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Aging-related induction of inducible nitric oxide synthase is vasculo-protective to the arterial media

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Abstract

Objective: Aging associated erectile dysfunction (ED) is primarily caused by the reduction in smooth muscle cells (SMC) and an increase in collagen within the corpora cavernosa, assumed to result from an increase in reactive oxygen species (ROS). This is accompanied by the expression of inducible nitric oxide synthase (iNOS) to produce nitric oxide that scavenges ROS and inhibits collagen deposition. We investigated whether with aging similar processes occur within the arterial media SMC that share some common physiological functions with the cavernosal SMC. **Methods:** Aged (22–24 months) male Brown Norway rats received water with or without an inhibitor of iNOS activity (L-*N*-(iminoethyl)-lysine acetate [L-NIL], 0.1 g/l), for 3 weeks. Young (3 months) untreated rats were used as control (n=5 per group). Tissue sections from the penis, abdominal aorta, femoral and brachial arteries were stained for collagen, SMC, iNOS, ROS plasminogen activator inhibitor (PAI) and apoptosis, and evaluated by quantitative image analysis. ROS were also determined in fresh tissue and whole blood by the GSH/GSSG ratio. **Results:** It was observed that most aging-induced changes in the media of the arterial tree from the aorta to the resistance arteries in the penis are similar to what occurs in the corpora cavernosa, i.e. a decrease in the SMC/collagen ratio and an increase in ROS and iNOS, and specifically in the case of the resistance arteries, an increase in SMC apoptosis and PAI. iNOS inhibition by L-NIL further increased ROS and decreased the SMC/collagen ratio in the media. **Conclusions:** These observations suggest that ED and arteriosclerosis in the aging male may share a common etiology, and that the expression of iNOS by the SMC is an attempt to counteract this fibrosis.

Keywords: Fibrosis; Arteries; Erectile dysfunction; Oxygen radicals; Smooth muscle

1. Introduction

Erectile dysfunction (ED) and cardiovascular disease, particularly hypertension, are both prevalent in the aging male [1,2]. One of the underlying causes of hypertension is arteriosclerosis, or arterial stiffness, due to an acquired fibrosis of the media of the arterial wall [3–6]. Arteriosclerosis is significantly associated with aging, and is recognized by an increase in collagen, and in some cases by a loss of smooth muscle cells (SMC) within the arterial media, which results in a decrease in the SMC/collagen ratio, often accompanied by endothelial dysfunction [7].

The pathogenesis of aging associated ED, both in the human and rat, is mostly related to the loss of SMC in the penile corpora cavernosa by apoptosis with a corresponding increase in collagen fibers [2,8–10]. The clinical result of this aging process in the penis is defective cavernosal SMC relaxation leading to veno-occlusive dysfunction [3,11], the most common cause of ED. In the arterial tree, excessive collagen deposition in the media, with or without loss of SMC, leads to defective vasorelaxation and clinically may present as hypertension [3–5]. Because the penis can be considered a specialized extension of the vascular tree, the common alterations observed in the SMC of both the penis and peripheral vascular system in the aging male leading to

Abbreviations: NO, nitric oxide; iNOS, inducible NOS; L-NIL, L-iminoethyl-L-lysine; SMC, smooth muscle cell; PAI, plasminogen activator inhibitor; ED, erectile dysfunction

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ED and hypertension, respectively, suggest that both conditions may share a common etiology.

One of the prevalent views of peripheral vascular disease is that it is caused by oxidative damage to the arterial wall by free radicals that cause lipid peroxidation and other alterations, the reactive oxygen species (ROS) [7,12]. These compounds are mainly produced by xanthine oxidase, NADPH oxidase, as well as mitochondrial enzymes, and counteracted by heme-oxygenase I and superoxide dismutase (SOD), that can reduce ROS by acting as endogenous antioxidants. In addition to causing endothelial damage, ROS are known stimulators of collagen deposition and SMC proliferation [7,12] in the vascular wall. Xanthine oxidase and SOD are present in the penile corpora cavernosa [13], and oxidative stress due to ROS has been postulated to be central to impaired cavernosal function in aging-related ED [13,14].

