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Oxidative stress associated with middle aging leads to sympathetic hyperactivity and downregulation of soluble guanylyl cyclase in corpus cavernosum

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Silva FH, Lanaro C, Leiria LO, Rodrigues RL, Davel AP, Claudino MA, Toque HA, Antunes E. Oxidative stress associated with middle aging leads to sympathetic hyperactivity and downregulation of soluble guanylyl cyclase in corpus cavernosum. Am J Physiol Heart Circ Physiol 307: H1393–H1400, 2014. First published September 12, 2014; doi:10.1152/ajpheart.00708.2013.—Impairment of nitric oxide (NO)-mediated cavernosal relaxations in middle age contributes to erectile dysfunction. However, little information is available about the alterations of sympathetic neurotransmission and contraction in erectile tissue at middle age. This study aimed to evaluate the alterations of the contractile machinery associated with tyrosine hydroxylase (TH) in rat corpus cavernosum (RCC) at middle age, focusing on the role of superoxide anion. Male Wistar young (3.5-mo) and middle-aged (10-mo) rats were used. Electrical-field stimulation (EFS)- and phenylephrine-induced contractions were obtained in RCC strips. Levels of reactive-oxygen species (ROS) and TH mRNA expression, as well as protein expressions for α1/β1 subunits of soluble guanylyl cyclase (sGC), in RCC were evaluated. The neurogenic contractile responses elicited by EFS (4–32 Hz) were greater in RCC from the middle-aged group that was accompanied by elevated TH mRNA expression (P < 0.01). Phenylephrine-induced contractions were also greater in the middle-aged group. A 62% increase in ROS generation in RCC from middle-aged rats was observed. The mRNA expression for the α1A-adrenoceptor remained unchanged among groups. Protein levels of α1/β1 sGC subunits were decreased in RCC from the middle-aged compared with young group. The NADPH oxidase inhibitor apocynin (85 mg·rat−1·day−1, 4 wk) fully restored the enhanced ROS production, TH mRNA expressions, and α1/β1 sGC expression, indicating that excess of superoxide anion plays a major role in the sympathetic hyperactivity and hypercontractility in erectile tissue at middle age. Reduction of oxidative stress by dietary antioxidants may be an interesting approach to treat erectile dysfunction in aging population.

antioxidant therapy; apocynin; NADPH oxidase; reactive-oxygen species; superoxide anion; tyrosine hydroxylase

increased intracavernosal blood flow, and veno-occlusive function. The degree of contraction of corpus cavernosum (CC) smooth muscle determines the function states of penile flaccidity, tumescence, erection, or detumescence (1). The balance between contractile and relaxant effects involves neurotransmitters and other endogenous agents (1). Nitric oxide (NO) released from nitrergic nerves and endothelial cells is considered the most important mediator of penile erection. Upon NO binding to the heme of the H-NOX domain in the β-subunit, soluble guanylyl cyclase (sGC) is activated several hundred fold, resulting in accumulation of intracellular cyclic guanosine monophosphate (cGMP) and subsequent activation of cGMP-dependent protein kinase (PKG), which leads to smooth muscle relaxation (15). Penile vessels and cavernosal smooth muscle also receive a rich adrenergic innervation that maintains the penis in a flaccid state mainly via a tonic activity of norepinephrine derived from the sympathetic nerves (1). Therefore, enhanced CC contractile responses appear to contribute to erectile dysfunction (ED).

ED is characterized by a persistent inability to achieve and/or maintain an erection sufficient for satisfactory sexual performance (12). Aging is critically involved in ED in humans (11). A number of experimental studies have assessed the age-related ED in different experimental conditions, but little information is available on alterations in the contractile mechanisms of erectile tissue at the middle age (2). In cavernosal tissues, decreased availability of NO formation and increased oxidant production appear to contribute to ED (4, 17, 21). The NADPH oxidase complex is a major source of superoxide in vascular cells, including CC (19). Recently, ED in middle-aged rats was associated with upregulation of NADPH oxidase subunit gp91phox and downregulation of neuronal and phosphorylated endothelial nitric oxide synthase (nNOS/p-eNOS at Ser1177) in cavernosal smooth muscle (45).

