Vasodilating Mechanisms of Testosterone

Authors   **O. Yildiz, M. Seyrek**

Affiliation   Department of Pharmacology, Gulhane School of Medicine, Ankara, Turkey

Abstract
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The increased incidence of cardiovascular disease in man compared with premenopausal women suggests an unfavourable effect of male sex hormone testosterone on the cardiovascular system. However, numerous clinical and epidemiological studies reported a controversial relationship between testosterone and cardiovascular disease. Furthermore, an increasing amount of evidence indicate that testosterone can exert acute vasorelaxing effects, *via* non-genomic mechanisms. These effects involve primarily the vascular smooth muscle, without requiring the presence of endothelium, although an endothelial contribution is apparent in some studies. To date, the mechanism behind the vasodilatory action of testosterone is still under debate and might be through either activation of K^+ channels or blockade of Ca^{2+} channels in vascular muscle cells. The purpose of this article is to review the evidence regarding the vasodilating effect of testosterone as well as its mechanism of action.

Correspondence
O. Yildiz
Department of Pharmacology · Gulhane School of Medicine · 06018 Ankara · Turkey
Tel.: + 90/312/30 44 76 6
Fax: + 90/312/30 42 15 0
oyildiz@gata.edu.tr

Introduction
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In men, the risk of developing coronary artery disease and hypertension is much higher than in premenopausal women (Messerli et al., 1987; Khaw et al., 1988). However, cardiovascular protection during the reproductive age is lost after menopause, and is recovered in postmenopausal women who receive estrogen replacement therapy (Stampfer et al., 1991). This has led to supposition that estrogens are beneficial and androgens are detrimental to the cardiovascular system.

There is now a plethora of evidence that estrogens may play an important cardiovascular protective role not only by means of long-term systemic effects, like those on serum lipid profile, but also by means of direct effects of vascular structure and function, such as inhibition of vascular growth modulation of vascular tone (Simonchini and Genazzani, 2003; Cho et al., 2003). Of the effects previously studied, the vasodilating effect induced by estrogens has been well-documented. The mechanism of action of estrogens appears to involve genomic and non-genomic mechanisms that cause increase and release of vasodilatory agents such as nitric oxide (NO) and prostacyclin, adenosine, cAMP and cGMP (Belfort et al., 1996; Darkow et al., 1997; Dubey and Jackson, 2001) Potassium–channel opening and calcium antagonistic actions have also been reported (White et al., 1995; Valverde et al., 1999; White et al., 2002; Han et al., 2006).

On the other hand, testosterone has classically been considered to exert an unfavourable effect upon the cardiovascular system. However, numerous clinical and epidemiological studies reported a controversial relationship between testosterone and cardiovascular disease. For example, low circulating testosterone levels in men are positively correlated with numerous risk factors for coronary heart disease (English et al., 1997 and 2000a). Evidence also supports a beneficial effect of testosterone on the blood lipid profile and against atheroma formation (English et al., 1997; Duell et al., 1990; Khaw et al., 1991; Hromadowa et al., 1991a and b; Alexandersen et al., 1999). Furthermore, acute intracoronary or intravenous infusion of testosterone also provides rapid improvements in myocardial ischemia (Rosano et al., 1999; Webb et al., 1999a). Finally, testosterone replacement therapy improves myocardial ischemia in patients with coronary artery disease, an effect presumably due to testosterone -induced coronary vasodila-

tation (English et al., 2000b). Previous animal studies have demonstrated that testosterone acts as a direct coronary vasodilator in a variety of species, including rabbit, dog, pig, and rat, both in vivo (Chou et al., 1996) and in vitro (Yue et al., 1995; Murphy and Khalil, 1999; Crews and Khalil 1999a and b; English et al., 2000c; English et al., 2002). Testosterone is also reported to exhibit a vasodilatory action in thoracic aortae (Yue et al., 1995; Perusquia et al., 1996; Honda et al., 1999; Ding and Stallone, 2001) and in vessels isolated from the mesenteric (Tep-areenan et al., 2002) and pulmonary (English et al., 2001; Jones et al., 2002) vasculature of experimental animals and more recently in isolated human pulmonary and mesenteric arteries (Jones et al., 2003a). We have recently shown that testosterone relaxes human internal mammary (IMA) and radial (RA) but not umbilical arteries (UA) (Yildiz et al., 2005a).

