Reactive Oxygen Species: players in the cardiovascular effects of testosterone.

Rita C Tostes¹, Fernando S Carneiro¹, Maria Helena C Carvalho² and Jane F. Reckelhoff³

¹University of Sao Paulo, Ribeirao Preto Medical School, Ribeirao Preto – SP, Brazil
²University of Sao Paulo, Institute of Biomedical Sciences, Sao Paulo – SP, Brazil
³University of Mississippi Medical Center, Women's Health Research Center, Jackson – MS, USA

Running Title: ROS and testosterone

Correspondence to: Rita C Tostes
Rita C Tostes, PhD, FAHA
Dept. of Pharmacology
Ribeirao Preto Medical School - University of Sao Paulo
Av Bandeirantes 3900
Ribeirao Preto - SP 14049-900
Brazil
Phone: +55 16 3602-4529 / 3602-3181
E-mail: rtostes@usp.br

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Abstract

Androgens are essential for the development and maintenance of male reproductive tissues and sexual function and for overall health and well-being. Testosterone, the predominant and most important androgen, not only affects the male reproductive system, but also influences the activity of many other organs. In the cardiovascular system, the actions of testosterone are still controversial, ranging from protective to deleterious effects. While early studies showed that testosterone-replacement therapy exerted beneficial effects on cardiovascular disease, some recent safety studies point to a positive association between endogenous and supraphysiological levels of androgens/testosterone and cardiovascular disease risk. Among the possible mechanisms involved in the actions of testosterone on the cardiovascular system, indirect actions (changes in the lipid profile, insulin sensitivity, and hemostatic mechanisms, modulation of the sympathetic nervous system and renin-angiotensin-aldosterone system) as well as direct actions (modulatory effects on pro-inflammatory enzymes, on the generation of reactive oxygen species, nitric oxide bioavailability, and on vasoconstrictor signaling pathways) have been reported. This mini-review focuses on evidence indicating that testosterone has pro-oxidative actions that may contribute to its deleterious actions in the cardiovascular system. The controversial effects of testosterone on ROS generation/oxidant status, both pro-oxidant and antioxidant, in the cardiovascular system and in cells/tissues of other systems are reviewed.

Key-words: cardiovascular, pro-oxidant, antioxidant
TESTOSTERONE

Testosterone is the predominant and most important androgen, playing a major role in the development of male reproductive tissues (130, 135). “Androgen” is a broad term for any natural or synthetic compound that primarily influences the growth and development of the male reproductive system, including the activity of the accessory male sex organs and development of male secondary sex characteristics (21, 135). Androgens are also essential for health and well-being, and their beneficial and stimulant effects have been known for a long time, since the seminal studies from Brown-Séquard in the nineteenth century:

... in the seminal fluid, as secreted by the testicles, a substance or several substances exist which, entering the blood by resorption, have a most essential use in giving strength to the nervous system and to other parts ...

Other androgens include androstenedione, 5α-dihydrotestosterone (DHT) - which is produced from testosterone by the enzyme 5α-reductase, dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and synthetic androgens in their many steroid ester variations (testosterone cypionate, decanoate, undecanoate, enanthate, propionate, heptylate, caproate, phenylpropionate, isocaproate, acetate, etc) (21, 135).

Testosterone plays a major role in the development of male reproductive tissues and is found in mammals, reptiles, birds, and other vertebrates. In men, testosterone also promotes secondary sexual characteristics, such as increased muscle, bone mass, and the growth of body hair (128, 130, 135).

More than 95% of testosterone is produced by the testes and secreted by the Leydig cells (interstitial cells of the testes), after a series of enzymatic reactions using the cholesterol molecule. Small amounts of testosterone are also secreted by the zona reticularis of the adrenal glands (130, 135). Although adult human males produce about ten times more testosterone than their female counterparts, females are very sensitive to the hormone. In women, the thecal cells of the ovaries synthesize testosterone, as does the placenta, and the adrenal cortex (135, 141).

DHT is also very important in male development. DHT in embryonic development causes differentiation of the penis, scrotum, prostate and their maturation during puberty and maintenance during adult life. Later in life, DHT contributes to male balding, prostate growth and sebaceous gland activity. DHT is more biologically active than testosterone since it binds to the androgen receptor with a 15-fold higher affinity than testosterone, but circulates at a significantly lower level.

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1 Testosterone, from "testo" = testes + "ster" = sterol + "one" = ketone, was isolated by Karoly David, Elizabeth Dingemanse, Janos Freud and Ernst Laqueur from tonnes of bull testes [quoted from Oettel, 2004 135. Oettel M. The endocrine pharmacology of testosterone therapy in men. Naturwissenschaften 91: 66-76, 2004.].
than testosterone. DHT may be considered a hormone with mainly paracrine/autocrine actions in the reproductive target tissues, not being directly secreted into the bloodstream. Thus, testosterone is the most common sex steroid in circulation of men (6, 21, 128, 135).

In men, whereas around 7% of testosterone is reduced to DHT by the cytochrome P450 enzyme, 5α-reductase (an enzyme highly expressed in male accessory sex organs and hair follicles), small amounts (around 0.5%) are converted into estradiol by aromatase (CYP19A1, an enzyme expressed in the brain, liver, adipose and cardiovascular tissues) (21, 135).

Through classic cytosolic androgen receptors (AR) or membrane receptors, testosterone induces genomic and non-genomic effects, respectively. The genomic or classical effects of testosterone depend on its binding to the AR, which acts as a transcription factor upon binding with the androgen response element (ARE), and modulates gene transcription and protein synthesis. Unlike testosterone-mediated genomic effects, non-genomic effects are rapidly produced, can be elicited even when androgens are prevented from entering the cell, do not require the association of AR to DNA, are insensitive to the inhibition of RNA and protein synthesis, and involve the activation of various signaling pathways, including calcium (Ca²⁺), nitric oxide (NO), protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) (62, 165). While a membrane receptor has been identified for testosterone, it is not clear that it mediates all the rapid, non-genomic effects of androgens in the cardiovascular system and other non-reproductive tissues.