NO is a physiological negative modulator of sympathetic neurotransmission (10, 43). Vasoconstriction produced by sympathetic nerve stimulation is enhanced by NO synthesis inhibition that in part reflects the removal of the relaxation normally caused by NO and in part is secondary to an increased release of norepinephrine from sympathetic nerves (25, 31). Reduction in nNOS expression and nitrergic innervation ap-
pears to contribute to the enhanced adrenergic neurotransmission in the mesenteric vascular bed of hypertensive rats (26). Hyperactivity of the sympathetic nervous system in spontaneously hypertensive rats is accompanied by increased oxidative stress and reduced NO bioavailability (30). Moreover, the increased oxidative stress leads to a reduction of sGC expression, impairing cGMP-dependent vasorelaxation (16, 46). Thus we hypothesized that increased oxidative stress impairs the biological activity of NO/sGC and enhances sympathetic neurotransmission in CC from middle-aged rats. Inhibition of oxidant levels by the NADPH oxidase inhibitor apocynin (85 mg·rat⁻¹·day⁻¹, 4 wk), and cavernosal segments were prepared as described above. Next, tissues were embedded in a freezing medium and transverse sections (30 μm) of frozen tissue were obtained on a cryostat, collected on glass slides, and equilibrated for 10 min in Hanks’ solution (in mM: 1.6 CaCl₂, 1.0 MgSO₄, 145.0 NaCl, 5.0 KCl, 0.5 NaH₂PO₄, 10.0 dextrose, and 10.0 HEPES pH 7.4) at 37°C. Fresh Hanks’ solution containing DHE (2 × 10⁻⁶ M) was topically applied to each tissue section, and the slices were incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained with an optical microscope (BX51, Olympus) equipped with filter to rhodamine and camera (DP-72, Olympus), using a 20× objective. The number of nuclei labeled with ethidium bromide (EB-positive nuclei) along CC was automatically counted using ImageJ software (National Institutes of Health, Bethesda, MD) and expressed as labeled nuclei per millimeters squared.

Western blotting. Cavernosal tissues were homogenized in a sodium dodecyl sulfate (SDS) lysis buffer with a Polyclon PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY) and centrifuged (12,000 g, 4°C, 20 min). Blotting was performed in SDS-PAGE. Primary antibodies were anti-α-opioid (1:10,000; Abcam, Cambridge, UK), anti-sGC α₁-path (1:100; Abcam), or anti-sGC β₁-path (1:1000, Novus Biologicals, Oakville, ON, Canada). Detection using specific antibodies, horseradish peroxidase-conjugated secondary antibodies, and luminol solution was performed. Densitometry was performed using the Scion Image software (Scion, Frederick, MD), and results were normalized to α-actin protein and expressed as arbitrary unit.

Real-time RT-PCR. Total RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA) from rat CC samples. Three-microgram RNA samples were incubated with 1 U DNaseI (Invitrogen, Rockville, MD) for 15 min at room temperature, and EDTA was added to a final concentration of 2 mM to stop the reaction. The DNaseI enzyme was subsequently inactivated by incubation at 65°C for 5 min. DNaseI-treated RNA samples were then reverse transcribed with Superscript III and RNAseOut (Invitrogen) for 50 min at 50°C, 15 min 70°C. cDNA samples were quantified using a Nanodrop spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE). Primers were designed using the PrimerExpress program (Applied Biosystems, Foster City, CA) (Table 1). The ideal concentration of use was determined for each pair of primers and the amplification efficiency was calculated according to the equation $E^{1-1(\text{slope})}$ to confirm the

