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Testosterone induces apoptosis in vascular smooth muscle cells via extrinsic apoptotic pathway with mitochondria-generated reactive oxygen species involvement

Rheure Alves Moreira Lopes,1 Karla Bianca Neves,2 Cezar Rangel Pestana,2 Andréia Z. Chignalia,1 Camila Zilottto Zanotto,1 Andréia Z. Chignalia,1 Yara Maria Valim,2 Leonardo R. Silveira,1,3 Carlos Curti,2 and Rita C. Tostes1

1Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Brazil; 2Faculty of Pharmaceutical Sciences of Ribeirao Preto University of Sao Paulo, Ribeirao Preto, Brazil; and 3School of Physical Education and Sports of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Brazil

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Lopes RA, Neves KB, Pestana CR, Queiroz AL, Zanotto CZ, Chignalia AZ, Valim YM, Silveira LR, Curti C, Tostes RC. Testosterone induces apoptosis in vascular smooth muscle cells via extrinsic apoptotic pathway with mitochondria-generated reactive oxygen species involvement. Am J Physiol Heart Circ Physiol 306: H1485–H1494, 2014. First published March 21, 2014; doi:10.1152/ajpheart.00809.2013.—Testosterone exerts both beneficial and harmful effects on the cardiovascular system. Considering that testosterone induces reactive oxygen species (ROS) generation and ROS activate cell death signaling pathways, we tested the hypothesis that testosterone induces apoptosis in vascular smooth muscle cells (VSMCs) via mitochondria-dependent ROS generation. Potential mechanisms were addressed. Cultured VSMCs were stimulated with testosterone (10−7 mol/l) or vehicle (2–12 h) in the presence of flutamide (10−5 mol/l), CCCP (10−6 mol/l), mimetic manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP; 3 × 10−5 mol/l), Z-Ile-Glu(O-Me)-Thr-Asp(O-Me) fluoromethyl ketone (Z-IETD-FMK; 10−5 mol/l), or vehicle. ROS were determined with lucigenin and dichlorodihydrofluorescein; apoptosis, with annexin V and calcein; O2 consumption, with a Clark-type electrode, and procaspases, caspases, cytochrome c, Bax, and Bcl-2 levels by immunoblotting. Testosterone induced ROS generation (relative light units/ng protein, 2 h; 162.6 ± 16 vs. 100) and procaspase-3 activation [arbitrary units, (AU), 6 h; 166.2 ± 19 vs. 100]. CCCP, MnTMPyP, and flutamide abolished these effects. Testosterone increased annexin-V fluorescence (AU, 197.6 ± 21.5 vs. 100) and decreased calcein fluorescence (AU, 34.4 ± 6.4 vs. 100), and O2 consumption (nmol O2/min, 18.6 ± 2.0 vs. 34.4 ± 3.9). Testosterone also reduced Bax-to-Bcl-2 ratio but not cytochrome-c release from mitochondria. Moreover, testosterone (6 h) induced cleavage of procaspase 8 (AU, 161.1 ± 13.5 vs. 100) and increased gene expression of Fas ligand (2ΔΔCt, 3.6 ± 1.2 vs. 0.7 ± 0.5), and TNF-α (1.7 ± 0.4 vs. 0.3 ± 0.1). CCCP, MnTMPyP, and flutamide abolished these effects. These data indicate that testosterone induces apoptosis in VSMCs via the extrinsic apoptotic pathway with the involvement of androgen receptor activation and mitochondria-generated ROS.

SEX HORMONES, including testosterone, have important extragonadal actions. Accordingly, androgen receptors are widely distributed and expressed in cells/tissues of the central nervous, renal, and cardiovascular systems. In the vasculature, androgen receptors are expressed by vascular smooth muscle cells (VSMCs) and endothelial cells (44, 47). Testosterone induces genomic and nongenomic effects through either classical cytosolic or membrane androgen receptors, respectively (2).

In the cardiovascular system, testosterone has multifaceted actions, ranging from protective to deleterious effects (39, 52). Testosterone induces vasodilatation; decreases total cholesterol, low-density lipoprotein, and triglycerides levels; and inhibits fatty streak formation, indicating that androgens may have protective effects against atherosclerosis (45, 46, 55). On the other hand, supraphysiological doses of testosterone reduce high-density lipoprotein and promote unfavorable changes in the lipid profile (26). Testosterone also stimulates thromboxane synthase as well as cyclooxygenase-1 and -2 in rat thoracic aorta and mesenteric arteries (10, 59); induces reactive oxygen species (ROS) generation in VSMCs (11), which may decrease nitric oxide bioavailability; and increases blood pressure and promotes renal dysfunction (30, 51, 53).

