. Conversely, a similar study demonstrated that testosterone therapy had no effect on serum TNFα concentrations in men with chronic heart failure (Pugh et al. 2005) and testosterone replacement in hypogonadal men with T2DM had no effect on the TNFα, IL6 or CRP levels (Kapoor et al. 2007). Likewise, in a prospective study of older men, the administration of DHT or human chorionic gonadotrophin (to stimulate testosterone synthesis from the Leydig cells) did not significantly alter the high-sensitivity CRP, soluble vascular cell adhesion molecule 1 (sVCAM-1) or soluble intracellular adhesion molecule (sICAM) levels (Ng et al. 2002).

TRT has been shown in some studies to lower serum levels of the adipocytokine, adiponectin, particularly in hypogonadal men with T2DM (Lanfranco et al. 2004, Page et al. 2005, Kapoor et al. 2007). Higher levels of serum adiponectin have been shown to lower cardiovascular risk. The interventional studies were short-term and may reflect the reduction in total fat mass as also observed
with a reduction in leptin. The long-term effect of testosterone on adiponectin is not known.

In addition to influencing the systemic markers of inflammation, testosterone has been shown to inhibit TNFα, IL1β and IL6 released from cultured peripheral blood monocytes isolated from androgen-deficient men with T2DM (Corrales et al. 2006; Fig. 5). The physiological and supraphysiological concentrations of testosterone reduced the expression and secretion of TNFα and IL1β in monocyte-derived macrophages obtained from a CHD age-relevant population, although no effects were observed on IL6 and CRP expression (Corcoran et al. 2010). In another study, IL6 production was shown to be reduced in isolated human monocytes from a small healthy male population following in vitro testosterone treatment (Kanda et al. 1996).

Animal studies support the reported beneficial effects of androgen supplementation on atherosclerosis in males, with evidence for AR-dependent and -independent actions, resulting in the underlying mechanisms being difficult to elucidate (Bruck et al. 1997, Alexandersen et al. 1999, Nathan et al. 2001, Nettleship et al. 2007a). The administration of physiological levels of testosterone significantly reduced high-cholesterol diet-induced fatty streak formation in the aortic root of Tfm mice, whereby the prominent lesional cells were demonstrated to be macrophages (Nettleship et al. 2007a, Kelly et al. 2012). In addition, a recent study using ARKO mice on an apolipoprotein E-deficient background (which are testosterone deficient) has shown that replacing testosterone to physiological levels reduced atherosclerotic lesion area and complexity compared with placebo, but not to the same extent as that observed in testosterone-treated orchidectomised wild-type controls (Bourghardt et al. 2010). Although consistent with AR-dependent and -independent mechanisms, the individual contribution of these pathways to the effect of testosterone on atherogenesis currently remains unknown.

The anti-inflammatory effects of testosterone may therefore act on the vasculature by influencing both systemic inflammation and leukocyte activation to reduce localised atheroma development in the susceptible regions of blood vessels. A direct anti-inflammatory effect of testosterone on the cells of the vasculature has also been proposed, although the evidence is limited and often contradictory.

**Influence of testosterone on vascular cell inflammation**

Research suggests that the expression of VCAM-1, as induced by pro-inflammatory cytokines such as TNFα or interferon γ (IFNγ) in endothelial cells, can be attenuated by treatment with testosterone (Hatakeyama et al. 2002, Mukherjee et al. 2002). Testosterone also inhibits the production of pro-inflammatory cytokines such as IL6, IL1β and TNFα in a range of cell types including human endothelial cells (Hatakeyama et al. 2002).

TNFα-induced VCAM-1 expression in HAECS was inhibited by testosterone treatment, suggesting that androgens play an important role in the prevention of atherogenesis (Hatakeyama et al. 2002). This study additionally described the inhibition of nuclear factor κB (NFκB) activation, not only as a potential mechanism for decreased VCAM-1 expression, but also as a prospective modulator of several other inflammatory gene targets known to be activated by this transcription factor. The promoter regions of the genes encoding ICAM-1, VCAM-1 and E-selectin all contain at least one κB site required for cytokine gene activation (Neish et al. 1992, Kaszubska et al. 1993, Hou et al. 1994, Read et al. 1994). Norata et al. (2006) furthered this notion by elegantly demonstrating a decreased inflammatory response to TNFα and lipopolysaccharide (LPS) in human endothelial cells when treated with DHT. This included a reduction in VCAM-1 and ICAM-1 expression as well as a decreased IL6, monocyte