### Materials and Methods

**Animals.** All animal care and experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA) and followed the Guide for the Care and Use of Laboratory Animals. Male Wistar rats (3.5- and 10-mo old; young and middle-aged, respectively) were provided by Central Animal House Services (CEMIB) of Universidade de Campinas (UNICAMP). Middle age in rats (10-mo old animals) was defined according to previous studies (40). Animals were housed in temperature-controlled facilities on a 12-h light-dark cycle with ad libitum food and water access. Young and middle-aged rats were treated orally with apocynin during 4 wk (85 mg·rat⁻¹·day⁻¹, given in the drinking water) (45). The average weights of dry cavernosal strips from young and middle-aged rats were 116 ± 4 and 122 ± 4 mg, respectively.

**Functional studies in cavernosal strips and concentration-response curves.** Rats were anesthetized with isoflurane and exsanguinated. Strips of rat CC were mounted in a 10-ml organ system containing Krebs solution at 37°C continuously bubbled with a mixture of 95% O₂ and 5% CO₂ (pH 7.4) and vertically suspended between two metal hooks. One hook was connected to a force transducer and the other acted as a fixed attachment point. Tissues were allowed to equilibrate for 60 min under a resting tension of 5 mM. Isometric force was recorded using a PowerLab 400 data acquisition system (Software LabChart, version 7.0; AD Instrument). Cumulative concentration-response curves to the contractile agent phenylephrine (α₁-adrenergic receptor agonist, 10⁻⁵–10⁻⁴ M) were obtained in cavernosal strips. Nonlinear regression analysis to determine the EC₅₀ was carried out using GraphPad Prism (GraphPad Software, San Diego, CA) with the constraint that Φ = 0. All concentration-response data were evaluated for a fit to a logistics function in the form: $E = E_{\text{max}}[1 + (10^x)^n] / [1 + (10^x)^n]$, where $E$ is the maximum response produced by agonists; $x$ is the logarithm of the EC₅₀; the concentration of drug that produces a half-maximal response; $n$ is a curve-fitting parameter that defines the slope of the concentration-response line; and $Φ$ is the response observed in the absence of added drug.

**Electrical-field stimulation.** Electrical-field stimulation (EFS) was applied in strips placed between two platinum ring electrodes connected to a Grass S88 stimulator (Astro-Med, West Warwick, RI). EFS was conducted at a 50-V, 1-ms pulse width and trains of stimuli lasting 10 s at varying frequencies (4–32 Hz). Frequency-response relationships were investigated at supramaximal voltage in all preparations stimulated electrically. Data were calculated in milliNewtons.

**Measurement of reactive-oxygen species.** The oxidative fluorescent dye dihydroethidine (DHE) was used to evaluate in situ reactive-oxygen species (ROS) generation (14). Cavernosal segments from young and middle-aged groups were equilibrated for 30 min in Krebs solution at 37°C continuously bubbled with a mixture of 95% O₂-5% CO₂ (pH 7.4). Tissues were then incubated with superoxide dismutase (SOD; 75 U/ml; 15 min) or apocynin (10⁻⁴ M; 30 min). In separate experiments, young and middle-aged rats were treated orally with apocynin (85 mg·rat⁻¹·day⁻¹, 4 wk), and cavernosal segments were prepared as described above. Next, tissues were embedded in a freezing medium and transverse sections (30 μm) of frozen tissue were obtained on a cryostat, collected on glass slides, and equilibrated for 10 min in Hanks’ solution (in mM: 1.6 CaCl₂, 1.0 MgSO₄, 145.0 NaCl, 5.0 KCl, 0.5 NaH₂PO₄, 10.0 dextrose, and 10.0 HEPES pH 7.4) at 37°C. Fresh Hanks’ solution containing DHE (2 × 10⁻⁶ M) was topically applied to each tissue section, and the slices were incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained with an optical microscope (BX51, Olympus) equipped with filter to rhodamine and camera (DP-72, Olympus), using a 20× objective. The number of nuclei labeled with ethidium bromide (EB-positive nuclei) along CC was automatically counted using ImageJ software (National Institutes of Health, Bethesda, MD) and expressed as labeled nuclei per millimeters squared.