ROS play a major role in various biological responses, such as host defense, activation of transcription factors, and modulation of kinases and ions transport systems activity (17, 62). However, in a situation of uncontrolled ROS generation or impaired ROS inactivation, termed oxidative stress, the excessive oxidants availability activates signaling pathways that have been implicated in the development of organ damage in cardiovascular and metabolic diseases including arterial hypertension, atherosclerosis, heart failure, diabetes, and stroke (57, 61).

The mitochondrial respiratory chain is considered an important source of ROS, mainly superoxide anion (O2•−), which is quickly converted into H2O2. It is estimated that 1–4% of the O2 is reduced to O2•−, especially by complexes I and III of the mitochondrial respiratory chain (4, 34). Both ROS and mitochondria play an important role in apoptosis
induction either under physiological or pathological conditions. The role of mitochondria in ROS-mediated apoptosis is not entirely clear, but oxidative stress in association with disruption of the mitochondrial membrane potential may contribute to release of cytochrome c from the organelles (36, 58).

Two pathways are responsible for initiating apoptosis, one mediated by cell death receptors located on the membrane surface, termed extrinsic pathway, and another mediated by mitochondria, termed intrinsic pathway. Both pathways are modulated by activation of caspases, which trigger the cellular alterations characteristic of apoptosis (56). The induction of the intrinsic pathway depends on the release of cytochrome c from mitochondria to the cytosol, where it forms a complex with the apoptotic protease-activating factor 1, ATP, and procaspase 9, termed apoptosisome, with consequent activation of procaspase 9 (28, 38). The cell death receptors, associated with activation of the extrinsic pathway, possess intracellular death domains, such as CD95, tumor necrosis factor-related apoptosis-inducing ligand-R1 and -R2, which are activated by tumor necrosis factors (TNFs). Upon binding to their receptors, TNF activates Fas-associated protein with a death domain, which recruits and activates procaspase 8 and procaspase 10 (28, 31, 67).

We previously reported that testosterone induces ROS generation via NADPH oxidase-dependent mechanisms in cultured VSMCs by both genomic and nongenomic effects, an effect not abolished in the presence of the aromatase inhibitor anastrozole (11). However, little is known about the effects of testosterone on mitochondria-generated ROS and apoptosis in VSMCs. Therefore, considering that 1) testosterone induces ROS generation in VSMCs, 2) mitochondria are important source of ROS, and 3) ROS modulate biological effects such as migration, proliferation, cell death and senescence, in this study we tested the hypothesis that testosterone induces apoptosis in VSMCs with mitochondria-generated ROS involvement and addressed potential mechanisms.

MATERIALS AND METHODS

Approach to the problem. Proapoptotic effects of testosterone were determined in cultured VSMCs from male Wistar rats. Testosterone-induced ROS generation in VSMCs was confirmed by performing assays with lucigenin and the 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) probe. To address the effects of testosterone on mitochondrial function, the measurement of oxygen consumption was measured with a Clark-type oxygen electrode disc. Testosterone-induced apoptosis was determined by phosphatidylerosine-binding protein annexin V conjugated with Cy3 and calcein fluorescence assays. All protocols were performed in the presence and absence of specific inhibitors to determine whether androgen receptor activation, ROS generation, or mitochondria contribute to the effects of testosterone. Activation of intrinsic and extrinsic apoptosis pathways was determined by Western blot analysis.

Animals. Male, 12-wk-old Wistar rats were housed in individually ventilated cages (3 rats per cage; 1,147 cm²) in a room with controlled humidity (50 ± 10%) and temperature (21 ± 2°C), and 12-h:12-h light-dark cycles. Animals had free access to food (Nuvilab-CR1 rat chow pellets, Nuvital, Curitiba, Brazil) and potable tap water. All experimental procedures performed in this study were approved by the Ethics Committee on Animal Experiments of the Ribeirao Preto Medical School, University of Sao Paulo (protocol no. 018/2011) and are in accordance with the “Guidelines of the Brazilian College of Animal Experimentation.”

Cell culture. Male Wistar rats were euthanized in a carbon dioxide (CO2) chamber. VSMCs from the mesenteric bed were isolated and characterized as previously described (11). Briefly, arteries were cleaned of adipose and connective tissue and VSMCs were dissociated by digestion of arteriolar arcades with an enzymatic solution, consisting of (in mg/ml) 2 collagenase, 0.12 elastase, 0.36 soybean trypsin inhibitor, and 2 bovine serum albumin type I in Ham’s F-12 culture medium. Cells were incubated for 45 min at 37°C and then filtered through a 100-μm nylon mesh. The cell suspension was centrifuged at 2,000 g and resuspended in Dulbecco’s modified Eagle’s medium ( Gibco, Carlsbad, CA), supplemented with 10% bovine serum, 2 × 10⁻³ mol/l glutamine, 2 × 10⁻² mol/l HEPES (pH 7.4), and antibiotics. VSMCs were identified by determination the expression of α-actin by fluorescence microscopy, and the absence of endothelial cells was confirmed by assessment of von Willebrand factor by real-time polymerase chain reaction (PCR; data not shown). Subconfluent cell cultures were rendered quiescent by serum deprivation for 24 h before experimentation. Low-passage cells (passages 4–7) from at least four different primary cultures were used in our experiments.