Mechanisms of testosterone-induced vasodilation
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Involvement of the androgen receptor (AR)
Numerous experimental studies have been conducted to make clear the mechanisms of testosterone-induced vasodilation. Testosterone is classically known to regulate cellular function *via* interaction with the nuclear AR. In the bloodstream, most of circulating testosterone is bound to serum proteins, mainly sex hormone binding protein (SHBG) and albumin. In target cells testosterone can directly bind to the nuclear AR, or it is converted to dihydrotestosterone, which binds in turn to the AR. The hormone-receptor complex directly interacts with the nuclear DNA, modulating the transcription of androgen responsive genes. This process of protein synthesis usually takes at least 40 min to hours.

In trying to explain the vasodilatory mechanism of action of testosterone, the initial studies focused on the degree of involvement these classical steroid transcription mediated pathways played in the response (Yue et al., 1995; Chou et al., 1996; Ding and Stallone, 2001; Tep-areenan et al., 2002; Jones et al., 2002). In this respect, it has been shown that the latency for the vasodilating effect of testosterone is fast and reversible. It seems unlikely that testosterone-mediated vasodilatation would involve classical AR and genomic signalling pathway, because the response is evident within minutes of application and is maximal by 20 min, not hours. Moreover, additional substantial evidence is provided by findings that testosterone-induced dilatation is not attenuated either by pre-treatment with the AR blocker flutamide (Yue et al., 1995; Chou et al., 1996; Ding and Stallone, 2001; Tep-areenan et al., 2002; Jones et al., 2002), or by the covalent linkage of testosterone analogues to albumin, thus preventing endocytosis into the smooth muscle cell (Ding and Stallone, 2001; English et al., 2000c). In addition, blockers of transcription (e.g. actinomycin D) and translation (e.g. cycloheximide) does not inhibit testosterone-induced vasodilation (Teoh et al., 2000). Furthermore, it has also been demonstrated that testosterone-mediated vasodilatation is maintained in vessels isolated from testicular feminized mice, which lack a functional AR (Jones et al., 2003b). Previously, it was proposed that the relative polarity of the testosterone molecule determined its own vasorelaxation efficacy (Ding and Stallone, 2001). In our laboratory, we have tested the efficacy of various testosterone analogs in human IMA and RA (Yildiz et al., 2005a and unpublished data). Testosterone propionate is the most polar, least lipid soluble and smallest molecular weight (MW: 344.5) among

the other esterified testosterone analogs we have tested. Our data support the suggestion that testosterone-induced vasorelaxation is a non-genomic and structurally specific effect of the testosterone molecule, which is enhanced in more polar analogs that have a lower permeability to the vascular smooth muscle cell membrane (Ding and Stallone, 2001).

Involvement of aromatization
Potential conversion of testosterone to estrogen is another important concept to be addressed. In this respect, previous studies have demonstrated that inhibition of aromatase activity or estrogen receptor antagonism had no effect of testosterone–induced vasodilation (Yue et al., 1995; Tep-areenan et al., 2002). Moreover, similar vasodilatation to testosterone was triggered by non-aromatizable dihydrotestosterone (Deenadayalu et al., 2001). In our laboratory, we used tamoxifen, an estrogen receptor antagonist and protein kinase C inhibitor, and letrozole, a non-steroidal aromatase inhibitor, to test the role of aromatisation in testosterone-induced relaxation in IMA.
While tamoxifen inhibited testosterone- induced relaxation, letrozole had no effect (Gul et al., 2004).
This suggested that aromatisation did not occur in human IMA, thus estrogen did not contribute on testosterone-induced relaxation.