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### Table 1. Sequence and ideal concentration for the primers used in quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene (Concentration)</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>TH (150 nM)</td>
<td>Foward 5'-AGGTCCTGAGACTCCGGTGCTCA-3'</td>
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<tr>
<td></td>
<td>Reverse 5'-TGGGCATTGAACTTCCTCG-3'</td>
</tr>
<tr>
<td>α₁A-Adr (300 nM)</td>
<td>Foward 5'-CGAGTCTACGATAGACCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCTGGTTGCACTTTTCTC-3'</td>
</tr>
<tr>
<td>β-Actin (70 nM)</td>
<td>Foward 5'-GCAATGACGGTTCGAGAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TATGTTCATGGATCCACAGGAT-3'</td>
</tr>
<tr>
<td>GAPDH (50 nM)</td>
<td>Foward 5'-CTTGGCAAGTATGATGACTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGGGCACAGTGGCGTTACTG-3'</td>
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accuracy and reproducibility of the reactions. Amplification specificity was verified by running a dissociation protocol. Quantitative RT-PCRs were performed in duplicate, using 6 μl SYBR Green Master Mix (Applied Biosystems), 10 ng cDNA, and ideal quantities of each primer in a final volume of 12 μl. Samples were run in MicroAmp Optical 96-well plates (Applied Biosystems) in a 7500 Fast Real Time PCR System (Applied Biosystems). Gene expression was quantified using the Gnorm program. Two replicas were run on the plate for each sample, and each sample was run twice independently. Results are expressed as mRNA levels of each gene studied, normalized according to β-actin and GAPDH expressions.

**Drugs and chemicals.** Apocynin, atropine, guanethidine, DHE, N^6^-nitro-L-arginine methyl ester hydrochloride (L-NAME), superoxide dismutase, phenylephrine, and prazosin were obtained from Sigma-Aldrich (St. Louis, MO). All reagents used were of analytical grade. Stock solutions were prepared in deionized water and stored in aliquots at −20°C. Dilutions were prepared immediately before use.

**Statistical analysis.** Data are expressed as means ± SE of n experiments. The program Instat (GraphPad Software) was used for statistical analysis. One-way ANOVA followed by a Tukey test was used in all groups. P < 0.05 was accepted as significant.

**RESULTS**

**Contractile responses induced by adrenergic nerve stimulation and phenylephrine in rat CC.** EFS produced frequency-dependent CC contractions in cavernosal strips (4–32 Hz) in both young and middle-aged rats (Fig. 1A). EFS-induced contractions were fully abolished by either the α-adrenoceptor antagonist prazosin (10⁻⁶ M; n = 3) or the sympathoinhibitory drug guanethidine (3 × 10⁻⁵ M; n = 10), confirming that nerve-induced cavernosal contractile responses are mediated by norepinephrine release.

In the cavernosal strips from middle-aged rats, EFS-induced contractions were significantly (P < 0.05) increased at all frequencies tested when compared with the young group (Fig. 1A). Oral treatment with the NADPH oxidase inhibitor apocynin (85 mg·rat⁻¹·day⁻¹, 4 wk) normalized the enhanced neurogenic CC contractions in middle-aged rats, with no significant changes in the young group (Fig. 1A).