Lucigenin-enhanced chemiluminescence. VSMCs were stimulated with testosterone (98% purity, 10⁻⁷ mol/l, 120 min to 12 h; Sigma, St. Louis, MO). In some experiments, cells were preexposed for 30 min to CCCP (98% purity, 10⁻⁶ mol/l; Tocris, Ellisville, MO), flavumide (98% purity, 10⁻⁵ mol/l; Sigma), or mimetic manganese(III) tetraakis[1-methyl-4-pyridyl]porphyrin (MnTMPyP; 95% purity, 3 × 10⁻⁵ mol/l; Enzo Life Sciences, Farmingdale, NY), CCCP, MnTMPyP, and flavumide effects on ROS generation, without testosterone, were also assessed. After stimulation, cells were washed and harvested in lysis buffer, consisting of 2 × 10⁻² mol/l of potassium phosphate monobasic (KH₂PO₄), 10⁻³ mol/l of ethylene glycol tetraacetic acid (EGTA), 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, and 10⁻³ mol/l of phenylmethanesulfonyl fluoride (PMSF). A sample (50 μl) was added to a suspension containing 175 μl of assay buffer, consisting of 5 × 10⁻² mol/l of KH₂PO₄, 10⁻³ mol/l of EGTA, and 15 × 10⁻² mol/l of sucrose, and 5 × 10⁻⁶ mol/l lucigenin (98% purity, Sigma). NADPH (98% purity, 10⁻⁴ mol/l; Sigma) was added to the suspension containing lucigenin. Luminescence was measured for 3 min by a luminometer (AutoLumat LB 953, Berthold) before and after addition of NADPH (98% purity, Sigma). Activity measured with buffer alone was subtracted from each test reading to yield net activity, which was expressed as relative light units of protein (% of control).

CM-H2DCFDA and calcein assay. After treatments, cells were harvested by trypsinization and resuspended in phosphate-buffered saline (PBS) for analysis of mitochondrial parameters. Intracellular ROS production was monitored by the oxidation of the cell permeant CM-H2DCFDA (Life Technology, Grand Island, NY). The nonfluorescent product CM-H2DCFDA is trapped after the cleavage of diacetate group by cellular esterases and becomes fluorescent when oxidized by ROS to CM-H2DCFDA. The cells were incubated with 2 × 10⁻⁶ mol/l CM-H2DCFDA for 30 min, and fluorescence intensity was analyzed at 503-/527-nm excitation/emission wavelengths. Cell permeability was demonstrated by calcein fluorescence method, using acetoxymethyl calcein ester (calcein AM), which enters the cells and becomes fluorescent upon deesterification. This assay was performed by loading cells with calcein AM (10⁻⁶ mol/l, Molecular Probes, Carlsbad, CA) for 30 min, and fluorescence intensity was analyzed at 503-/527-nm excitation/emission wavelengths.

Phosphatidylerosine externalization. Phosphatidylerosine translocation from the inner to the outer leaflet of the plasma membrane is one of the early features of apoptosis (21). Cell surface phosphatidylerosine was detected by phosphatidylerosine-binding protein annexin V conjugated with Cy3 using the commercially available annexin V (Cy3 apoptosis detection kit, 98% purity, Sigma). Cells were then processed according to the kit instructions, and fluorescence intensity was analyzed at 503-/527-nm excitation/emission wavelengths. Data were
collected in three individual experiments, each in quadruplicate, and used to calculate the respective means and the standard error. VSMCs stimulated with staurosporine (95% purity, 10⁻⁷ mol/l, Sigma) were used as positive control.

Cellular respiration. Cells were harvested by trypsinization and resuspended in PBS (1 ml) medium using a thermostated recirculating water bath at 37°C. The rate of oxygen consumption was measured with Clark-type oxygen electrode disc, Hansatech. After baseline assessment of oxygen consumption, oligomycin (95% purity, 1 µg/ml, Sigma) and CCCP (10⁻⁶ mol/l) were added as internal controls. The results were expressed in nmol of O₂/min and normalized by protein concentration.