Involvement of endogenous vasodilators
A number of studies have investigated the involvement of dilator prostanoids and endothelium derived nitric oxide (Yue et al., 1995; Chou et al., 1996; Costarella et al., 1996; Honda et al., 1999; Ding and Stallone, 2001; Tep-areenan et al., 2002; Jones et al., 2002; Tep-areenan et al., 2003). However, some of these studies report that indomethacin, an inhibitor of cyclo-oxygenase responsible for the synthesis of the cyclic endoperoxide from arachidonate, has no inhibitory effect upon testosterone mediated relaxation (Chou et al., 1996; Yue et al., 1995; Honda et al., Jones et al., 2002). Similarly, testosterone–induced vasodilatation has been reported to be preserved in endothelial denuded vessels (Yue et al., 1995; Murphy and Khalil, 1999; Crews and Khalil 1999a and b; Deenadayalu et al., 2001; Perusquia et al., 1996; Honda et al., 1999; Hishikawa et al., 1995) or in the presence of inhibitors of nitric oxide syntase or guanylate cylase (Yue et al., 1995; Deenadayalu et al., 2001; Honda et al., 1999; Jones et al., 2002). In addition, previous studies also showed that testosterone had no effects on NOS activity in human and bovine aortic endothelial cells (Hishikawa et al., 1995; Goetz et al., 1999). In canine coronary artery (English et al., 1997) and rat mesenteric arterial bed (Tep-areenan et al., 2002) testosterone causes acute endothelium-dependent relaxation, mediated by nitric oxide. However, nitric oxide synthase (NOS) inhibition has no effect on relaxations to testosterone in rabbit coronary artery (Yue et al., 1995), rat aorta (Honda et al., 1999) and pulmonary artery (Jones et al., 2002). In addition, other studies with canine, rabbit, and pig coronary arteries (Yue et al., 1995; Chou et al., 1996; Deenadayalu et al., 2001) and rat thoracic aorta and mesenteric arterial bed (Honda et al., 1999; Ding and Stallone et al., 2001; Tep-areenan et al., 2002) have reported that endothelium and NO - independent vasorelaxations induced by testosterone. We have recently shown that testosterone –induced vasodilatation in human internal mammary and radial arteries is preserved in endothelial denuded vessels or in the presence of inhibitors of nitric oxide syntase or guanylate cylase (Yildiz et al., 2005a and unpublished observation).

Potassium – channel opening and calcium antagonistic actions

A number of studies have provided evidence of a modulatory role for testosterone upon potassium channel function. Chou et al. (1996) and Honda et al. (1999) previously showed that glibenclamide, an ATP-sensitive K^+ channel (K_{ATP}) inhibitor, reduced vasorelaxation to testosterone in dog coronary artery and rat thoracic aorta, respectively. On the other hand, Ding and Stallone (2001) demonstrated that neither incubation with glibenclamide nor tetraetylammonium (TEA), a non-selective K^+ channel inhibitor, had any effect on the vasodilatory response to testosterone in rat thoracic aorta. But they showed that incubation with 4-aminopyridine (4-AP), a voltage-gated K^+ channel (K_V) inhibitor, significantly reduced vasorelaxation to testosterone and they therefore proposed that testosterone acts *via* K_V channel opening action. Deenadayalu et al. (2001) recently demonstrated that TEA inhibited vasorelaxation to testosterone in pig coronary arteries. Moreover, Deenadayalu et al. (2001) confirmed that testosterone opened large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) in single pig coronary myocytes by direct evidence from patch-clamp studies. In our recent study, we have also observed an inhibitory effect of TEA, but not glibenclamide and 4-AP, on vasorelaxation to testosterone, suggesting that testosterone –mediated vasodilatation may occur *via* activation of BK_{Ca} channels in human internal mammary artery (Yildiz et al., 2005a). However in human radial artery, glibenclamide, but no other K^+ channel inhibitors, reduced vasorelaxation to testosterone, suggesting involvement of K_{ATP} channels (unpublished observations).

Interestingly, Deenadayalu et al. (2001) reported a decrease in the response to testosterone under similar conditions with our study in IMA (Yildiz et al., 2005a), namely less testosterone relaxation after KCl-precontraction compared to that after $PGF_2\alpha$-precontraction. In our study, we have found a significant reduction in the efficacy of testosterone in vessels precontracted with KCl compared to $PGF_2\alpha$. An explanation of these results may lie with the potassium channel function which is compromised in conjunction with the vasodilatory efficacy of testosterone. An alternative explanation of the dilatory action testosterone may lie with the types of calcium channels involved. $PGF_2\alpha$ acts primarily at the prostanoid receptors gated directly to receptor-operated calcium channels (ROCCs) (Tosun et al., 1997). This receptor stimulation also results in phosphatidylinositol 4,5 bisphosphate hydrolysis (Raymond et al., 1983) with resultant generation of inositol triphospate, which triggers calcium release from the intracellular stores (Berridge, 1987). The high extracellular potassium gradient generated by milimolar KCl triggers membrane depolarization with subsequent activation of voltage -operated calcium channels (VOCCs). Recently, Jones et al. (Jones et al., 2003c and 2004) reviewed the literature and proposed that the variance in the testosterone –induced dilatation following preconconstiction with KCl and $PGF_2\alpha$, could be due to a differing inhibitory efficiacy of testosterone upon ROCCs and VOCCs.