Next, cavernosal strips were incubated simultaneously with the NOS inhibitor L-NAME (10⁻⁴ M) plus the muscarinic...
receptor antagonist atropine (10⁻⁶ M) before EFS was performed. This pharmacological strategy allows the evaluation of the adrenergic nerve-mediated responses in conditions of complete absence of NO (9). Under this condition, an increased contractile response at about 30–40% was observed in CC of both young and middle-aged rats. However, the EFS-induced neurogenic contractions (4–32 Hz) remained significantly greater in CC from middle-aged (P < 0.05) compared with young rats (Fig. 1B). The increased neurogenic contractions evoked by EFS were attenuated by apocynin treatment in CC of the middle-aged group. No significant changes in CC evoked by EFS were attenuated by apocynin treatment in young rats (Fig. 1B). The increased neurogenic contractions compared with middle-aged group. However, the EFS-induced contractile response at about 30–40% was observed in CC of middle-aged rats and to identify potential sources of ROS formation, we performed DHE imaging of fresh frozen sections of CC preparation of young and middle-aged rats. Under identical reaction conditions, the DHE signal was 62% much more intense in transversal cross section of CC middle-aged than the young rats (P < 0.01). This increase in DHE staining was blocked by treatment with apocynin in both middle-aged (by 73%) and in the young group (by 68%). Likewise, in vitro preincubation of CC with either SOD (75 U/ml, 15 min) or apocynin (100 μM, 30 min) nearly abolished the increased ROS levels in both young and middle-aged groups (Fig. 3, A and B). These findings indicate that superoxide production in aged tissues involves NADPH oxidase source (Fig. 3, A and B).

mRNA expression for tyrosine hydroxylase and α1A-adrenoceptor. The mRNA expression for the tyrosine hydroxylase in cavernosal tissues was 57% higher (P < 0.05) in middle-aged CC compared with the young group. Treatment with apocynin attenuated the increased mRNA for tyrosine hydroxylase in middle-aged rats, whereas no changes were observed in CC of young rats (Fig. 4, A and B). The mRNA expression for the α1A-adrenoceptor remained unchanged among groups (Fig. 4, A and B).

Protein expression for α1- and β1-subunits of sGC. Measurement of cavernosal sGC protein expression for α1- and β1-subunits of sGC in middle-aged rats were reduced by 44 and 62% compared with those of the young group, respectively. Treatment with apocynin restored the protein levels of α1- and β1-subunits in the middle-aged group (Fig. 5, A and B). In young rats, the protein expression for β1-subunits of sGC was not affected by apocynin treatment; however, the protein expression for α1-subunits of sGC was significantly increased by apocynin (P < 0.05).

DISCUSSION

In the present study, we show that CC from middle-aged rats displays increased sympathetic neurotransmission and α1-adrenoceptor-mediated contractile responses, which are associated with increased mRNA expression of tyrosine hydroxylase. Decreased expression of α1- and β1-subunits of sGC in cavernosal smooth muscle of middle-aged rats was also observed. Moreover, prolonged treatment with apocynin restores the functional and molecular alterations in CC from middle-aged rats, indicating that increased superoxide anion generation plays a major role in the pathophysiological alterations of CC at the middle age.

In the penile flaccid state, sympathetic neural activity predominates; the trabecular smooth muscles, which support the vascular sinuses, are tonically contracted permitting only a small amount of arterial inflow. Catecholamines cause concent-
tion-dependent contractions in CC and penile arteries and veins (1). The \( \alpha_1 \)-adrenoceptor subtype is functionally predominant since its activation by phenylephrine potently contracts human CC (11). In fact, intracavernous injection of \( \alpha \)-adrenergic blockers causes tumescence and erection while \( \alpha \)-adrenergic agonists cause detumescence (5). ED in obese (8, 48) and hypertensive animals (9) was associated with increased EFS-induced contractions in the erectile tissue. In our study the neurogenic contractions were also significantly greater in middle-aged compared with young rats, suggesting an increased nerve-evoked norepinephrine release in CC. Norepinephrine is synthesized from the amino acid precursor \( L \)-tyrosine. Tyrosine hydroxylase is the first rate-limiting enzyme in catecholamine synthesis that catalyzes the conversion of tyrosine to \( L \)-dihydroxyphenylalanine (DOPA). This latter is converted to dopamine by DOPA decarboxylase, which in turn is converted to norepinephrine by dopamine \( \beta \)-hydroxylase (36). Increased tyrosine hydroxylase immunostaining in arteries and veins of kidney and heart has been related with sympathetic hyperactivity and has proven to be a valuable technique to evaluate regional patterns of sympathetic activity (6). An increase in immunostaining for tyrosine hydrolase was also found in CC of diabetic rats (34). Therefore, we evaluated the tyrosine hydroxylase expression in CC tissues in both young and middle-aged groups. Accordingly, in our study, the tyrosine hydroxylase mRNA expression was increased in the middle-aged group compared with the young group, which is consistent with the

