

## Testosterone, tissue factor inhibition and vascular aging

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Aging is associated with important changes in the cardiovascular system. A key feature is the development of endothelial dysfunction, with the concomitance of enhanced vascular tone, increased production of reactive oxygen species, a fall in fibrinolytic capacity and a rise in vascular and blood procoagulant activity (1). In parallel, the ability to repair vessel injury is impaired by a decreased number and function of circulating progenitor cells.

The decline in sex hormone levels with age in both men and women has been thought to contribute to the increased prevalence of endothelial dysfunction, cardiovascular diseases and cardiac-related mortality with age. More specifically, the higher prevalence of ischaemic heart disease in men compared to pre-menopausal age-matched women has suggested that oestrogens, more than testosterone, may exert cardiovascular protection. However, prospective, cross-sectional, and interventional studies have not confirmed these hypotheses (2–4), and have even fuelled the notion of an “oestrogen-androgen paradox” whereby oestrogens may enhance and testosterone may attenuate athero-thrombotic risk (5).

*In vivo* thrombin generation is largely dependent on tissue factor (TF), a fully-functional 45 kDa membrane glycoprotein expressed in vascular smooth muscle cells and adventitial fibroblasts (6). A circulating, often encrypted form can be found on white cells, platelets, and shed microparticles (6). Endothelial cells may express TF

upon pro-inflammatory cytokine activation (7). At sites of vessel injury/activation, TF may thus come into contact with blood and interact with FVIIa, initiating coagulation (6). Generated thrombin further induces TF expression on the surface of activated platelets, leading to the propagation of coagulation (6).

The initiation of coagulation by TF is a regulated process, controlled mainly by tissue-factor pathway inhibitor (TFPI). This 276-amino acid, reversible, serine protease is produced almost entirely by endothelial cells (6). TFPI contains Kunitz domains that mimic the substrate of the target protease: after binding and inhibiting FXa through one Kunitz domain, TFPI subsequently inhibits the TF-FVIIa complex through another domain (6), limiting thrombus formation to the site of TF exposure. In plasma, only 10% of TFPI is in a free active form, the majority being bound to low-density lipoproteins with little inhibitory function (6). Heparin and shear stress upregulate the expression of TFPI (6), whose free plasma levels correlate with age and with markers of endothelial dysfunction (8).

Testosterone is produced in men mainly by Leydig cells, with the adrenal cortex contributing minimally (9). Total serum concentrations approximate 20–30 nM in adult men up to 55 years of age, gradually declining thereafter to about 15 nM (9). Levels below 10–12 nM suggest a condition of hypogonadism (9). Circadian variations account for evening nadirs and morning peaks (9). Up to 60–80% of testosterone is tightly bound to the sex hormone-binding globulin (SHBG), with only 1–3% unbound as free fraction and the remainder loosely linked to albumin (9). The bioavailable fraction typically comprises free and albumin-bound testosterone, but experimental studies show that cellular internalisation of SHBG-bound testosterone also occurs (10).

Testosterone and its main metabolite, dihydrotestosterone, exert genomic and

nongenomic effects (11). Genomic effects occur within hours. They are mediated by the nuclear androgen receptor that acts as a ligand-inducible transcription factor, leading to activation of gene expression (11). Nongenomic effects typically occur within seconds or minutes. They are mediated either by non-transcriptional effects of the androgen receptor or by specific plasma membrane receptors, such as the SHBG receptor (11). They involve intracellular second messengers, including increased intracellular free calcium or activation of kinase-signalling cascades, and may also modulate the cell's transcriptional activity (11). Testosterone's conversion to oestradiol by aromatase leads to activation of oestrogen receptors and to hypothalamic feedback on luteinising (LH) and follicle stimulating (FSH) hormones.