AR are widely distributed in several cells/tissues, including vascular smooth muscle cells and endothelial cells (70). Activation of AR may vary depending on whether naturally produced or commercially available hormones are being used, as well as whether metabolic products of androgens are being generated (e.g. testosterone is metabolized by 5α-reductase to the more potent AR ligand, DHT; alternatively, testosterone is metabolized by aromatase (CYP19A1) to the primary form of estrogen, 17β-estradiol). The effects of AR activation also may vary depending on whether androgen effects are tested in tissues/cells derived from male or female animals, or whether an underlying disease is present (49, 113, 188).

Testosterone supplements are mainly used in the treatment of hypogonadism, i.e. in males with very low levels or no endogenous testosterone production. Appropriate use for hypogonadism is known as hormone replacement therapy (testosterone replacement therapy) and aims to maintain serum testosterone levels in the normal range (14).

Due to its anabolic effects, testosterone has also been used in many other conditions, including infertility, lack of libido or erectile dysfunction, osteoporosis, to stimulate penile enlargement and height growth, to promote bone marrow stimulation and to reverse the effects of anemia, and even appetite stimulation (14, 141). High dose testosterone is administered to female-to-male transsexuals, and very low doses are used to treat female androgen insufficiency-
associated symptoms (reduced libido, diminished well-being, and lowered mood or low energy state) in post-menopausal women (14, 141). Women, who experience surgical menopause, have adrenal insufficiency or pituitary insufficiency, or those who experience premature ovarian failure, also have reduced androgen production and may undergo androgen replacement therapy, either with DHEA or testosterone (141). Finally, testosterone and other anabolic steroids are used by athletes in order to enhance muscle development, strength, or endurance, i.e. to improve performance (12). Since most circulating testosterone is bound to sex hormone-binding globulin (SHBG), with a small fraction bound to albumin, SHBG-binding abnormalities can lead to either increased or decreased bioavailable testosterone levels, such as with aging.

Other common causes of pronounced elevations of testosterone include genetic conditions (e.g. congenital adrenal hyperplasia) and adrenal, testicular, and ovarian tumors. Excessive production of testosterone during childhood induces premature puberty in boys and masculinization in girls. Mild-to-moderate testosterone elevations are usually asymptomatic in adult males, but can cause varying degrees of virilization, including hirsutism, acne, oligo-amenorrhea, or infertility in adult females (134, 141). The polycystic ovary syndrome (PCOS) is the most frequent endocrinopathy (hirsutism, acne, menstrual disturbances, insulin resistance and, frequently, obesity, elevated blood pressure) and is characterized by hyperandrogenemia in women of reproductive age (192).

IS TESTOSTERONE A GOOD OR A BAD GUY FOR CARDIOVASCULAR SYSTEMS OF MEN AND/OR WOMEN? THE DIFFERENCES MAY BE RELATED TO ENDOGENOUS LEVELS vs. SUPPLEMENTATION.

In addition to its effects on the male reproductive system or sexual function, testosterone influences the activity of other organs and systems. Studies on the effects of testosterone in the cardiovascular system were initially reported in the late 1930s into the early 1940s (48, 75, 80, 110, 198). From these initial studies until nowadays, the cardiovascular actions of testosterone (androgens) are still controversial, ranging from protective to deleterious effects.

Numerous studies in the literature demonstrate increased cardiovascular risk and mortality with testosterone deficiency. In addition, testosterone therapy has been demonstrated to attenuate cardiovascular risk factors and also cardiovascular outcomes. Khaw and co-workers conducted a prospective study with 11,606 men, aged 40-79 years, and mean follow up of 7 years. Testosterone baseline levels were inversely related to mortality due to all causes, cardiovascular diseases and cancer (100). The same trend was also observed for coronary heart disease,

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3 Testosterone use is considered to be a form of doping in most sports and in many countries. Along with other anabolic androgenic steroids, testosterone is a controlled substance and its non-medical use is considered drug abuse.
although statistical significance was not achieved (100). Similarly, Hyde and co-workers studied 4,249 men with 5.1 years of follow-up on average. Lower free testosterone levels were associated with all-cause mortality (HR= 1.62; 95% CI= 1.20-2.19, for 100 vs 280 pmol/liter) and also predicted cardiovascular disease mortality (HR= 1.71; 95% CI= 1.12-2.62, for 100 vs 280 pmol/liter) (90). Prospective data from the European Male Aging Study on 2,599 men, aged 40-79 years, in 8 European countries, demonstrated that late-onset hypogonadism increases the risk of all-cause mortality by 5-fold. In addition, men with testosterone levels less than 8 nmol/L had a 2-fold higher risk of mortality compared to eugonadal men, and a 3-fold higher risk when presenting three symptoms of abnormal sexual function (irrespective of serum testosterone) (154). In type 2 diabetic patients, low testosterone levels also predict an increase in all-cause mortality. In diabetic men, testosterone replacement therapy reduced mortality to 8.4% compared to 19.2% in the untreated group (p=0.002) (133). Shores and co-workers found similar results with testosterone treatment in men with low testosterone levels in whom the mortality was decreased to 10.3% in testosterone-treated group compared with 20.7% in the untreated group. Furthermore, testosterone treatment decreased the risk of death (HR= 1.71; 95% CI= 1.12-2.62, for 100 vs 280 pmol/liter) when adjusted for age, body mass index, testosterone level, medical morbidity, diabetes and coronary heart disease (173).

Maintaining normal testosterone levels in elderly men improves many parameters that are linked to increased cardiovascular disease risk. Men whose testosterone levels are slightly above average are less likely to have hypertension, to experience a myocardial infarction, to be obese, and less likely to rate their own health as fair or poor (17, 179). Accordingly, testosterone increases lean body mass, decreases visceral fat mass, total cholesterol, low density lipoprotein and triglyceride levels, and inhibits fatty streak formation (128).

Testosterone deficiency has also been linked to increased mortality due to all-cause and/or cardiovascular disease in different groups of patients with myocardial infarction (127), coronary heart disease (117), erectile dysfunction (40), diabetes (133, 150), renal disease (24) and patients referred to coronary angiography (109). In addition to the several studies that have demonstrated an association between low testosterone levels and coronary artery disease (46, 53, 87, 164), other evidence demonstrated that low testosterone is not only associated with coronary artery disease, but also with its severity (46, 111, 145, 164).