**Figure 5**

Potential cell-specific anti-inflammatory effects of testosterone. Androgens are known anti-inflammatory agents, but the specific actions of testosterone on vascular and immune cells have not been inconsistent. Testosterone has been shown, however, to reduce pro-inflammatory cytokine production and adhesion molecule expression in a number of athero-relevant cell types including monocytes, macrophages, endothelial cells, smooth muscle cells and foam cells. Testosterone may also inhibit the formation of foam cells (open triangle). IL, interleukin; TNF, tumour necrosis factor; MCP, monocyte chemoattractant protein; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; LOX-1, lectin-like oxidised LDL receptor-1; SR-B1, scavenger receptor-B1. *Effects also observed with DHT.
chemoattractant protein 1 (MCP1 (CCL2)) and TNF-α release. The authors have recently extended these findings by demonstrating that DHT can reduce TNF-α-induced MCP1 expression in endothelial cells (Norata et al. 2010). Although these studies were performed in HUVECs, and therefore may not be reflective of the behaviour of other endothelial cell types, the inhibition of VCAM-1 expression supports the findings of previous investigations (Hatakeyama et al. 2002). Zhang et al. (2002), however, found no effect of testosterone on TNF-α receptor expression in TNF-α-stimulated endothelial cells and, contradictory to the proposed atheroprotective role of testosterone, demonstrated androgen enhancement of TNF-α-induced E-selectin and VCAM-1 upregulation. Several studies have also demonstrated that the cytokine-stimulated upregulation of adhesion molecules is increased in cultured endothelial cells upon treatment with androgens (McCrohon et al. 1999, Mukherjee et al. 2002, Death et al. 2004).

### Potential signalling mechanisms of testosterone in vascular inflammation

The conflicting experimental observations regarding testosterone may in part be due to differences in the cell type studied, the need for often supraphysiological treatment concentrations in vitro and differences in the inflammatory stimuli or target investigated. The key to unravelling the link between testosterone and its role in atherosclerosis may lay in the understanding of testosterone signalling and the cross-talk between receptors and intracellular events that result in pro- and/or anti-inflammatory actions in athero-sensitive cells.

#### AR mechanisms

Through direct AR antagonism, Zhang et al. (2002) completely abrogated the androgen enhancement of TNF-α-induced E-selectin and VCAM-1 upregulation, indicating that testosterone functions through the AR to modulate adhesion molecule expression. In support of this, McCrohon et al. (1999) reported that DHT increased the expression of VCAM-1 in HUVECs without cytokine influence and that this effect was antagonised by the blockade of the AR. A recent study using cultured human stromal cells derived from benign prostatic hyperplasia patient biopsy samples demonstrated that a pre-treatment with DHT reduced the cytokine-stimulated inflammatory response (Vignozzi et al. 2012). Indeed, DHT inhibited NFκB activation and thereby suppressed the secretion of several inflammatory/growth factors, with the most pronounced effects on IL8, IL6 and basic fibroblast growth factor. Norata et al. (2006) also demonstrated that DHT could inhibit an LPS-induced upregulation of MCP1 in HUVECs, an effect that was abolished by AR antagonism. The authors additionally reported that the upregulation of MCP1 expression by TNF-α stimulation was partially, but significantly, inhibited by DHT treatment, interestingly an effect that was not altered by AR blockade. Both NFκB and AR act at the transcriptional level and have been experimentally found to be antagonistic to each other, limiting gene expression to the relative nuclear presence of either factor (McKay & Cidlowski 1998). As the AR and NFκB are mutual antagonists, their interaction and influence on functions can be bidirectional, with inflammatory agents that activate NFκB interfering with normal androgen signalling as well as the AR interrupting NFκB inflammatory transcription. If this is the case, prolonged exposure of vascular cells to the inflammatory activation of NFκB associated with atherosclerosis may reduce or alter any potentially protective effects of testosterone. In addition, physical interactions of NFκB subunits and promoter regions of AR gene have been observed (Supakar et al. 1995), suggesting a possible feedback loop, whereby inflammatory activities in cells promote increased androgen sensitivity through increased receptor synthesis. The in vivo significance of these interactions, however, remains to be established. In addition to the interaction of testosterone with NFκB, preliminary data propose that DHT and IFNγ also modulate each other’s signalling through interaction at the transcriptional level, suggesting that androgens down-regulate IFN-induced genes (Bettoun et al. 2005).