Besides antioxidants, nitric oxide (NO), synthesized by nitric oxide synthase (NOS), also quenches ROS in the vasculature, as shown by the increase in ROS levels and the development of cardiac and renal fibrosis and vascular stiffness when there is long-term systemic blockade of NOS activity with NOS inhibitors [15,16]. In fact, the ROS-quenching and anti-fibrotic effects of NO are not limited to the SMC and can be demonstrated in other non-vascular conditions [17,18]. In this process, NO reduces ROS levels through the formation of peroxynitrite [7,13,17–19], thereby increasing the NO/ROS ratio. NO is also postulated to not only inhibit collagen synthesis directly, but to favor collagen degradation by stimulating metalloproteinases and down-regulating expression of their inhibitors, such as the plasminogen activator inhibitor (PAI) [20]. The predominance of nitrosative pathways over oxidative stress is proposed to be protective against fibrosis [17,18], ED [13], atherosclerosis and hypertension [19,21].

The NO/ROS balance also directly modulates the relaxation of the vascular and penile SMC. The NO produced by the endothelial NOS in the vascular endothelium controls blood pressure by relaxing the arterial SMC [19,22]. In the penile corpora cavernosa, NO as a mediator of penile erection is produced by the neuronal NOS, specifically the PnNOS variant, localized in the nerve terminals, and to a lesser extent by endothelial NOS in the lacunar and sinusoidal endothelium of the penis [22]. In experimental animals, reduction in NOS levels in the vasculature and penile corpora is associated with hypertension [19] and with ED, respectively [9,22]. If oxidative stress becomes excessive, the reaction of ROS with NO to form peroxynitrite reduces NO concentration in the tissues, which would lead to hypertension and ED by impairing NO dependent SMC relaxation.

It is still unknown to what extent these neuronal and endothelial NOS isoforms would participate in producing NO as an antifibrotic mechanism. In contrast, more direct evidence has emerged recently on the role of the inducible isoform of NOS (iNOS) [23] in reducing ROS and modulating the SMC/collagen ratio in different tissues. iNOS is spontaneously induced in the corpora cavernosa [10] and brain [24] during aging, and in certain fibrotic conditions [17,18]. In the vasculature, iNOS is also induced in the media in aging-related arterial stiffness [25–27], transplant arteriosclerosis [28], and atherosclerosis [29,30], and it is assumed to inhibit collagen deposition and prevent medial hyperplasia via induction of SMC apoptosis and/or inhibition of SMC replication [19,23,29]. The specific inhibition of iNOS activity by L-*N*-(iminoethyl)-lysine acetate (L-NIL) [17,18,30], or the blockade of iNOS expression in the iNOS knockout mouse [29,31], causes fibrosis in vascular and non-vascular tissues, a decrease in NO/peroxynitrite levels, an increase in ROS, and a reduction in the SMC/collagen ratio.

Despite the fact that a certain predominance of the nitrosative over the oxidative pathways may preserve the normal integrity and function of blood vessels and corpora cavernosa, an excessive production of NO and peroxynitrite may also induce apoptosis and cell loss [10,17,18,23]. Depending on the context, this may be beneficial by preventing media hyperplasia in atherosclerosis and restenosis and ameliorate fibrosis in other systems [17–19,30]. But excessive peroxynitrite may also be noxious, if it leads to a loss of SMC and the subsequent impairment of tissue relaxation. We propose that during aging, iNOS induction in the vasculature is not restricted to the cavernosal SMC [10] and large arteries [25–27], but is generalized to the wall of the entire peripheral vascular tree. This process would aim to counteract oxidative stress and metalloproteinase inhibition, and the subsequent decrease in the SMC/collagen ratio that causes loss of compliance and NO-induced vasorelaxation. To test this hypothesis, we have examined large and small (resistance) arteries in both young and aged rats for SMC/collagen ratio, iNOS, peroxynitrite, heme-oxygenase I, SOD, PAI and SMC apoptosis, and determined how these parameters were affected in aged rats when iNOS activity was specifically inhibited with L-NIL.

2. Materials and methods

2.1. Animal treatment and tissue processing

Five young (3 months) and 10 aged (22–24 months) male Brown Norway rats were obtained from the NIH/NIA colony (Harlan Sprague–Dawley, San Diego, CA), and maintained under controlled temperature and lighting, in accordance to NIH guides. One half of the aged animals (n=5 per group) were treated for 3 weeks with L-NIL at 0.1 g/l in the drinking water, while the rest of the animals received plain drinking water [17,18]. Animals were anesthetized, pretreated with heparin and perfused through the left ventricle with saline followed by 10% formalin [10,17,18]. The abdominal aorta, brachial and femoral neurovascular bundles as well as the penis, denuded of its skin, were removed and post-fixed overnight in 10% formalin, and washed and stored in PBS at 4 °C until paraffin embedding.