Svartberg and co-workers demonstrated an inverse association between total testosterone levels and intima-media thickness after adjusting for smoking, physical activity, blood pressure and lipid levels, in a population-based and cross-sectional study of 1, 482 men. However, these changes were not independent of body mass index (182). Similarly, Vikan and co-workers studying 2,290 men found an inverse association between testosterone levels and total carotid plaque area even after adjusting for age, systolic blood pressure, smoking and use of lipid-
lowering drugs (196). In addition to these studies, testosterone therapy increased time delay to angina onset evoked by a treadmill test in three randomized, placebo-controlled studies (54, 163, 199). These results corroborate other studies in the literature suggesting that testosterone causes brachial and coronary artery vasodilation (98, 136, 200). Finally, testosterone deficiency has been shown as an independent risk factor for worse outcomes in congestive heart failure patients. Toma and colleagues conducted a meta-analysis of four randomized controlled trials. Testosterone therapy for 52 weeks improved peak oxygen consumption, 6-minute walk test, and incremental shuttle walk test. Importantly, no adverse cardiovascular events were observed (186).

Further support to protective effects of testosterone comes from a very recent and large observational cohort, where testosterone therapy was used in 83,010 men with documented low testosterone levels. In this study, total testosterone levels were assessed after testosterone replacement therapy was introduced. The subjects were categorized into three groups: 1) men who received testosterone therapy and total testosterone levels were normalized; 2) men who received testosterone therapy and total testosterone levels were not normalized; and 3) men who did not receive testosterone therapy. The all-cause mortality, the risk of myocardial infarction and stroke were higher in untreated men and in testosterone-treated men without normalization of testosterone levels when compared to testosterone-treated men whose hormone levels were normalized (171).

Taken together, this group of scientific studies and clinical evidence strongly suggests that testosterone at physiological levels promotes cardiovascular health and decreases morbidity and mortality in men under several conditions.

Although early studies showed that testosterone-replacement therapy did not increase the incidence of cardiovascular disease or cardiac events, such as myocardial infarction, stroke, or angina in men (59, 78, 79), recent safety studies point to a positive association between endogenous androgen and cardiovascular disease risk, as noted below. In addition, a group of studies failed to show any association between low testosterone levels with coronary artery disease (5, 10, 26, 97).

In women, testosterone may have different effects than in men. In a cross-sectional study evaluating 344 women, aged 65–98 years, high total testosterone levels were associated with a threefold greater risk of coronary heart disease. In addition, a significant association was found between high total and free testosterone levels and an adverse metabolic profile, i.e. insulin resistance and abdominal obesity (143).

Insulin resistance is considered the major player in the metabolic abnormalities and increased cardiovascular risk associated with PCOS. However, androgen excess, as well as a proinflammatory profile, further aggravates the cardiovascular and metabolic aberrations in women with PCOS (38, 153). Women with PCOS harbor a two-fold increased risk for arterial disease
(coronary heart and stroke), independent of their body mass index (43). In a study with 390
postmenopausal women followed for 5 years, 79% of women that presented clinical features of
PCOS experienced a 5-year cardiovascular disease event-free survival versus 89% of women
without PCOS, independent of hypertension, diabetes status, or other cardiovascular disease risk
factors (172).

In another study with 200 nondiabetic postmenopausal women, half of them with
cardiovascular disease and half serving as matched controls, the free androgen index (FAI) was
significantly higher in women with cardiovascular disease compared with controls (139).

Studies in men who already have cardiovascular disease show different responses to
testosterone supplementation. Concerns about the risk of heart attacks with testosterone therapy
were highlighted in a clinical trial involving 209 men, with an average age of 74 and with a high
prevalence of hypertension, diabetes, hyperlipidemia, and obesity. Patients were scheduled to
receive either testosterone gel or placebo for 6 months. The data and safety monitoring board
recommended discontinuation of the trial due to a significantly higher rate of adverse
cardiovascular events in the testosterone group compared with the placebo group (a total of 23
subjects in the testosterone group, as compared with 5 in the placebo group, had cardiovascular-
related adverse events) (13).

Very recent clinical trials further raised concerns about the safety of testosterone therapy.
Another randomized clinical trial that aimed to assess the association between testosterone
therapy and all-cause mortality, myocardial infarction, or stroke among male veterans with low
testosterone levels (<300 ng/dL) who underwent coronary angiography in the Veterans Affairs
(VA) system between 2005 and 2011, was stopped prematurely due to adverse cardiovascular
events (195). The use of testosterone therapy in this retrospective national cohort study was
significantly associated with increased risk of adverse outcomes, the rate of adverse events was
25.7% among the 1223 patients that started testosterone therapy, compared to a rate of 19.9%
among the 7,489 men who were not using testosterone (195).

Early this year, a cohort study on the risk of acute non-fatal myocardial infarction showed
that older men, and younger men with pre-existing diagnosed heart disease, may be twice as
likely to suffer a non-fatal heart attack following initiation of testosterone therapy. The study
involved 55,593 men who received an initial prescription for low dose testosterone between 2006
and 2010 (60). The authors reported that the incidence rate of myocardial infarction in men, aged
65 years and older, in the 90 days following an initial prescription of testosterone (post-prescription
interval) was higher than the incidence rate in the one year prior to the initial prescription (pre-
prescription interval) with a post/pre rate of 2.19. The study also showed that the post/pre rate for
testosterone prescription increased with age from 0.95 for men under age 55 years to 3.43 for
men aged ≥ 75 years (60).
Increasing the debate on the beneficial / deleterious effects of testosterone, Morgentaler and co-workers have raised a series of methodological problems and limitations in these studies and pointed that the conclusions drawn from these trials should be taken cautiously (131).