In contrast, Death et al. (2004) support a role of the AR in the enhancement of pro-inflammatory cytokine-stimulated expression of adhesion molecules. The treatment of HUVECs with DHT and IL1β significantly increased VCAM-1 expression, and this was blocked by AR antagonism with hydroxyflutamide. The authors further demonstrated that DHT increased VCAM-1 promoter activity via NFκB activation, although not via direct interactions between the AR and NFκB. Instead, DHT was demonstrated to decrease the level of inhibitory κB (IκB) protein through heightened degradation, thus activating NFκB and subsequent VCAM-1 expression.

#### ER mechanisms

Mukherjee et al. (2002) showed that testosterone could attenuate TNF-α-induced VCAM-1 upregulation in HUVECs, an action that was abolished by both ER antagonism and aromatase inhibition.
This study utilised HUVECs of female origin and may not be directly comparable to investigations with male endothelial cells. In parallel, E2 can inhibit IL1-induced ICAM-1, VCAM-1 and E-selectin expression in HUVECs isolated from a female foetus (Caulin-Glaser et al. 1996). Xing et al. (2007) reported that E2 inhibits the mRNA expression of Icam1, Vcam1, P-selectin and MCP1 in rat aortic SMCs and further described that this effect was attenuated by ERα blockade. Additionally, Nakagami et al. (2010) have recently demonstrated that E2 inhibits lipoprotein-α (an atherogenic lipoprotein)-induced expression of VCAM-1, ICAM-1 and E-selectin in bovine aortic endothelial cells and that IL1β, TNFα and MCP1 in macrophages differentiated from the THP-1 monocyte cell line. Indeed, E2 inhibits MCP1 expression in rat thoracic aorta VSMCs (Jiang et al. 2010) and inhibits human monocyte migration towards MCP1 in the culture, an effect that was abolished by ER blockade (Yamada et al. 1996). The inhibition of monocyte migration was not demonstrated with testosterone treatment, suggesting that the ER effects are independent of testosterone conversion (Yamada et al. 1996). Aromatase expression, however, was not assessed in these cells.

Simoncini et al. (2000b) also showed that E2 inhibited the expression of VCAM-1 when induced by LPS, IL1α or TNFα. The authors considered this effect to be via decreased NFκB activity. Indeed, the ER has been shown to inhibit NFκB activity in several cell lines (Kalaitzidis & Gilmore 2005). The ER can directly bind the subunits of NFκB in vitro and inhibit transcriptional activity possibly by preventing DNA binding or by inhibiting essential coactivator protein association preventing their interaction with NFκB (Stein & Yang 1995). However, ER–NFκB complexes have not been reported. The ER has been suggested to influence NFκB activities indirectly through the inhibition of IkB kinase (IKK) complex that regulates IkB degradation (Simoncini et al. 2000a,b). Norata et al. (2010) suggest that part of the testosterone-mediated atheroprotective effects could depend on ER activation mediated by the testosterone/DHT 3β-derivative, 3β-Adiol. Indeed, TNFα-induced induction of ICAM-1, VCAM-1 and E-selectin as well as MCP1 and IL6 was significantly reduced by a pre-incubation with 3β-Adiol in HUVECs (Norata et al. 2010). These effects were inhibited by selective ERβ antagonism. In the same study, 3β-Adiol also reduced LPS-induced gene expression of IL6, TNFα, cyclooxygenase 2 (COX2 (PTGS2)), CD40, CX3CR1, plasminogen activator inhibitor-1, MMP9, resistin, pentraxin-3 and MCP1 in the monocytic cell line U937 (Norata et al. 2010). This study suggests that testosterone metabolites, other than those generated through aromatisation, could exert anti-inflammatory effects that are mediated by ER activation.