The effects of testosterone on male sexual maturation, increased muscle mass, and bone density are well known (12). In contrast, the metabolic, vascular and haemostatic effects are less well appreciated and somewhat controversial. Testosterone administration to hypogonadal men stimulates lean body mass and insulin sensitivity, and reduces visceral fat and total cholesterol (12). *In vitro*, testosterone induces relaxation of vascular muscle cells through K-channel opening or calcium antagonist effects (13); moreover, it suppresses pro-inflammatory cytokine effects on human aortic endothelial cells and the production of interleukin-6 in human monocytes (4, 14). In human umbilical vein endothelial cells, physiological testosterone concentrations increase mRNA and protein expression of tissue-type plasminogen activator (t-PA) and TFPI, while reducing the expression of plasminogen activator inhibitor type 1 (15). These effects are inhibited by flutamide, a selective testosterone receptor antagonist, and suggest promotion by testosterone of endogenous fibrinolysis and anticoagulant activity *in vivo*, through genomic pathways. Conversely, supraphysiological concentrations

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lead to decreased t-PA and TFPI expression (15), in keeping with the results of studies showing that androgen abuse in athletes predisposes to thrombotic vascular complications (16).

In the November 2009 issue of *Thrombosis and Haemostasis*, Agledahl et al. reported the results of a double-blind randomised study of repeated intramuscular injections of 1000 mg testosterone undecanoate or placebo (5 injections over 10 months) to 26 elderly men (mean age 69 years) with low baseline serum testosterone ( $\leq 11$  nM) (4). The effects on plasma free TFPI antigen, on the time-course of thrombin generation and on clotting assays were assessed at baseline and three months after the last injection (4). Previous observations of low serum testosterone with reduced plasma free TFPI and faster TF-initiation of coagulation in older men suggested that testosterone might enhance TFPI synthesis not only *in vitro* but also *in vivo* (4).

The results of this trial are “negative”, as no significant change in TFPI concentrations, TF-dependent thrombin generation, nor other thrombogram or clotting parameters were found at follow-up in the testosterone versus placebo-treated group – nor versus baseline – despite significantly increased serum testosterone levels and a pronounced negative feedback on serum LH and FSH in the active-treatment group (4).

Given the relative shortage of rigorous, prospective, controlled studies assessing the benefits and risks of testosterone-replacement therapy in men, the authors should be commended for a well-designed, careful, longitudinal trial. The investigators not only measured the serum concentrations of total testosterone, SHBG, LH and FSH in morning samples taken at the same time of day, but also calculated the free testosterone fraction. Functional coagulation assays were performed in parallel to TFPI and other antigenic measures. Limitations, on the other hand, include, possibly, the lack of albumin in the calculation of free testosterone, the lack of serial TFPI measures, especially soon after each testosterone injection, and the small sample size. The authors did not pre-spec-

ify the primary or secondary endpoints, and sample size calculations were based on expected changes in TFPI; thus, the absence of inter- and intra-group differences regarding other variables may stem from lack of statistical power.

The question of whether the optimal timing of testosterone administration (e.g. closer to the initial decline of endogenous production) may have been missed in this trial leading to negative results (related to a possible progressive peripheral hypo-responsiveness to falling testosterone concentrations) remains unanswered. However, in the study by Agledahl et al. (4), the pronounced negative feedback on LH and FSH concentrations observed in the active-treatment group strongly suggests maintained responsiveness – at least of certain target cells – to testosterone. Moreover, the known inverse relation between androgen receptor tissue expression and serum testosterone levels suggests hyper-responsiveness in hypogonadal states (17).

Aging-associated endothelial dysfunction may be responsible for the absence of effective endothelial cell reservoirs of TFPI and lack of efficacy of testosterone in promoting increased TFPI plasma levels, despite an adequate regimen of administration. Alternatively or additionally, the short-term effects of testosterone on TFPI expression *in vitro* may not translate into a similar, sustained, long-term effect *in vivo*, particularly if endothelial physiology is altered by age and cardiovascular risk factors. Does this trial encourage further attempts to administer testosterone to promote anticoagulation in older men? We believe that stronger pathophysiological data on the cardiovascular role of testosterone (and its metabolites) and on the functional significance of parameters of coagulation (including plasma free TFPI) should be gained as a prelude to further similar clinical trials.

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