Finally, a number of lawsuits, alleging a significantly increased rate of stroke and heart attack in elderly men using testosterone supplements, are currently underway against testosterone manufacturers, and the Food and Drug Administration added a warning regarding deep vein thrombosis and pulmonary embolism among testosterone users (57, 86). The number of lawsuits is rapidly increasing since it has been alleged that the manufacturers of testosterone supplements and prescriptions failed to adequately investigate the potential side effects of testosterone therapy, yet aggressively marketed the products in a way that resulted in the widespread over-use of testosterone supplements among men who may have had no real medical need for testosterone treatments but may have had underlying cardiovascular diseases.

Non-medical testosterone use (or testosterone abuse) has been shown to increase arterial blood pressure and to induce left ventricular hypertrophy, myocardial infarction due to coronary vasospasm or thrombosis (4, 56, 61, 69, 83, 125).

POSSIBLE MECHANISMS RESPONSIBLE FOR DELETERIOUS EFFECTS OF TESTOSTERONE SUPPLEMENTS

Among the possible mechanisms involved in the deleterious actions of supraphysiological doses of testosterone, both direct actions in the vasculature and indirect actions have been reported. Unfavorable changes in the lipid profile (88), pro-coagulatory effects, activation of the sympathetic nervous system and renin-angiotensin-aldosterone system, and insulin resistance are some of the indirect actions of testosterone that can affect the vasculature (25, 52, 66, 76, 105, 119). Among the direct effects of testosterone, stimulation of pro-inflammatory enzymes in the vasculature [e.g. thromboxane synthase, as well as cyclooxygenase-1 (COX-1) and COX-2 in rat thoracic aorta and mesenteric arteries (31, 177)], reactive oxygen species (ROS) generation in vascular smooth muscle cells (33), that may decrease nitric oxide (NO) bioavailability and lead to increased blood pressure and renal dysfunction (36, 95, 155, 159), activation of vasoconstrictor signaling pathways [protein kinase C (PKC), mitogen-activated protein kinases (MAPKs)] and increased vasoconstriction have been reported (34, 35, 99). It is worth mentioning that the beneficial effects of testosterone have been linked to modulatory effects on some of the same pathways mentioned above adding to the controversial nature of testosterone supplementation.

In the next section, we will focus on evidence indicating that testosterone has pro-oxidative actions that may contribute to the deleterious actions of testosterone in the cardiovascular system.
OTHER MECHANISMS: TESTOSTERONE AND ROS GENERATION / OXIDATIVE STATUS

Since comprehensive and excellent recent reviews have highlighted the effects of ROS in the cardiovascular system as well as the implications of ROS in cardiovascular diseases (142, 146, 170, 189, 190), only key points on ROS generation will be included in this mini-review.

ROS play a major role in various biological responses, such as host defense, activation of transcription factor, modulation of kinase and ion transport systems activity (47, 170). ROS and reactive nitrogen species (RNS) are products of cellular metabolism, and are well recognized for their dual roles as both deleterious and beneficial species. Although ROS, such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical, are generated as the natural byproducts of normal oxygen metabolism, they can create oxidative damage via interaction with other bio-molecules (47, 170). In the situation of uncontrolled ROS generation or impaired ROS inactivation, termed oxidative stress, the excessive availability of oxidants activates signaling pathways that have been implicated in the development of organ damage in cardiovascular and metabolic diseases, including hypertension, atherosclerosis, heart failure, diabetes and stroke (189, 190). Increased ROS production has been directly linked to vascular remodeling, endothelial dysfunction, altered vasoconstrictor responses, inflammation and modifications of the extracellular matrix, all important features of cardiovascular disease pathophysiology (142, 189).

ROS can be produced from complexes in the cell membrane [nicotinamide adenine dinucleotide phosphate NAD(P)H-oxidase or Nox], cellular organelles (peroxisomes and mitochondria), and in the cytoplasm (by xanthine oxidase). Furthermore, low levels of tetrahydrobiopterin (BH$_4$) and L-arginine, the rate limiting cofactor and substrate for endothelial nitric oxide synthase (eNOS), respectively, can cause the uncoupling of eNOS, resulting in decreased NO production and increased ROS generation (47, 189).

NAD(P)H oxidases (Nox) are the main source of ROS in the vasculature. They function as electron transport chains across membranes, using NAD(P)H as the electron donor to reduce molecular oxygen to superoxide, and participate not only in normal cell function, but also trigger the development of injury in pathological conditions (170, 189). The Nox family is composed of catalytic subunits (Nox1-5, Duox1, and Duox2), and the docking subunit p22phox, all present in the cell membrane. The regulatory subunits Nox organizer 1 (Noxo1), Nox activator 1 (Noxa1), p67phox, p47phox, and p40phox, are located in the cytosol. Activation of Rac1/2 regulates the translocation and assembly of the NAD(P)H oxidase subunits in the plasma membrane, and is a key event in Nox activation (19, 51, 142, 146).

Antioxidants, on the other hand, act by scavenging or chain-breaking ROS and RNS. Antioxidants are molecules that accept or donate electrons and, as a consequence, can convert ROS and RNS into less reactive products, thus neutralizing them. Antioxidants can be enzymes, such as superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, and
glutathione transferase, as well as non-enzymatic endogenous molecules, such as α-tocopherol, β-carotene, glutathione, ascorbic acid, adenosine, lipoic acid, coenzyme Q, and lactoferrin.

Synthetic molecules, such as thiols, ebselen (selenium-based peroxide scavenger), idebenone (coenzyme Q-analogue), mitoQ (mitochondrial-targeted ubiquinone), and α-tocopheryl-succinate nanoparticles, as well as plant-derived molecules, such as polyphenol compounds (including quercitin, myricetin, catechins, anthocyanins), proanthocyanidin, and lycopene, are also antioxidants (68, 149). Considering that redox homeostasis of the cell is ensured by a delicate balance between ROS production and its antioxidant capacity, it is also important to consider the effects of androgens upon cell antioxidant systems. To make it easier for the reader, the effects of testosterone on the main components that influence ROS generation and degradation (or that control the cell redox status) will be discussed.