Fig. 3. Reactive-oxygen species levels through dihydroethidine (DHE)-induced fluorescence in corpus cavernosum from young and middle-aged rats in the presence of either apocynin (Ap. I.; 100 \( \mu \)M) or superoxide dismutase in vitro (SOD I.; 75 U/ml). Another group of young and middle-aged rats were treated orally with apocynin (Ap. T.; 85 mg·rat\(^{-1}\)·day\(^{-1}\), 4 wk). Representative (A) and quantitative analysis (B) for DHE-fluorescence photomicrographs of microscopic sections of corpus cavernosum. Data represent the means ± SE of 5 experiments. *\( P < 0.01 \), compared with young group. #P < 0.05, compared with middle-aged group.

Fig. 4. mRNA expressions of tyrosine hydroxylase (A) and \( \alpha_1 \)-adrenoceptor (B) in corpus cavernosum isolated from young and middle-aged rats, treated or not with apocynin (85 mg·rat\(^{-1}\)·day\(^{-1}\), 4 wk). The mRNA expression level of each gene was normalized by GAPDH and \( \beta \)-actin expression. Values are expressed in arbitrary units. Data represent the means SE for 6 rats each group. *\( P < 0.05 \), compared with young group. #P < 0.05, compared with middle-aged group.
Recently, apocynin, administered at a single oral dose of 50
mg/kg to rats, was shown to achieve a maximal plasma concentration ($C_{max}$) of 8 ± 2 µM (50). Therefore, it is unlikely that such a high concentration of apocynin to act as ROS scavenger is found in plasma of rats treated for 4 wk with this drug.

NADPH oxidase has been shown to be localized in sympathetic nerve fibers and its endings, as well as in periarterial nerves, indicating a superoxide-mediated mechanism in peripheral neurovascular control (7). In our study, the superoxide production in middle-aged rats is thus likely to enhance tyrosine hydroxylase expression, causing a higher norepinephrine production/release under EFS stimulation. The greater nerve-stimulated norepinephrine release in hearts (28) and mesenteric arterial bed (30) of spontaneously hypertensive rats has been attributed to increased oxidative stress in the erectile tissue (1). We previously reported that ED in middle-aged rats is associated with sympathetic hyperactivity, which was restored by the antioxidant N-acetylcysteine (30). Incubation of CC with l-NAME (nonspecific NOS inhibitor) in the presence or not of atropine (nonselective muscarinic antagonist) fully abolishes the EFS-induced cavernosal relaxations (41). In our present study, using l-NAME (together with atropine) to produce a full NO inhibition under EFS stimulation, we observed higher CC contractions in all groups, confirming that the NO-cGMP pathway normally refrains the smooth muscle contractile machinery (41). Interestingly, however, in the middle-aged group, the cavernosal strips from l-NAME- and atropine-treated preparations continued to display an augmented contractile response to EFS, reinforcing that the sympathetic hyperactivity is rather due to a state of oxidative stress. In the young CC group, despite apocynin treatment reduced the ROS production/release under EFS stimulation. The greater adrenergic-induced cavernosal contractions in middle-aged rats is thus likely to enhance tyrosine hydroxylase expression, causing a higher norepinephrine production/release under EFS stimulation. The greater nerve-stimulated norepinephrine release in hearts (28) and mesenteric arterial bed (30) of spontaneously hypertensive rats has been attributed to increased oxidative stress in the erectile tissue (1). We previously reported that ED in middle-aged rats is associated with sympathetic hyperactivity, which was restored by the antioxidant N-acetylcysteine (30). Incubation of CC with l-NAME (nonspecific NOS inhibitor) in the presence or not of atropine (nonselective muscarinic antagonist) fully abolishes the EFS-induced cavernosal relaxations (41). In our present study, using l-NAME (together with atropine) to produce a full NO inhibition under EFS stimulation, we observed higher CC contractions in all groups, confirming that the NO-cGMP pathway normally refrains the smooth muscle contractile machinery (41). Interestingly, however, in the middle-aged group, the cavernosal strips from l-NAME- and atropine-treated preparations continued to display an augmented contractile response to EFS, reinforcing that the sympathetic hyperactivity is rather due to a state of oxidative stress. In the young CC group, despite apocynin treatment reduced the ROS production/release under EFS stimulation.