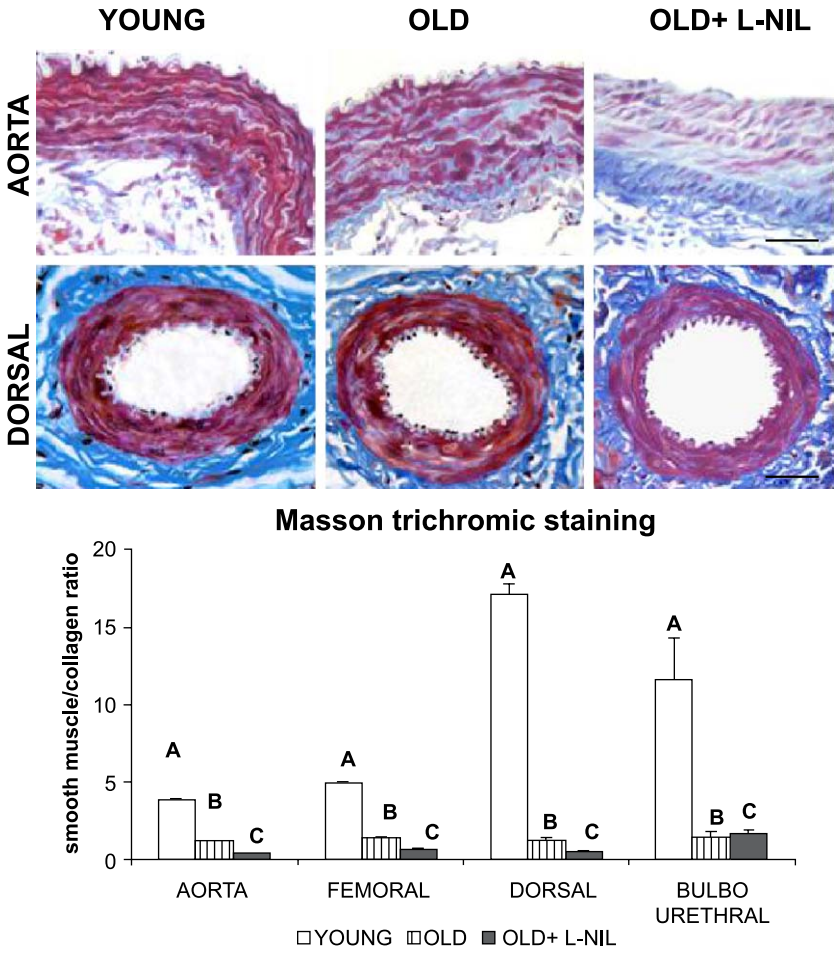

Fig. 1. Intensification by iNOS blockade of aging-related fibrosis in the arterial media. Old male rats were given L-NIL for 3 weeks, or left untreated. Young untreated rats served as controls. Tissue sections were obtained from penis (to visualize the dorsal and bulbourethral penile arteries) and from the aorta and femoral artery, and stained with Masson (SMC: red, collagen: blue). Top: micrographs from selected arteries and tissue sections, as indicated. Bar=50 μm. Bottom: Quantitative image analysis (QIA) expressed as ratios of areas occupied by SMC and collagen, as means±S.E.M. Aorta: A vs. B, C, $p<0.001$; B vs. C, $p<0.01$; femoral: A vs. B, C, $p<0.001$; B vs. C, $p<0.01$; dorsal: A vs. B, C, $p<0.001$; B vs. $p<0.05$; bulbourethral: A vs. B, C, $p<0.001$; B vs. C, NS.

2.2. Histochemical detection of collagen and SMC and arterial morphometry

SMC and collagen fibers within the corporal tissue and vascular tree were estimated by Masson trichromic staining (Sigma Diagnostic, St. Louis, MO) [17,18,32] in sections adjacent to those used for immunohistochemical staining, followed by image analysis to measure the ratio between SMC (red) and collagen fibers (blue). The results were expressed as red/blue ratios per area (see below). In the arterial tree, the intima/media thickness (IMT), and the diameter of the lumen were also measured.