NAD(P)H oxidase

Our group reported that testosterone induces ROS generation in cultured VSMC, with greater production of ROS in cells from hypertensive as compared to normotensive animals (33). Testosterone effects are not due to conversion of testosterone to 17β-estradiol, since the aromatase inhibitor, anastrazole, has no effect on ROS formation. While testosterone effects on steady-state mRNA levels of NAD(P)H oxidase subunits Nox1 and p22phox are not evident, testosterone upregulates the expression of Nox4 only in VSMC from normotensive but not from hypertensive animals (33). Although vascular NAD(P)H oxidases have been generally implicated in excessive and deleterious ROS formation, Nox4 (unlike Nox1) has been shown to exhibit protective effects. Nox4 maintains VSMC in a differentiated state, counteracting proliferation and vascular hypertrophy (39); Nox4-derived H₂O₂ mediates vasodilation through hyperpolarization (156); and mice overexpressing Nox4 exhibit increased vasodilator function and lower blood pressure (20). Therefore, the lack of stimulatory effects of testosterone on Nox4 expression in cells from hypertensive rats might indirectly promote VSMC growth under hypertensive but not normotensive conditions.

The non-genomic stimulatory effects of testosterone on ROS generation in VSMC from hypertensive animals are insensitive to the AR antagonist, flutamide, and lead to phosphorylation of the non-receptor tyrosine kinase, c-Src (33). Of importance, c-Src mediates vascular contraction and hypertrophy, and is upregulated in experimental polygenic hypertension (23, 191). It is possible that testosterone actions on VSMC may further aggravate vascular dysfunction associated with cardiovascular diseases (e.g. hypertension). Further support to this idea is the fact that testosterone abolishes the beneficial effect of estrogen treatment in the aorta of ovariectomized spontaneously hypertensive rats (SHR) by increasing ROS production through p47phox phosphorylation (41). On the other hand, a genetic vascular predisposition may lead to
Testosterone metabolites may play a role in ROS generation in the cardiovascular system. The cytochrome P4501B1 (CYP1B1) also metabolizes testosterone into 6β-hydroxytestosterone, 2α-, 15α- and 16α-hydroxytestosterone (178). 6β-hydroxytestosterone contributes to angiotensin II-induced hypertension and its associated cardiac damage. All changes are accompanied by increased NAD(P)H oxidase activity and ROS generation (147). Similarly, rats with DHT-induced hypertension display increased expression of p47phox, gp91phox and, consequently, increased superoxide generation in renal interlobar arteries (175). In addition, female rats treated with DHT (to mimic/imitate PCOS condition) display increased mRNA expression of gp91phox, p22phox, p47phox and NOX4 in the renal cortex (202). Increased circulating androgens, such as dehydroepiandrosterone (DHEA) in healthy, ovulating women, as occurs in women with PCOS, also increases leukocyte ROS generation, p47(phox) gene expression, and plasma TBARS (72). These studies highlight the possible effects of testosterone and its metabolites inducing NAD(P)H oxidase-dependent ROS production in the cardiovascular system. However, it is not clear if these effects are directly produced by testosterone or indirectly through testosterone-induced cytokine release, eicosanoid production, or upregulation of vasoactive substances. Therefore, more studies are warranted to investigate these possibilities.

Considering that some actions of testosterone do not rely on AR activation, i.e. AR-independent effects, it is possible that an as yet unidentified receptor may be involved. As suggested by Barton and colleagues, the orphan G protein-coupled receptor GPRC6A, that has been shown to mediate non-genomic responses to testosterone, might be a possible candidate to mediate the rapid, AR-independent effects of testosterone on ROS generation in VSMC (11). In addition, testosterone induces Duox1 activation through GPRC6A in keratinocytes that leads to H₂O₂ generation (104).

Mitochondrial ROS and Androgens

The mitochondrial respiratory chain is also an important source of ROS, mainly superoxide, that is quickly converted into H₂O₂. It is estimated that 1-4% of oxygen is reduced to superoxide, especially by complexes I and III of the mitochondrial respiratory chain (18, 30, 45, 203). Mitochondrial dysfunction contributes to inflammation, cell senescence, and apoptosis and plays an important role in vascular disease (203). Our group recently reported that testosterone, in addition to its effects on NAD(P)H activity, also induces mitochondrial-associated ROS generation and apoptosis in VSMC via activation of AR (115).

Apoptosis, or programmed cell death, occurs in multicellular organisms, and includes a series of biochemical events (blebbing, cell shrinkage, nuclear fragmentation, chromatin
condensation, and chromosomal DNA fragmentation) that lead to characteristic cell changes and death. Apoptosis deregulation is considered a pathogenetic process in a variety of human diseases and is an important mechanism underlying the changes observed in hypertension, diabetes and arteriosclerosis (73). As ROS are important activators of the apoptotic cascade, it is reasonable to assume that testosterone-induced ROS may modulate the apoptosis-associated damage in these diseases.

Two pathways are responsible for initiating apoptosis, one mediated by cell death receptors located on the membrane surface, termed the extrinsic pathway, and another mediated by mitochondria, termed the intrinsic pathway. Both pathways are modulated by activation of caspases, which trigger the cellular alterations characteristic of apoptosis (169). In VSMC, testosterone-induced apoptosis is associated with a decrease in the ratio of Bax/Bcl-2 (pro-apoptotic and anti-apoptotic proteins) in total cell homogenates as well as in the mitochondrial fraction. Since there is no evident cytochrome c release from mitochondria to cytosol and procaspase 8 is activated and gene expression of two cell death receptor ligands is increased, it seems that testosterone activates the extrinsic pathway of apoptosis in VSMC, in association with AR activation and mitochondrial-generated ROS (115). In prostate cancer cell lines, in addition to directly inducing ROS generation (166), testosterone enhances H$_2$O$_2$-induced apoptosis (91), indicating that under oxidative stress conditions, androgen signaling may further enhance apoptosis and DNA damage response.