Real-time PCR. Quiescent VSMCs were stimulated with testosterone (10⁻⁷ mol/l for 6 h). mRNA expression was quantified by real-time PCR, which was performed as previously described (11). Briefly, total RNA extracted from VSMCs (TRizol) was used with RNase-free DNase I, and 2 µg of RNA were reverse-transcribed in a reaction containing oligo dT (100 µg/ml), 10⁻² mol/l dNTP, 5 × first-strand buffer, and 2 µl of 200 U M-MLV reverse transcriptase. For real-time PCR amplification, 2 µl of each reverse transcription product was diluted in a reaction buffer, containing 5 µl SYBR Green PCR master mix and 900 nmol/l primers in a final volume of 10 µl per sample. We analyzed the following genes (Life Technologies): von Willebrand factor (XM_342759.4), TNF (NM_012675.3), Fas ligand (FasL; NM_012908.1), smoothelin (NM_001013049.2), and β-actin (Rn00667869_m1), used as internal control. The reaction conditions were as follows: two incubations at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of three steps; i.e., 15 s denaturation at 95°C, 60 s annealing at 60°C, and 15 s at 72°C. β-Actin gene was used as the housekeeping gene. The data were analyzed by 2ΔΔCt method, and the results are expressed relatively to control (100%, time 0 h).

Isolation of mitochondria and cytosolic fraction. After stimulation with testosterone, the culture medium was discarded and cells were washed three times with PBS and resuspended in 50 µl of ice-cold buffer A, consisting of (in mol/l) 0.02 HEPES (pH 7.5), 0.0015 MgCl₂, 10 KCl, 0.001 ethylenediamine tetraacetic acid (EDTA), 0.001 EGTA, 0.001 dithiothreitol, 0.0001 PMSF, 0.25 sucrose, and protease inhibitor 1 × SIGMAFAST. Thereafter, resuspended cells were centrifuged at 10,000 g for 15 min at 4°C, and the pellet (mitochondrial fraction) was then resuspended in buffer A. The supernatant was centrifuged at 130,000 g for 1 h at 4°C, and the resulting supernatant was considered the cytosolic fraction. To evaluate the efficiency of method, voltage-dependent anion channel expression, a mitochondrial marker was evaluated in the cytosolic and mitochondrial fraction (data not shown).

Immunoblotting. Quiescent VSMCs were stimulated with testosterone (10⁻⁷ mol/l) for 2–12 h. Whenever necessary, cells were preincubated for 30 min with CCCP (10⁻⁶ mol/l), flutamide (10⁻⁵ mol/l), MnTMPyP (3 × 10⁻⁵ mol/l), or Z-Ile-Glu(O-Me)-Thr-Asp(O-Me) fluoromethyl ketone (Z-IETD-FMK; 10⁻³ mol/l; Cell Signaling, Danvers, MA). Cells were harvested in lysis buffer, consisting of (in mol/l) 5 × 10⁻² sodium pyrophosphate, 5 × 10⁻² NaF, 5 × 10⁻³ NaCl, 5 × 10⁻³ EDTA, 5 × 10⁻³ EGTA, 2 × 10⁻⁶ HEPES, 2 × 10⁻³ sodium orthovanadate (Na₃V ᵃ₀₃), and 5 × 10⁻⁵ PMSF and 0.5% Triton X-100 and 1 mg/ml leupeptin-aptrospin-pepsatin. Proteins were extracted, separated by electrophoresis on a polyacrylamide gel (20–80 µg) and transferred to a nitrocellulose membrane, as previously described (11). Non-specific binding sites were blocked with 1% bovine serum albumin in Tris-buffered saline solution with 1% of Tween 20 for 1 h at room temperature. Antibodies were then incubated with specific antibodies, overnight at 4°C. Membranes were washed three times with TBS-Tween 20 and incubated with specific secondary antibodies for 1 h at room temperature. Signals were revealed after reaction with enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent Prime), and the images were captured in ImageQuant 350 (GE Healthcare, Piscataky, NJ).

RESULTS

Cultured VSMCs were characterized, using immunofluorescence, by the presence of α-actin, characteristic of smooth muscle cells, and by the absence of von Willebrand factor, characteristic of endothelial cells. The absence of endothelial contamination was confirmed by determining mRNA expression for von Willebrand factor, using real-time PCR. Cultured VSMCs also expressed smoothelin mRNA, a smooth muscle differentiation marker at levels comparable to native smooth muscle cells (from mesenteric arteries). Endothelium-intact aortas were used as a positive control for the presence of von Willebrand factor (data not shown).