ED in cardiovascular and endocrine-metabolic diseases has been attributed to increased oxidative stress in the erectile tissue (1). We previously reported that ED in middle-aged rats is associated with upregulation of NADPH oxidase subunit gp91phox in cavernosal smooth muscle (45). In the present study we enlarged these observations by showing an elevated ROS production and tyrosine hydroxylase mRNA expression, as well as greater EFS-induced cavernosal contractions, in the erectile tissue from middle-aged animals. Moreover, the present study shows that 4-wk treatment of middle-aged rats with the NADPH oxidase inhibitor apocynin normalized the ROS levels and restored the tyrosine hydroxylase mRNA expression and hence the sympathetic cavernosal contractions. In vitro incubation of CC with SOD (or apocynin) prevented the elevated ROS levels in CC from middle-aged rats, confirming the involvement of superoxide. Altogether, the greater EFS-induced cavernosal contractions in middle-aged rats appear to be a consequence of an increased NADPH oxidase-dependent superoxide production in aged tissues. Apocynin at high concentrations (>100 µM) can act as a ROS scavenger (18). Recently, apocynin, administered at a single oral dose of 50
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in CC of middle-aged rats, suggesting that excess of ROS acts to favor the oxidized NO-insensitive state of sGC.

In men, a previous study reported that an increased norepinephrine levels in cavernosal tissue during sexual arousal is associated with organic ED (2). In addition, human CC contractions induced by the α1-adrenoceptor agonist phenylephrine progressively increase with age (11). In our study, the maximal contractile response to phenylephrine was greater in the middle-aged group, which was also restored by apocynin treatment. Therefore, increased ROS levels and hence impairment of sGC-cGMP pathway in the cavernosal smooth muscle are likely to favor the contractile responses downstream the α1-adrenoceptor. This is consistent with our findings showing that α1A-adrenoceptor mRNA expression remains unchanged between groups, thus excluding a role for upregulation of these receptors in mediating the sympathetic hyperactivity in middle-aged rats. Collectively, our data suggest that increased contractile responses to sympathetic activation in CC of middle-aged rats may make penile tumescence more difficult to occur. Excess of superoxide in middle-aged rats may occur at intracellular levels of both sympathetic nerves and cavernosal smooth muscle cells. In the former it promotes a higher tyrosine hydroxylase expression, whereas in the latter it reduces NO bioavailability and sGC expression, which in turn causes ED by mechanisms involving impairment of relaxations (45) and facilitation of cavernosal contraction.

**Conclusions.** Our study shows that ED seen in middle-aged rats is associated with upregulation of tyrosine hydroxylase mRNA expression and increased sympathetic-induced contractions, along with α1-adrenoceptor-mediated cavernosal vasoconstriction. Downregulation of GCs (α1- and β1-subunits) in cavernosal smooth muscle also accounts for ED in middle-aged rats. Moreover, apocynin treatment normalized all the functional and molecular alterations, demonstrating a major role for the elevated oxidative stress contributing to ED in middle-aged rats. Therefore, reduction of oxidative stress by dietary antioxidants may be an interesting approach to manage ED and improvement of the quality of life in aging population.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