Although studies on the role of testosterone and/or androgens upon mitochondrial ROS generation in the cardiovascular system are scarce, there are few studies in the literature showing this possibility in other cells. DHT induces mitochondrial ROS production through p66Shc protein in AR-positive prostate cancer cells (193). Similarly, methyltrienolone (synthetic AR agonist) induces mitochondrial ROS generation in prostate cancer cells through mitochondrial fatty acid oxidation (112). In addition, testosterone increases mitochondrial oxygen consumption, membrane potential and ROS production in leukocytes from female-to-male transsexuals (194). Testosterone, DHEA and 3α-androstanediol increase mitochondrial ROS levels, oxygen consumption rate and ATP levels in neuroblastoma cells. Moreover, testosterone increases mitochondrial respiratory capacity (74). Conversely, stanozolol (an anabolic androgenic steroid) decreases mitochondrial ROS and oxidative stress induced by acute exercise in rat skeletal muscle (167). Considering the anabolic effects of testosterone and its related androgens and also their effects upon mitochondrial metabolism, it is likely that androgens can modulate mitochondrial ROS generation. However, there are very few studies that have tested this hypothesis whereas all the aforementioned studies point to a possible modulation of mitochondrial ROS by androgens. However, caution must be taken when interpreting these results since they were performed under pathological, supraphysiological doses of androgens. Therefore, whether these effects of...
androgens are produced physiologically, and if so, what relevance do they have for the cardiovascular system remains unknown.

Similarly, how testosterone-induced ROS preferentially induces vasodilation, VSMC migration or interferes with mitochondrial function is not known, but may be related to the theory that location of ROS generation is of major importance. In addition, it is not clear why testosterone activates different sources of ROS-generating enzymes/organelles in these studies. Figure 1 summarizes signaling pathways implicated in testosterone effects on ROS generation/cellular oxidant status in the cardiovascular system.

Xanthine oxidase

Testosterone-induced ROS generation is also coupled to a commonly described effect of testosterone in the vasculature: vasodilation. Testosterone stimulates the generation of superoxide and NO, to produce peroxynitrite, which has direct vasodilator effects. Furthermore, the vasodilator influence of testosterone is inhibited by scavenging peroxynitrite, indicating the latter may play an important role in testosterone vascular effects. Xanthine oxidase was identified as the likely source of testosterone-stimulated superoxide production via activation of AR and the PI3 kinase-Akt signaling cascade (152). Similarly to VSMC, in the renal macula densa-like cell line MMDD1, testosterone induces superoxide generation via xanthine oxidase-, NAD(P)H oxidase- and AR-dependent pathways. This effect is associated with an increase in tubuloglomerular feedback (TGF), which results in lower tubular perfusion and altered control of renal microcirculation, potentially contributing to the higher prevalence of hypertension and renal injury in males (65). On the other hand, in an ischemia/reperfusion model, testosterone exhibits a protective effect in spinal cord by decreasing xanthine oxidase activity (77), and in kidneys of aged rats (24 months), testosterone treatment decreases xanthine oxidase activity (201).

Cyclooxygenase

There are three isoforms of cyclooxygenase, namely COX1, COX2 and COX3. In most tissues, COX1 is constitutively expressed. COX2, initially thought to be expressed only during an inflammatory process, has been shown to be constitutively expressed in cardiovascular, renal and central nervous systems. COX-3 is a splice variant of COX-1 with all catalytic features of COX-1 and COX-2. These enzymes oxidize arachidonic acid into prostaglandins, which play a crucial role in vascular tone control (58, 185). In recent years, studies have shown that ROS activate COX, and that COX and its products induce ROS generation. This reciprocal association was mainly identified in hypertensive conditions (123), and in endotoxic shock (197).

Androgens modulate COX1 and COX2 expression, and some of the effects of androgens rely on COX activation/inhibition. Castration dramatically decreases COX1 and COX2 mRNA
expression in rat epididymis (31). Testosterone-induced hyperplasia is decreased by treatment with flavocoxid [a dual inhibitor of COX and 5-lipoxygenase (5-LOX)] (3). Testosterone-induced relaxation is decreased in diabetic animals with COX inhibition (122). The relaxation effect of testosterone on renal arteries is also mediated by COX enzymes, and possibly, via modulation of thromboxane A2 production in smooth muscle cells and prostacyclin in endothelial cells (121). Despite the evidence showing a positive modulation of COX2 by androgens, Martorell and co-workers found increased COX2 expression in aorta of orchidectomized rats (124).

Testosterone metabolites also modulate COX enzymes. DHT treatment in rats or ex-vivo incubation of pial arteries with DHT upregulates COX2 expression and increases constriction of middle cerebral arteries (71). In human coronary smooth muscle cells, DHT also increases COX2 expression. Interestingly, DHT attenuates the increases in COX2 expression in coronary arteries produced by either LPS or IL-1β, suggesting a differential role for DHT under inflammatory conditions (137). The same protective effect of DHT was demonstrated in cerebral arteries under hypoxic conditions (207).

Considering the number of reports demonstrating the effects of androgens on COX expression and that COX is a source of ROS, the lack of studies evaluating COX-derived ROS by androgens in the cardiovascular system is surprising, specially taking into account the importance of ROS for vascular tone control. Our group recently demonstrated that COX2-dependent ROS production is obligatory for testosterone-induced leukocyte migration (32), which may contribute to inflammation and vascular dysfunction. However, more studies on the role of androgens modulating COX-derived ROS and its implications for the cardiovascular system are warranted.

**Other sources of ROS**

Uncoupled eNOS and 5-LOX are also potential sources of ROS in the vasculature. eNOS uncoupling decreases NO production and increases ROS generation (47, 189). Angiotensin-II-induced ROS production in smooth muscle cells is partly dependent on 5-LOX and, its product, leukotriene B4 (116). Although uncoupled eNOS and 5-LOX have been implicated in ROS generation and several aspects of cardiovascular disease, the effects of testosterone on ROS production by these enzymes still need to be determined.

**Antioxidant status**

The main function of antioxidants is to prevent and/or delay protein and lipid oxidation, DNA mutation and, ultimately, cellular oxidative damage. However, when ROS production is maintained at high levels, the system defenses against ROS can be overwhelmed, which may lead to disease conditions. Fortunately, there are many endogenous factors that can serve as modulators of the production and actions of ROS. The enzymatic antioxidant system is considered “the first line of
defense” against ROS production, and is composed of superoxide dismutase (SOD) that depletes superoxide, catalase (CAT) that decomposes H$_2$O$_2$, and the glutathione peroxidase/glutathione reductase system. Thiols and low molecular weight antioxidants such as tocopherols, ascorbate, retinols, urate and reduced glutathione represent “the second line of defense” (149).