Testosterone induces ROS generation in VSMCs with involvement of mitochondria and androgen receptors. Measurement of ROS by lucigenin assay showed that testosterone at 10⁻⁷ mol/l increased ROS generation in VSMCs after 2 h of stimulation (Fig. 1A). To determine whether ROS generation induced by testosterone involves mitochondria and androgen receptors, the assays were performed in the presence of an uncoupler of oxidative phosphorylation, CCCP, or an androgen receptor antagonist, flutamide, respectively. As shown in Fig. 1, testosterone-induced ROS generation was inhibited in the presence of either CCCP (10⁻⁶ mol/l, Fig. 1, A and B) or flutamide (10⁻⁵ mol/l; Fig. 1A). The SOD2 mimetic MnT-MpyP (3 × 10⁻⁵ mol/l) also inhibited ROS generation. CCCP, flutamide, and MnT-MpyP alone did not change ROS generation (Fig. 1C). Androgen receptor expression was confirmed in cultured VSMCs by Western blot analysis, as shown in Fig. 1D.

Testosterone effects on mitochondrial respiration in VSMCs were next determined. As shown in Fig. 2, testosterone (10⁻⁷ mol/l, 6 h) decreased basal respiration of mitochondria and did not affect either the decrease or the increase in O₂ consumption induced by oligomycin and CCCP, respectively.

Testosterone induces procaspase-3 activation by mechanisms involving mitochondria, ROS generation, and activation of androgen receptors. Since oxidative stress induces apoptosis, the effects of testosterone on procaspase-3 cleavage, an index of procaspase-3 activation, were determined. As shown in Fig. 3A, stimulation of VSMCs with testosterone for 6 and 12 h induced procaspase-3 cleavage. Considering that the apoptotic cascade can be triggered after few hours of ROS generation, the mechanisms involved in testosterone-induced procaspase-3 cleavage were assessed after 6 h of stimulation with testosterone by using pharmacological inhibitors. As shown in Fig. 3B, testosterone-induced caspase-3 activation was inhibited in the presence of flutamide (10⁻⁵ mol/l),
MnTMPyP (3 × 10⁻⁵ mol/l), or CCCP (10⁻⁶ mol/l). Flutamide, MnTMPyP, or CCCP alone did not change caspase-3 activity (Fig. 3C).

**Testosterone induces apoptosis in VSMCs.** The proapoptotic effects of testosterone in VSMCs were confirmed with the annexin-V probe. As shown in Fig. 4A, stimulation of VSMCs with 10⁻⁷ mol/l testosterone for 6 h increased annexin-V fluorescence. Staurosporine (10⁻⁶ mol/l) was used as a positive control. Cell viability after stimulation with testosterone was assessed with the calcine-AM probe. As shown in Fig. 4B, calcine fluorescence decreased after stimulation of VSMCs with testosterone (6 h). H₂O₂ (10⁻³ mol/l) was used as a positive control.

**Assessment of the intrinsic pathway in testosterone-induced apoptosis in VSMCs.** The activation of the intrinsic apoptosis pathway was addressed through the levels of Bax and Bcl-2 proteins, which are involved in the permeability of the outer mitochondrial membrane. Bax (proapoptotic)-to-Bcl-2 (anti-apoptotic) ratio was assessed in VSMCs by Western blot analysis, both in total cell homogenate and mitochondrial fraction. As shown in Fig. 5A, Bax-to-Bcl-2 ratio was decreased in the total cell homogenate of VSMCs after stimulation with testosterone (12 h). Figure 5B shows that testosterone also decreased Bax-to-Bcl-2 ratio in the mitochondrial fraction at all times (6 and 12 h).

In addition, testosterone did not alter cytochrome-c levels in VSMCs, either in the cytoplasmic or mitochondrial cell fractions. As shown in Fig. 6, the cytoplasmic-to-mitochondrial cytochrome c ratio in cells stimulated with testosterone was similar to the control. cell viability after stimulation with testosterone was assessed with the calcine-AM probe. As shown in Fig. 4B, calcine fluorescence decreased after stimulation of VSMCs with testosterone (6 h). H₂O₂ (10⁻³ mol/l) was used as a positive control.

**Assessment of the extrinsic pathway in testosterone-induced apoptosis in VSMCs.** To verify whether testosterone activates the extrinsic apoptosis pathway in VSMCs, the androgen effects on procaspase-8 cleavage were determined. Figure 7A shows that testosterone (10⁻⁷ mol/l, 6 h) induces procaspase-8 cleavage. As shown in Fig. 7B, testosterone-induced procaspase-3 activation was inhibited in the presence of the caspase-8 inhibitor Z-IETD-FMK (10⁻⁵ mol/l). To validate Z-IETD-FMK effect, Jurkat cells were stimulated with staurosporine (caspase-8-positive control). As shown in Fig. 7C,
Stauroporine-induced caspase-8 cleavage was abrogated by Z-IETD-FMK.