A number of previous studies in isolated vessel preparations have supported the idea that testosterone acts as a Ca^{2+} channel antagonist, by inhibiting VOCCs. It has previously been demonstrated that testosterone inhibits VOCCs in isolated rat pulmonary (Jones et al., 2002), rat coronary (English et al., 2002) and porcine coronary arteries (Crews and Khalil, 1999b). These studies have indicated that of the multiple pathways underlying Ca^{2+} signalling and homeostasis in vascular smooth muscle, testo-

sterone appears specifically to target voltage-gated Ca^{2+} entry via L-type Ca^{2+} channels, whilst agonist mobilization of Ca^{2+} and consequent capacitative calcium entry remain unaffected (Jones et al., 2002; English et al., 2002; Crews and Khalil, 1999b; Scragg et al., 2004). Moreover, Scragg et al. (2004) have employed whole-cell patch clamp recordings under non-physiological conditions and they have shown that testosterone inhibits L-type Ca^{2+} channels. In recent study, Hall et al. (2006) have employed fluorometric recordings and they have provided evidence that testosterone can block both native and recombinant L-type voltage-gated Ca^{2+} channels with IC_{50} values within or close to levels of circulating testosterone concentrations. VOCC inhibition by testosterone is rapid (within 2 minutes), making it unlikely to be mediated through a genomic effect. Hall et al. (2006) have also observed that nifedipine, a L-type Ca^{2+} channel blocker, blocks the effects of testosterone and they have proposed that some of the beneficial cardiovascular effects of testosterone arise from its ability to act like a dihydropyridine antihypertensive to suppress Ca^{2+} entry and so promote vasodilation. In our laboratory, we have observed that testosterone at concentrations of $100 \mu M$ and higher inhibited $CaCl_2$ –induced contractions in human IMA (Yildiz et al., 2005a). Consistent with our observation, it has been recently demonstrated that testosterone, at high concentrations, inhibits Ca^{2+} influx in rat aorta (Tep-areenan et al., 2003).

To date, the mechanism behind the vasodilatory action of testosterone is still under debate and might be through either activation of K^+ channels or direct blockade of Ca^{2+} channels in vascular muscle cells. Deenadayalu et al. (2001) convincingly demonstrated that testosterone-mediated vasodilatation occurs via activation of K^+ channels by direct evidence from patch-clamp study. On the other hand, only the study of Hall et al. (2006) has provided evidence for a direct calcium antagonistic effect of testosterone, so far. Indeed, either mechanism would allow vasodilatation from reduced Ca^{2+} influx through VOCCs (◉ **Fig. 1**).