Besides increasing ROS, a single supraphysiological dose of testosterone decreases SOD, CAT, and glutathione peroxidase expression in human endothelial cells (176). Moreover, testosterone decreases the total antioxidant capacity in urine (176). On the other hand, Zhang and co-workers demonstrated that castration decreased SOD and glutathione peroxidase activity and increased malondialdehyde (MDA) levels, an indicator of lipid peroxidation, in murine cardiomyocytes. Testosterone replacement to physiological levels restored all changes by AR-independent mechanisms (206). In addition, the same research group demonstrated that cardiomyocytes from testicular feminized (Tfm) mice displayed decreased SOD and glutathione peroxidase activities combined with increased MDA levels. Testosterone replacement normalized these changes (205). Similarly, Klapcinska and colleagues showed that castration decreases SOD, CAT, glutathione peroxidase, and glutathione reductase activity in rat left ventriculars. In contrast to the Zhang studies, castration did not increase lipid peroxidation and testosterone replacement did not restore antioxidant enzymes activities in this study (103). SOD activity was decreased in cardiac muscle of castrated rats, but CAT and glutathione were not changed (9). In humans, hypogonadism is associated to decreased plasma antioxidant capacity. After testosterone replacement and eugonadal re-establishment, the antioxidant capacity returns to normal values, suggesting that normal levels of testosterone are essential to maintain the antioxidant status (118).

Other androgens are also capable of modulating the antioxidant capacity and, therefore, the redox balance in the cardiovascular system. DHEA acute treatment (6h) increases SOD activity in rat heart homogenates. Conversely, DHEA treatment for 24h decreases SOD and increases glutathione-S-transferase activities (93). Nandrolone decanoate is frequently used by bodybuilders and recreational athletes to increase performance. Recently, Frankenfeld and co-workers demonstrated that nandrolone decanoate treatment increases cardiac NOX2 mRNA levels and H$_2$O$_2$ generation. In addition, these changes were accompanied by decreased renal CAT activity, total reduced thiol residues, and carnonyl content (64). Treatment with nandrolone decanoate also prevents the increase in cardiac expression of SOD, glutathione peroxidase and glutathione reductase induced by exercise in rats. Moreover, nandrolone decanoate treatment blocks around 50% of the exercise-induced cardiac tolerance to ischemic events (29). Similarly, turinabol treatment increases TBARS levels by 46% with slightly increases in CAT activity in rabbit plasma. Methandienone treatment reduces total antioxidant capacity by 46% (67). Interestingly, only high doses of the anabolic androgenic steroids were able to provoke these effects.
Table 1 summarizes the effects of testosterone and other androgens (or the lack of them) on ROS generation / oxidative status in various cell types. In addition to those mentioned above, and as shown in Table 1, testosterone-induced ROS generation has been reported in renal cells (37, 65, 144), in human prostate cancer cells (112, 148, 151, 181), and leukocytes, among others. However, in myocardial (50, 103, 206) and neuronal cells (2, 55, 85), testosterone also has antioxidant effects. It reinforces the notion that the effects of testosterone on ROS generation are controversial, mainly because androgens have pro-oxidant as well as anti-oxidant effects depending on the tissue/cell being studied (and sometimes even when the same cell type is being studied).

Summary

The role of testosterone in mediating or protecting against ROS and antioxidant capacity in the cardiovascular system is far from being clear, with testosterone presenting pro-oxidant as well as antioxidant effects. There are various possible contributing factors to these discrepant effects of testosterone in the cardiovascular system and in the other tissues and organs: 1) Acute vs. chronic differential effects are possibly due to activation of distinct sets of signaling pathways as reported with other hormones/neurotransmitters; 2) The initial metabolic/energetic/redox status of the cell; 3) global (or local) increases in testosterone may produce differential effects based on the specific cell types that are stimulated; 4) the concentrations of testosterone (physiological, supraphysiological); 5) the steroid ester used (testosterone cypionate, decanoate, undecanoate, enanthate, propionate, heptylate, caproate, phenylpropionate, isocaproate, acetate), which changes the compound solubility in water and slows the release of the parent steroid, i.e. changes absorption time. Different esters are also susceptible to the presence of native and selective esterases in the many complex biological/cellular environments and can mask specific functional groups. These processes may confer different responses to different esters; 6) the “sex” of the individual or cell/tissue where the effects of testosterone are being studied; and not least, 7) different animal species (mice, rats, humans, rabbits, birds) and the age, duration and characteristics of the diseases/conditions upon which testosterone effects are determined. The complexity of testosterone effects is evident, and further basic and clinical studies are required for a better understanding of the mechanisms by which testosterone has its biological activity independent of reproduction, that may be detrimental and/or beneficial to the cardiovascular system.
**Legend to Figure 1.**

**Testosterone effects on ROS generation/cellular oxidant status in the vascular system.**

Testosterone has been shown to increase ROS in smooth muscle cells via different cellular sources, such as activation of NAD(P)H oxidase, mitochondria, COX-2 and xanthine oxidase. The genomic action of testosterone induces c-Src and PI3K/Akt pathways, which in turn, activates NAD(P)H oxidase and xanthine oxidase, respectively. Testosterone may also increase ROS, via its non-genomic action, through GPRC6A receptor. Increased ROS production may lead to migration, apoptosis, hypertrophy and inflammation, causing vascular dysfunction.

AR: androgen receptor; T: testosterone; ARE: androgen responsive element; COX-2: cyclooxygenase-2; XO: xanthine oxidase; c-Src: Proto-oncogene tyrosine kinase Src; PI3K/Akt: Phosphoinositide-3-kinase/Protein kinase B; GPRC6A: G protein-coupled receptor, family C, group 6, member A.