As the extrinsic apoptotic pathway is activated by death receptors, such as Fas and TNF, testosterone-induced upregulation of FasL and TNF/ H9251 gene expression, ligands of the Fas and TNF receptors, respectively, were determined. As shown in Fig. 8, A and B, after 6 h of stimulation with testosterone, there was an increase in FasL and TNFα mRNA expression. These effects were abolished by flutamide, MnTMPyP, or CCCP.

**DISCUSSION**

We have reported that testosterone directly induces ROS generation in VSMCs in a concentration- and time-dependent manner via modulation of the NAD(P)H oxidase enzymatic complex and activation of MAPK and tyrosine kinase pathways (11). In addition to providing evidence on the involvement of mitochondria in testosterone-induced ROS generation, the present study shows that testosterone induces apoptosis of VSMCs and also elucidates mechanisms involved in the proapoptotic effects of the androgen. Succinctly, testosterone-induced ROS generation was inhibited in the presence of flutamide (androgen receptor antagonist, 10−5 mol/l), MnTMPyP (SOD2 mimetic, 3 × 10−5 mol/l), and CCCP (mitochondrial uncoupler, 10−6 mol/l). C: CCCP, MnTMPyP, and flutamide (Flu) effects on procaspase-3 activation after 6 h of stimulation. A–C: representative images of Western blot analysis (top) and corresponding densitometric analysis showing caspase 3-to-procaspase 3 ratio (bottom). Bars represent means ± SE; n = 4. *P < 0.05 vs. control; #P < 0.05 vs. Testo (1-way ANOVA with Tukey’s posttest).

In line with our data, testosterone-induced ROS generation has been reported in rodent VSMCs (11) and renal cells (22), as well as in human prostate cancer cells (60) and leukocytes (23, 63). Similarly to VSMCs, in the macula densa-like cell line MMDD1, testosterone induces O2− generation via activation of NAD(P)H oxidase and androgen receptor-dependent pathways. This effect is associated with an increase in tubulo-
glomerular feedback, 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 (22). In human leukocytes, testosterone undecanoate, used in female-to-male transsexuals, decreases mitochondrial O2 consumption and membrane potential and induces oxidative stress (63). Elevation of circulating androgens (dehydroepiandrosterone) in healthy ovulatory reproductive-age women, as occurs in women with polycystic ovary syndrome, also increases leukocyte ROS generation, p47phox gene expression, and plasma thiobarbituric acid reactive substances (23). In prostate cancer cell lines, in addition to directly inducing ROS generation (55), testosterone enhances H2O2-induced apoptosis (27), indicating that under oxidative stress conditions, androgen signaling may further enhance apoptosis and DNA damage response.

It is important to mention that antioxidant effects of testosterone have also been described. For example, testosterone reduces hyperglycemia-induced oxidative stress in pancreatic β-cells (25), decreases cardiac prooxidant state via androgen receptor-dependent mechanisms (18), and increases SOD activity in brain tissues (35). Of importance, testosterone-induced reduction of oxidative stress and endoplasmic reticulum stress in pancreatic β-cells occur only in high-glucose conditions; testosterone has no effects in low-normal glucose levels (25).

Similarly, testosterone has both pro- and antiapoptotic effects in different cells/tissues. In agreement with our results, Bowles et al. (5) showed that in VSMCs isolated from pig coronaries, testosterone treatment increases approximately fourfold caspase-3 activity, an effect inhibited by PKC-δ small interfering RNA. Decreased expression of TNF-α, IL-1β, IL-6, p38 MAPK, caspase 1, 3, and 11 and increased expression of the antiapoptotic protein Bcl-2 have been reported in castrated and flutamide-treated rats after acute myocardial ischemia-reperfusion (64), suggesting proapoptotic and proinflammatory effects of testosterone in the heart. In prostate cancer cells, testosterone has been shown to either promote (37) or inhibit (33) apoptosis. In C2C12 skeletal muscle cells, testosterone protects against H2O2-induced apoptosis by mechanisms that involve the apoptotic intrinsic pathway (50).