2.3. Immunohistochemical detection

The determinations of iNOS, nitrotyrosine, heme-oxygenase I, PAI-1 [32], manganese superoxide dismutase (MnSOD) and CuZn SOD (Cu/Zn SOD) [33] were carried out on 5 μm paraffin-embedded adjacent tissue sections, that were quenched for endogenous peroxidase activity after deparaffinization and rehydration. Sections were blocked with normal goat serum, and incubated with polyclonal IgG antibodies against mouse iNOS (1:500) (Transduction Laboratories, Lexington, KT), nitrotyrosine (1:100) (Upstate, Lake Placid, NY), Mn SOD and Cu/Zn SOD (Oxis Health Products, Portland, OR) (1:800 and 1:500, respectively),

Table 1
Effect of aging on arterial wall thickness and lumen diameter in large and small arteries in the rat n=5

	Young (μm)	Old (μm)	Old+L-NIL (μm)
Intima media thickness			
Aorta	73.4±8.6	93.0±6.6**	85.2±6.6**
Femoral	49.7±7.1	63.5±2.4	51.8±4.6
Penile dorsal	17.0±2.3	18.6±1.4	23.6±3.3
Bulbourethral	10.5±1.3	10.1±1.6	9.9±0.7
Lumen diameter			
Penile dorsal	100.8±13.7	118.7±6.6	105.5±16
Bulbourethral	40.1±5.6	42.6±6.8	47.3±6.6

**$p<0.01$ compared to young.

heme-oxygenase I (Stressgen, San Diego, CA) or PAI-1 (Abcam, Cambridge, UK). For negative controls, the first antibodies were replaced by IgG isotype. The detection was based on a secondary anti-rabbit biotinylated antibody (1:200) for iNOS and nytrotyrosine (Calbiochem, La Jolla, CA), or anti-sheep biotinylated antibody (1:200) for Cu/Zn and Mn SOD, followed by the ABC complex (1:100) (Calbiochem) and 3,3′ diaminobenzidine) (DAB) (Sigma). Sections were counterstained with hematoxylin.

The TUNEL assay [10,24] was performed in the adjacent matched tissue sections used for iNOS and nitrotyrosine staining, applying the Apoptag Oncor kit (Oncor, Gaithersburg, MD).

2.4. Quantitative image analysis

Immunoreactivity was quantified by image analysis [10,17,18,32] using the Image Pro 4.01 software (Media Cybernetics, Silver Spring, MD), coupled to an Olympus BHS microscope/VCC video camera. After images are calibrated for background lighting, optical density per area (OD/AREA) results are proportional to the unweighted average optical density which is used to determine the concentration of immunoreactive antigen. At least five sections per specimen were analyzed, with five fields per section and five animals per group. Each slide assayed had its corresponding negative control. In certain cases, the number of immunopositive cells was determined as a percentage of the total counterstained nuclei in a computerized grid. In the Masson staining, the ratio between SMC (red) and collagen fibers (blue) was obtained and expressed per area. The rate of programmed cell death (apoptotic index) was determined in the arterial media, as well as in the endothelium in the case of the dorsal penile artery, and expressed as the percentage of apoptotic cells within the total number of cells in a given area (non-apoptotic nuclei plus apoptotic cells).

2.5. Measurement of GSH/GSSG ratio in tissue homogenates and whole blood

Skin-denuded penile shaft, aorta and blood were obtained from a separate series of young and old rats, and old rats