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List of References


GPRC6A

NAD(P)H oxidase

Mitochondrion

c-Src

ROS

COX-2

XO

VASCULAR DYSFUNCTION

Migration
Apoptosis
Hypertrophy
Inflammation

PI3K/Akt

RNA

DNA

Nucleus

ARE
<table>
<thead>
<tr>
<th>Cell/tissue/animal</th>
<th>Effect on Redox status</th>
<th>Associated effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-oxidant</strong></td>
<td><strong>Anti-oxidant</strong></td>
<td></td>
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<tr>
<td>PCOS / menopause (Experimental models and patients)</td>
<td>↑</td>
<td>↓</td>
<td>↑ lipid peroxidation and ROS generation</td>
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<tr>
<td>Cardiovascular system</td>
<td></td>
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<tr>
<td>Heart / cardiac cells from experimental animal models [orchidectomized (ORX) and testosterone-treated animals, Testicular feminized (Tfm) mice e.g.]</td>
<td>↑</td>
<td>↓</td>
<td>↑ lipid peroxidation, MDA levels ↓ SOD, GPx, catalase, GR enzyme activities</td>
</tr>
<tr>
<td>Arteries from experimental animal models [andropause - follitropin receptor knockout (FORKO) male mice; androgen receptor knockout (ARKO) mice]</td>
<td>↑</td>
<td>↓</td>
<td>↑ superoxide anion production, lipid peroxidation, gene expression of NADPH oxidase components, phosphorylation of JNK and Smad2/3 ↓ NO availability, eNOS expression and phosphorylation Akt phosphorylation</td>
</tr>
<tr>
<td>Blood/Immune system cells</td>
<td></td>
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<tr>
<td>Leukocytes from individuals that received testosterone / androgens</td>
<td>↑</td>
<td>↓</td>
<td>↑ ROS production, p47(phox), plasma TBARS. ↓ GSH levels, (GSH)/(GSSG) ratio</td>
</tr>
<tr>
<td>Immune cells from experimental animals</td>
<td>↓</td>
<td>↓</td>
<td>↓ superoxide anion production, lipid peroxidation (TBARS) ↑ GSH-R activity, CoQ10 levels</td>
</tr>
<tr>
<td>Cell lines [HL-60 (Human promyelocytic leukemia cells), THP-1 cells (human monocytic cell line)]</td>
<td>↓</td>
<td>↓</td>
<td>no changes in oxidant/antioxidant status</td>
</tr>
<tr>
<td>Prostatic cells [TM3 Leydig cell line, 22rv1 cells (human prostate carcinoma cell line), AR-negative PC3 human prostatic cancer cell line, LNCaP cells (androgen-sensitive human prostate adenocarcinoma cells)].</td>
<td>↑</td>
<td>↓</td>
<td>↑ ROS generation, lipid peroxide contents, mitochondrial fatty acid oxidation ↓ activity of catalase, SOD, GSH-Px, GSH-ST, and GSH-R; levels of reduced GSH and Vitamin C.</td>
</tr>
<tr>
<td>Prostate (from testosterone-treated experimental animals)</td>
<td>↓</td>
<td>↓</td>
<td>↓ ROS-generating NAD(P)H oxidases expression [Nox1, gp91(phox), Nox4] ↑ activity of catalase, SODs, GSH-R, SOD2, GSH-Px1, thioredoxin, and peroxiredoxin 5 ↓ ROS-detoxifying enzymes (SOD2, GSH-Px1, thioredoxin, and peroxiredoxin 5)</td>
</tr>
<tr>
<td>Neuronal cells</td>
<td>↓</td>
<td>-</td>
<td>↑ aconitase activity (a ROS-sensitive mitochondrial enzyme)</td>
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<tr>
<td>[PC-12 cells (cell line derived from a pheochromocytoma of the rat adrenal medulla) and brain cells from experimental animals (cerebellar granule cells, astrocytes, N27 dopaminergic cells)]</td>
<td></td>
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<td>↑ expression of Nrf2, HO-1 and NQO1</td>
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<td></td>
<td></td>
<td></td>
<td>↑ activities of catalase, SOD and GSH-Px enzymes</td>
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<td></td>
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<td></td>
<td>↓ MDA levels</td>
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<tr>
<td>Kidneys and Renal cells</td>
<td>↑</td>
<td>-</td>
<td>↑ renal lipid peroxidation, H2O2 levels, urinary excretion of H2O2, superoxide anion formation</td>
</tr>
<tr>
<td>(from experimental animal models)</td>
<td></td>
<td></td>
<td>↓ expression and activity of MnSOD, catalase, SOD1, SOD2.</td>
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<tr>
<td>Skeletal muscle</td>
<td>↑</td>
<td>-</td>
<td>↑ TBARs, lipid peroxidation.</td>
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<td>(gastrocnemius soleus and extensor digitorum longus muscles e.g.)</td>
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<td>↑ MnSOD</td>
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<td>↓</td>
<td>↑</td>
<td>↑ nitrotyrosine levels, ER stress markers</td>
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<td>Pancreatic cells /diabetes-related conditions</td>
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<td>↑ MDA levels</td>
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<td>INS-1 cells (rat insulinoma cell line) and animal experimental models</td>
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<td>↑ activities of GSH and catalase</td>
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<tr>
<td>Bladder/urinary-genital tract</td>
<td>↓</td>
<td>-</td>
<td>↓ MDA levels</td>
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<td>Cavernous tissue from experimental animal models</td>
<td></td>
<td></td>
<td>↑ GPx, cGMP</td>
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</table>

**Abbreviations:** CoenzymeQ10 (CoQ10), copper-zinc superoxide dismutase (SOD1 or Cu/ZnSOD), endothelial NO synthase (eNOS), extracellular superoxide dismutase (SOD3 or ecSOD), glucose-6-phosphate dehydrogenase (G6PD), glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R), glutathione S-transferase (GSH-ST), heme oxygenase-1 (HO-1), hydrogen peroxide (H2O2), inducible nitric oxide synthase (iNOS), malondialdehyde (MDA), manganese superoxide dismutase (SOD2 or MnSOD), NAD(P)H:quinone oxidoreductase-1 (NQO1), nitric oxide (NO), nuclear factor erythroid 2-related factor 2 (Nrf2), polycystic ovary syndrome (PCOS).