These studies clearly indicate that testosterone exerts opposite effects on ROS generation and apoptosis, depending not only on the cell type but also on the cell functional status. Possible contributing factors to these discrepant effects of testosterone are briefly discussed. First, there is acute versus chronic effects of the androgen. It is possible that acute and chronic changes in testosterone levels lead to recruitment of a distinct set of signaling pathways, as reported with other
hormones/neurotransmitters. Second, the cellular status may interfere with testosterone actions. Removing or adding testosterone to cells may produce opposite/differential effects depending on the initial metabolic/energetic/redox status of the cell. Third, manipulations that globally (or locally) increase testosterone may produce differential effects based on the specific cell types that are being stimulated in the tissue/organ. For example, testosterone may have differential effects in isolated VSMCs versus VSMCs in contact with endothelial or inflammatory cells. Other factors that should be considered include 1) the concentrations of testosterone (physiological, supraphysiological); 2) the steroid ester (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, used in the different studies; and 3) the “sex” of the cell/tissue where the effects of testosterone are being addressed. Although most signaling pathways are common in cells/tissues derived from females and males, some pathways may show sex differences or yet cells/tissues derived from females and males may differentially respond to sex hormones (41, 42). The complexity of testosterone effects is evident, and further studies are required for a better understanding of the pro- or antioxidative/apoptotic effects of testosterone, especially in the cardiovascular system.

Our data showing that procaspase-3 cleavage and increased annexin-V fluorescence are inhibited in the presence of flutamide, MnTMPyP, or CCCP, implicate androgen receptors activation, ROS and mitochondria, respectively, in testosterone-induced apoptosis. As recently reviewed by Kaminskyy and Zhivotovsky (31a), ROS may differently affect cell fate (apoptotic or autophagic processes), depending on ROS levels, the source of ROS formation, and cellular localization. Whether ROS preferentially transduces a proapoptotic signal pathway over an antiapoptotic pathway may also be determined by the nature and strength of the stimulus (extrinsic factors), as well as by the predominant ROS generated in a particular cell type, the differential availability of certain tran-

Fig. 7. Testosterone induces procaspase-8 activation, and testosterone-induced procaspase-3 activation is inhibited by Z-Ile-Glu(O-ME)-Thr-Asp(O-Me) (Z-IETD) in VSMCs. A: testosterone effects on procaspase-8 activation, after 2 and 6 h of stimulation were determined by Western blot analysis and represented as caspase 8-to-procaspase 8 ratio. C: Jurkat cells stimulated with staurosporine (10−6 mol/l) were used to validate Z-IETD-fluoromethyl ketone (FMK) effect. B: testosterone effects on procaspase-3 activation (after 6 h of stimulation), represented by caspase 3-to-procaspase 3 ratio, was determined in presence of the caspase-8 inhibitor Z-IETD-FMK (10−8 mol/l). A and B: representative images of Western blot analysis (top) and corresponding densitometric analysis (bottom) showing caspase-to-procaspase ratio. Bars represent means ± SE; n = 5–11. *P < 0.05 vs. control; #P < 0.05 vs. Testo (1-way ANOVA with Tukey’s posttest).

Fig. 8. Testosterone upregulates TNF-α and Fas ligand (FasL) mRNA expression in VSMCs. Testosterone effects on mRNA expression were assessed by real-time PCR in cultured VSMCs after 6 h of stimulation in the presence or absence of CCCP (mitochondrial uncoupler, 10−6 mol/l), MnTMPyP (SOD2 mimetic, 3×10−5 mol/l), or flutamide (androgen receptor antagonist, 10−5 mol/l), which were added 30 min before testosterone. The figure shows 2ΔΔCt values for TNF-α (A) and FasL (B). Values were normalized by the corresponding β-actin mRNA expression. Bars represent means ± SE; n = 4–8. *P < 0.05 vs. control; #P < 0.05 vs. testosterone (1-way ANOVA with Tukey’s posttest).
scriptional factors and their cross talk with specific apoptotic substrates, and the availability of antioxidant enzymes (intrinsic factors) (31a).

There are at least two broad pathways that lead to apoptosis: the intrinsic and extrinsic pathways. Since the intrinsic pathway involves permeabilization of the outer mitochondrial membrane, expression of proteins involved in this process, such as Bax and Bcl-2, proapoptotic and antiapoptotic proteins, respectively (19), as well as cytochrome-c release from mitochondria to cytosol were addressed. Bax-to-Bcl-2 ratio decreased in total cell homogenates of VSMCs exposed to testosterone, suggesting, at least, the absence of increased outer mitochondrial membrane permeabilization. As the activity of these proteins depends on their interaction with the mitochondrial membrane, their levels were also determined in the mitochondrial fraction, where a decrease of Bax-to-Bcl-2 ratio was also observed. These results suggest inhibition of the intrinsic pathway of apoptosis or, at least, a lack of activation of this pathway in VSMCs exposed to testosterone. Indeed, testosterone did not promote the release of cytchrome c from mitochondria to cytosol, which, in agreement with previous data (12, 65), may be due to Bcl-2 overexpression.