In contrast, Aziz & Wakefield (1996) demonstrated that E2 enhanced the expression of E-selectin after TNFα stimulation of endothelial cells and supported similar findings from a previous study where VCAM-1 and ICAM-1 were additionally upregulated, promoting leukocyte adherence (Cid et al. 1994). Both these investigations reported a lack of oestrogen modulation on adhesion molecule expression following IL1β stimulation, whereas Zhang et al. (2002) reported that E2 enhanced TNFα-induced mRNA and surface endothelial cell expression of E-selectin and VCAM-1. The effect was completely abrogated by pre-incubating the cells with the oestrogen antagonist tamoxifen.

**AR- and ER-independent mechanisms** In Tfm mice (non-functional AR and low endogenous testosterone), serum TNFα and IL6 were significantly elevated compared with those in littermates receiving a normal diet, and testosterone treatment significantly reduced serum IL6 in Tfm mice receiving a high-cholesterol diet (Kelly et al. 2012). Conversely, Bourghardt et al. (2010) observed no effect of testosterone treatment on IL6 or other cytokines in ARKO mice. Fatty streak formation as an early indication of atherosclerosis was also shown to be increased in Tfm mice (Kelly et al. 2012). Testosterone treatment significantly reduced aortic lipid deposition and consequently inflammatory monocytes/macrophages present in these early lesions in Tfm mice compared with placebo. No differences were observed between animal groups for the expression of the atherogenic chemokine fractalkine (CX3CL1) and its receptor (CX3CR1) in the plaque area. Despite this lack of local anti-inflammatory effects of testosterone on specific atherogenic markers, this study supports a role for AR-independent effects in atheroprotection; however, the influence of the ER was not investigated. A similar study in Tfm mice demonstrated that the beneficial effects of testosterone treatment on fatty streak formation were only partially reduced by ERα blockade with fulvestrant and aromatase inhibition with anastrozole, suggesting that testosterone may also act via undetermined AR- and ER-independent mechanisms in atheroprotection (Nettleship et al. 2007a).

Using the non-aromatisable androgen DHT, Osterlund et al. (2010) demonstrated that human coronary artery smooth muscle cells (HCASMCs) attenuate their increased expression of the inflammatory
mediator COX2 under inflammatory conditions (LPS or IL1β) when co-treated with DHT. This effect of DHT was not altered by AR antagonism with bicalutamide. Interestingly, under normal conditions without pro-inflammatory stimulation, DHT alone increased COX2 levels compared with the vehicle and AR antagonism attenuated the response. The authors suggest that DHT differentially effects COX2 levels under physiological and pathophysiological conditions in human coronary artery smooth muscle cells and via AR-dependent and -independent mechanisms influenced by the physiological state of the cell (Osterlund et al. 2010). DHT was also shown to suppress hypoxia-induced upregulation of COX2 by inhibiting hypoxia-inducible factor 1α (HIF1α (HIF1A)) DNA binding and subsequent transcription of the COX2 gene in primary human brain VSMCs (Zuloaga & Gonzales 2011). Similarly, these effects were not reversed by AR blockade.

Consistent with a membrane mode of action, testosterone and DHT can activate second-messenger cascades through the SHBG receptor complex. Such an activation of the SHBG receptor transduces its signal via a G-protein, leading to the activation and rapid generation of cAMP (Rosner et al. 1999). In the case of testosterone ion channel modulation and subsequent second-messenger activation, relatively little is known about the ultimate cellular effect, but physiological genotrophic effects that impact on cell function are likely, and in cells relevant to the atherosclerotic process, these functions may be linked to inflammation. Ca2+ functions as a ubiquitous second-messenger molecule and may be involved in a mechanism by which testosterone influences intracellular signal cascades. The activation of these cascades can modulate the activity of transcription factors and subsequent gene expression. Whether these genes have an inflammatory function remains unclear.