Contradictory effects of testosterone on potassium channels

There is a complex-mixture of vessel-type dependent mechanisms reported in previous studies about the type of the K^+ channel involved in the vasodilatory action of testosterone. In rabbit, dog and pig coronary conduit arteries, testosterone acts via BK_{Ca} and K_V channel opening action, however testosterone - induced vasodilation occurs via K_{ATP} channel opening action in dog coronary resistance arteries (Yue et al., 1995; Chou et al., 1996; Deenadayalu et al., 2001). Accordingly, testosterone acts via BK_{Ca} channel opening action in an human conduit artery, the IMA (Yildiz et al., 2005a). However, RA shows a resistance vessel characteristics as elastomuscular wall structure and a small diameter, and testosterone - induced vasodilation occurs via K_{ATP} channel opening action in RA similar to resistance arteries (unpublished observations). Therefore, it may be suggested that testosterone preferentially stimulates the K_V and K_{Ca} channels in large conductance vessels, and the K_{ATP} channel in small resistance vessels. In fact, there are some differences in the vascular reactivity between large conductance and small resistance arteries Yokoshiki and Sperelakis, 2003. For example, it was reported that adenosine, a metabolic regulator of coronary blood flow (probably via activating K_{ATP} channels and inhibiting L-type Ca^{2+} current (t), preferentially blocked the action potential in the small coronary artery, but had no effect on the action potential
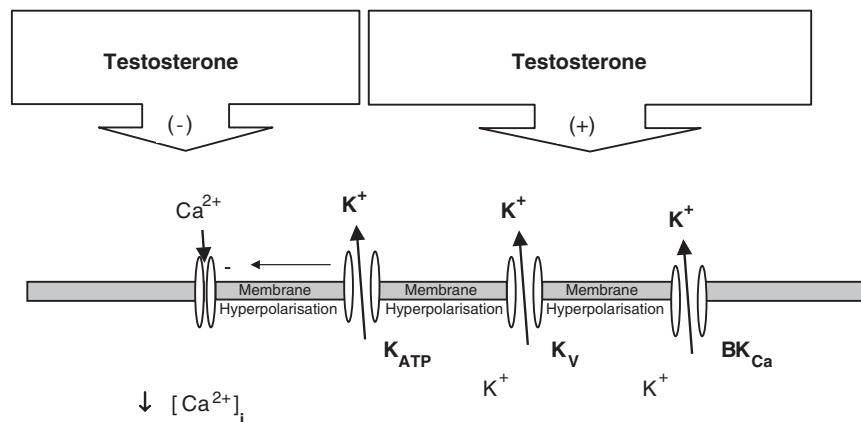
Fig. 1 Proposed model for actions of testosterone. The opening of K^+ channels in the cell membrane of smooth muscle cells in arteries by testosterone increases K^+ efflux, which causes membrane potential hyperpolarization. This closes voltage-operated calcium channels (VOCCs), decreasing Ca^{2+} entry, which leads to vasodilation. K_{ATP}: ATP-sensitive K^+ channel, BK_{Ca}: large-conductance Ca^{2+}-activated K^+ channel, K_V: a voltage-gated K^+ channel.

in the large coronary artery (Harder et al., 1979). Likewise, the coronary dilating actions levcromakalim, of a K_{ATP} channel opening drug, were heterogeneous, and it was more sensitive in small resistant coronary arteries (Sato et al., 1994). It was also reported that the resting tone of large coronary conductance vessels was little affected by glibenclamide (Shimizu et al., 2000), whereas that of small resistance vessels was affected. Hence, the tone of the small vessels is likely to be regulated by the glibenclamide-sensitive K_{ATP} channel (Yokoshiki et al., 1997). Another important finding is that testosterone relaxes human internal mammary and radial arteries but it has no vasodilatory effect on human umbilical arteries (HUA) (Yildiz et al., 2005a). HUA is unique, since it shows a conductance vessel characteristics as elastic wall structure and it has a small diameter. Functional K_v and BK_{Ca} channels are present in HUA (Yildiz et al., 2006a). However, this artery seems to lack the non-genomic signalling pathway involved in testosterone-induced vasodilation.

Potential factors influencing testosterone responses

An important point of the previous studies which should be addressed was that high concentrations of testosterone (in the micromolar range) were required to elicit vasodilation. Most of these studies were carried out in isolated vessel preparations, therefore the effects of testosterone might occur only at supra-physiological concentrations. There may be a concentration gradient from the perfusate down to the cell membrane as a result of impaired delivery of the testosterone molecule or binding to various proteins. There may be a consequence of access to smooth muscle ion channels; this could be restricted in more intact preparations lacking their natural blood supply as the lipophilic androgen would need to cross some barriers before reaching its presumed target on smooth muscle cells (Hall et al. 2006).

Another point of the previous studies which should be looked up was the latency for the vasodilating effect of testosterone. In most of the mechanistic studies cited in this review, free testosterone analogues were used, and the vasodilatory action of testosterone was rapid, occuring within minutes. As mentioned above, the covalent linkage of testosterone analogues to a macromolecule, such as albumin, has no effect upon the timescale and magnitude of the vasodilatory response to testosterone (English et al., 2000d; Ding and Stallone, 2001). Therefore, the conjugation of testosterone to albumin eliminates the permeability of testosterone to the cell membrane, and thereby, these findings again suggest a non-genomic action.