Since testosterone activated procaspase 8 in VSMCs, a central component involved in the apoptotic signaling pathway activated by death receptors, we hypothesized that testosterone-promoted cleavage of procaspase 3 in VSMCs resulted from the activation of the extrinsic pathway of apoptosis. Accordingly, the caspase-8 inhibitor Z-IETD-FMK decreased testosterone-induced activation of procaspase 3. In addition, testosterone increased gene expression of two cell death receptor ligands, an effect that was blocked by an androgen receptor antagonist, a SOD mimetic, or a mitochondrial uncoupler. Corroborating our data, in bone marrow macrophages, testosterone increases gene expression of FasL and TNF, which is accompanied by increased expression of caspase 8 (67). Of importance, cleavage of procaspase 3 is also observed in cultured bone marrow macrophages stimulated with testosterone (31). Together, these results suggest that testosterone activates the extrinsic pathway of apoptosis in VSMCs, in association with androgen receptor activation and mitochondria-generated ROS. The use of a single pharmacological inhibitor to establish androgen receptor involvement in the effects of testosterone represents a limitation of the present study.

At the physiological level, the ability of organisms to grow or repair a damaged tissue and, consequently, restore relevant biological functions is a fundamental and important aspect of biology. For example, healing of injured blood vessels, as in the reendothelialization of the luminal surface after endothelial denudation due to catheter-based interventional treatment, is a prerequisite for restoration of normal blood vessel functions. In addition, whereas angiogenesis, or new blood vessel growth, is important in the treatment of ischemic disorders such as occlusive coronary and peripheral arterial diseases, inhibition of angiogenesis is desired in diseases such as cancer and proliferative diabetic retinopathy. Apoptosis plays a role in vascular tissue morphogenesis and homeostasis and, consequently, in the regulation of vascular structure (24) and vascular remodeling (29).

Apoptosis deregulation is considered a pathogenetic process in a variety of human diseases and is an important mechanism underlying the changes observed in hypertension, inflammation, and arteriosclerosis (43). 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. Testosterone-induced ROS generation promotes migration of VSMCs, and this effect is increased in VSMCs of hypertensive rats (11), in agreement with the assumption that increased ROS levels play an important role in the pathophysiology of arterial hypertension (6, 49). Also, in line with our results, cardiomyocyte death is markedly greater in men than in women (20) and O2 production and vascular remodeling are higher in injured femoral arteries of male mice (48). It is possible that these events are associated with greater testosterone levels in males and testosterone effects on ROS generation and apoptosis. Accordingly, a recent study showed that sex differences in cardiac ischemia-reperfusion-induced injury is associated with a differential regulation of pro- and antiapoptotic proteins in male and female rats (8). Therefore, the present study may shed some light on the mechanisms involved in sex differences in organ injury.

In the cardiovascular system, both protective and deleterious actions of testosterone have been reported (3, 16, 21, 39, 52, 66). Epidemiological studies indicate a high prevalence of low-serum testosterone levels in men with cardiovascular disease and animal studies show that testosterone is atheroprotective (32). Testosterone-induced production of nitric oxide (3), relaxation of various vascular beds (16, 66), decrease of total cholesterol, low-density lipoprotein and triglycerides, and formation of endothelial progenitor cells (21) may account for the protective effects of the androgen in the cardiovascular system. However, reports of sudden cardiovascular death among male athletes abusing of anabolic steroids and the fact that in many experimental models of hypertension [spontaneously hypertensive rats (54), Dahl salt-sensitive rats (13), genetically hypertensive rats (1), and rats with deoxycorticosterone acetate-salt-induced hypertension (15)], males have higher levels of arterial pressure, which are abrogated by orchiectomy (9, 54), indicate that testosterone is detrimental to the cardiovascular system. In addition, testosterone has been shown to directly affect many of the mechanisms that control cardiovascular function, such as the renin-angiotensin-aldosterone system, sympathetic neural activity, endothelial function, and VSMC tone (7, 14, 40).

Finally, it is important to mention that findings from preclinical studies often differ from those in clinical setting and therefore should be cautiously interpreted. Therefore, it would be important to determine whether similar results are found in human VSMCs and whether these events take place in the vasculature of men undergoing treatment with testosterone or other androgens. Large, randomized, placebo-controlled trials are required to elucidate the clinical relevance of our findings.

In summary, the present data provide evidence for the involvement of mitochondria on testosterone-induced ROS generation and shows that testosterone leads to apoptosis of VSMCs via the extrinsic pathway of apoptosis. Of importance, we described a new mechanism by which testosterone influences vascular function and may play a role in cardiovascular diseases. The identification of biochemical markers activated by testosterone in VSMCs, such as ROS and apoptosis-related proteins, is of great interest.
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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TESTOSTERONE AND APOPTOSIS IN VASCULAR SMOOTH MUSCLE CELLS