Testosterone and cardiovascular safety

TRT to treat male hypogonadism has been available since 1939. Concerns have been raised in regard to the safety of TRT in men with CVD. There are, however, a number of systematic meta-analyses of clinical trials of TRT that have not demonstrated an increased risk of adverse cardiovascular events or mortality (Haddad et al. 2007, Fernández-Balsells et al. 2010, Corona et al. 2011, Carson & Rosano 2011). The TOM trial, which was designed to investigate the effect of TRT on frailty in elderly men, was terminated prematurely as a result of an increased incidence of cardiovascular-related events after 6 months in the treatment arm (Basaria et al. 2010). An elderly cohort of frail men with a high proportion having multiple comorbidities were given 100 mg/day testosterone gel, twice the recommended initiation dose of testosterone gel (50 mg/day), with six men having their dose increased to 150 mg/day. The cardiovascular-related events were heterogeneous and included self-reported syncope and oedema, the latter a known side effect of the supraphysiological levels of testosterone. The study was not powered to detect a statistically significant increase in cardiovascular events. A similar trial that also investigated the effect of testosterone therapy on fraility using a conventional testosterone dosage (50 mg/day) did not report any difference in cardiovascular events between the treatment and placebo arms (Srinivas-Shankar et al. 2010). Furthermore, trials of TRT in men with either chronic stable angina or chronic cardiac failure have also found no increase in either cardiovascular events or mortality in studies up to 12 months (English et al. 2000b, Malkin et al. 2006b, Caminiti et al. 2009). In addition, a median 5-year follow-up study of elderly community-dwelling men (n = 2416; 61–81 years old) found that men in the highest quartile of serum testosterone had a reduced number of cardiovascular events (HR 0.7) compared with men in the lower three quartiles (Ohlsson et al. 2011). Evidence may therefore suggest that low testosterone levels and testosterone levels above the normal range have an adverse effect on CVD, whereas testosterone levels titrated to within the mid- to upper-normal range have at least a neutral effect or, taking into account the knowledge of the beneficial effects of testosterone on a series of cardiovascular risk factors, there may possibly be a cardioprotective action. A large long-term ideally 5-year outcome study would be required to provide the evidence.

Summary

The effect of testosterone on human vascular function is a complex issue and may be dependent upon the underlying androgen and/or disease status. Although not definitive, the majority of studies suggest that testosterone may display both acute and chronic vasodilatory effects upon various vascular beds at both physiological and supraphysiological concentrations and via endothelium-dependent and -independent mechanisms (Manolakou et al. 2009). In addition, testosterone may also chronically condition vessel response to other vasoactive agents to influence reactivity, with treatment in testosterone-deficient men potentially restoring vascular function.
Concurrently, testosterone has demonstrated anti-inflammatory effects clinically and TRT can improve atherosclerosis assessed non-invasively in hypogonadal men and in animal studies. Although conflicting and contradictory experimental evidence exists, testosterone can influence cell-specific vascular inflammation (Fig. 5) and may potentially be a mechanism by which TRT protects against atherogenesis in animal models. Testosterone may, therefore, alleviate the haemodynamic symptoms of atherosclerosis and improve atherosclerotic outcomes associated with disturbed flow patterns and dysfunctional vascular reactivity and offer potential therapeutic benefits for CVD. The mechanism of the action of testosterone on vascular cells remains unknown but may include classical steroid receptor activation and modulation of gene transcription (genomic), AR-mediated activation of rapid intracellular signalling pathways (non-genomic), direct ion channel modulation and/or activation of a thus far unknown membrane receptor to elicit these effects on vascular function and vascular inflammation.

It is important to recognise that findings from preclinical studies do not always translate into normal human biology and the clinical setting and therefore necessitate a cautious interpretation. Some of the in vitro studies discussed in this review provide contradictory results, which may occur because of differing experimental conditions. However, the current scientific literature supports firstly the notion that testosterone is a vascular hormone that does affect vasoreactivity and secondly that testosterone can beneficially enhance biological processes involved in atheroprotection, in particular, lipid deposition and inflammation both within the arterial wall and in the circulation. Clinical studies have shown benefits for up to one year for cardiac ischaemia and its symptom of angina, cardiac failure and certain cardiovascular risk factors. While testosterone has therapeutic potential as a vascular hormone, further large randomised placebo-controlled trials are required to elucidate its long-term clinical relevance to cardiovascular health in men. Whether or not testosterone directly protects against atherosclerosis and reduces cardiovascular events including mortality therefore warrants further investigation, and the complex underlying vascular mechanisms of action require clarification.

Declaration of interest
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