The issue of how testosterone is solved and delivered is of great importance. Different studies in humans and animals have used different solvents in different concentrations (e.g. ethanol and dimethysulphoxide). Solvents have unselective effects on ion channel activity (Kunz et al., 2006), within miliseconds, which could interfere with selective actions of testosterone. In previous studies however, no significant relaxant effect of final concentrations of the solvents has been reported (Yue et al., 1995; Chou et al., 1996; Costarella et al., 1996; Honda et al., 1999; English et al., 2000d, Ding and Stallone, 2001; Tep-areenan et al., 2002; Jones et al., 2002; Tep-areenan et al., 2003). In our experiments, we did not let the final ethanol concentration in the bath to exceed 0.1 % (v/v), since it might induce relaxation itself (Yildiz et al., 2005a).

Variability of testosterone -induced vasodilatory responses

An interesting finding in our observations was the marked variability of responses to testosterone in various subjects in IMA (Yildiz et al., 2005a). We have recently investigated the relationship of this variability with cardiovascular risk factors (Yildiz et al., 2005b). Cumulative relaxations to testosterone after precontraction with KCl have been examined in IMA segments from patients with identified cardiovascular risk factors such as hypercholesterolemia, diabetes, hypertension, smoking, age, gender, body mass index (BMI), and number of occluded vessels. Testosterone responses were significantly diminished in subjects with 3 compared with 1 risk factor. Hypercholesterolemia has independently influenced testosterone responses by significantly decreasing its maximum, and smoking has significantly decreased the sensitivity to testosterone. In our study, aging is one of the factors related to diminished testosterone responses. In accordance, English et al. (2000b) have also demonstrated that aging reduces the testosterone –induced relaxation in coronary arteries from male Wistar rats in vitro. We have concluded that the variability observed in testosterone-induced vascular relaxations may in part be related to differences in risk factors present among the individuals studied (Yildiz et al., 2005b).

Clinical relevance and future research

Testosterone-induced vasodilation might protect against cardiovascular disease, possibly by arterial vasodilation and reduced blood pressure (Thompson and Khalil, 1999; Wu and von Eckardstein, 2003; Littleton-Kearney and Hurn, 2004); thus animal studies have shown that testosterone therapy improves coronary blood flow (Scheuer et al., 1987). In humans, testosterone

has been shown to cause a dose dependant vasodilation both in vitro and in vivo. When testosterone is instilled into the left coronary artery, vasodilation ensues and coronary flow increases (Webb et al., 1999a). More importantly, acute administration of intravenous testosterone improves exercise tolerance and reduces angina threshold in men with coronary artery disease (Rosano et al., 1999; Webb et al., 1999b). These effects were observed using supraphysiological doses, but chronic administration of low physiological replacement doses of testosterone over three months in men with chronic stable angina significantly improved exercise tolerance and angina threshold (English et al., 2000e).

As summarised in this review, several lines of evidence indicate that testosterone can exert acute vasorelaxing effects. This action of testosterone is independent of the classical androgen receptor which mediates its effects genomically, suggesting a nongenomic action. The majority of vasodilatory action of testosterone seems to be independent of the endothelium. Recent evidences suggest that the mechanism behind the vasodilatory action of testosterone is through either activation of K^+ channels or blockade of Ca^{2+} channels in vascular muscle cells. The variance in the species and the vascular preparations used may contribute to the complex-mixture of vessel-type dependent mechanisms reported in this review.

Mechanistic studies conducted in human preparations are important to extrapolate our knowledge about vasoactive substances to clinical cardiovascular situations. Therefore, to test the effects of testosterone as well as other vasoactive substances in different human arterial beds is of considerable interest (Yildiz et al., 1996a and b; Yildiz et al., 2006a and b). We have recently shown that testosterone induces vasodilation in human arteries such as IMA and RA, which are the grafts of choice for myocardial revascularization to replace diseased coronary vessels (Yildiz et al., 2005a). We have tried testosterone on UA and observed no response. Previously, Fausett et al also reported that testosterone had no relaxant effect on human umbilical artery (1999). Obviously, additional studies in human arteries would be of interest to make clear the mechanism of vasodilatory effect of testosterone in different vascular beds.

Taken together, these findings provide evidence for the marked vasodilating effect induced by testosterone. Further studies, especially in different human vascular preparations and with cellular patch-clamp techniques, are essential to understand the exact mechanism of the effect testosterone and to fully realise the therapeutic potential of testosterone therapy in male patients with coronary artery disease.

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