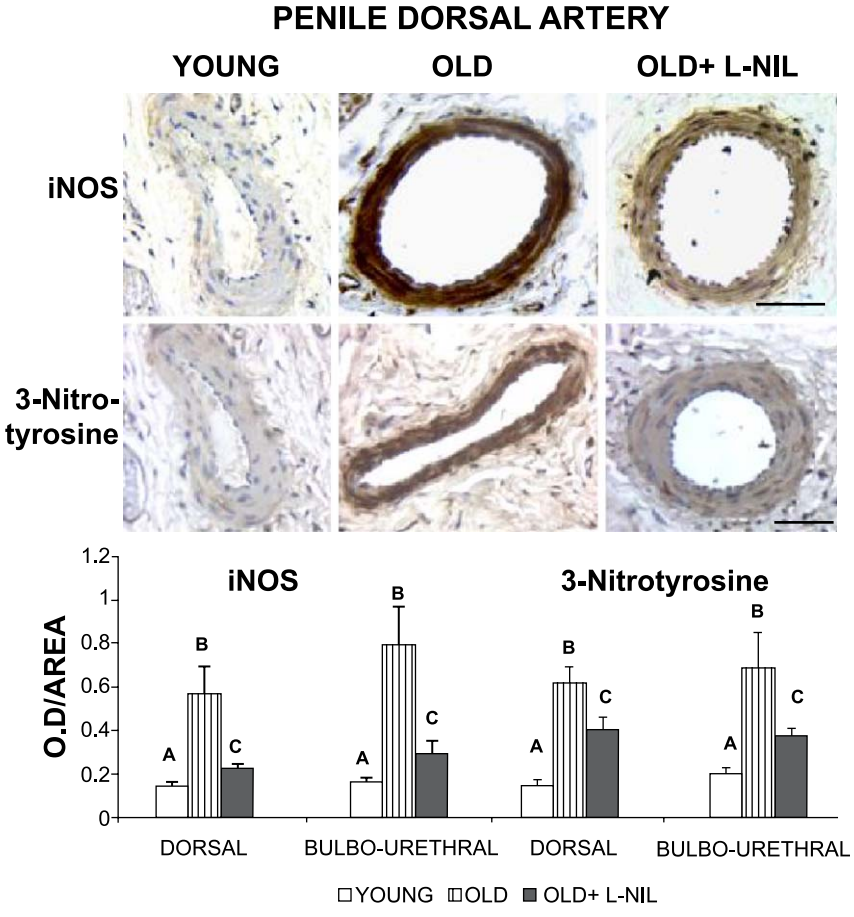

Fig. 2. Reduction by iNOS blockade of the aging-related stimulation of the nitrosative pathway in the media of the penile arteries. Sections were immunostained as indicated. Nitrotyrosine is a marker for peroxynitrite. Top: micrographs from selected arteries and tissue sections, as indicated. Bar=50 μm. Bottom: QIA as on Fig. 1, expressed as intensity of immunostaining per area, as means±S.E.M. For iNOS: dorsal: A vs. B, $p<0.05$; A vs. C, NS; B vs. C, $p<0.05$; bulbourethral: A vs. B, $p<0.05$; A vs. C, NS; B vs. C, $p<0.05$. For 3-nitrotyrosine: dorsal: A vs. B, $p<0.001$; A vs. C, $p<0.05$; B vs. C, $p<0.05$; bulbourethral: A vs. B, $p<0.01$; A vs. C, NS; B vs. C, $p<0.01$.

treated with L-NIL as above (n=5/group). Tissue was homogenized in 6 vol. of 5% metaphosphoric acid, and the whole blood was collected with or without 1-methyl-2 vinylpyridinium trifluoromethane sulfonate (M2VP) scavenger of reduced glutathione, described in the commercial kit protocol ("Bioxytech GSH/GSSG-412 kit" from Oxis Health Products). The omission or addition of M2VP allows the measurement of reduced (GSH) and oxidized (GSSG) glutathione, respectively. The spectrophotometric detection was recorded at 412 nm for 3 min after the addition of 3.8 μmol NADPH. The GSH/GSSG ratio is inversely related to ROS levels.

2.6. Statistical analysis

Values were expressed as mean±S.E.M. for n=5 animals/group. The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons among the different groups were analyzed by a single factor analysis of variance (ANOVA), followed by post-hoc comparisons with the Student–Newman–Keuls test, according to the Graph Pad Prism V4.0 program. A $p<0.05$ was considered significant.

3. Results

3.1. Intensification of aging-related fibrosis in the arterial media by iNOS inhibition

In order to determine whether aging per se is associated with an intensification of collagen deposition and a relative loss of SMC in the media from the aorta to the peripheral resistant arteries [3–6], Masson trichrome staining was performed on sections from the abdominal aorta, femoral and brachial arteries as well as from the penile shaft focusing on two peripheral putative resistance arteries: the bulbourethral and dorsal arteries of the penis. Fig. 1 (micrographies) shows that in the media of the dorsal penile artery, few collagen fibers were present in the young rats but were considerably increased in the aged animals, resembling the situation seen in the aorta. As expected from the hypothesis that iNOS may act as antifibrotic agent within the vascular tree, the administration of L-NIL, a specific inhibitor of iNOS activity, for 3 weeks to the aged rats led to a further increase in the collagen fibers within the media of the aorta and the dorsal penile artery.

Image analysis was performed in all arteries with the exception of the brachial (Fig. 1, bottom). In all vessels studied, there was a marked reduction in the SMC/collagen ratio with aging. Following iNOS blockade by L-NIL, there was a further exacerbation in the amount of collagen within the media (with the exception of the bulbourethral artery), suggesting that the decrease in NO production by the inhibition of iNOS leads to an intensification of the aging-related fibrosis. These alterations were not accompanied in the resistant arteries by a significant increase in the intima/media thickness (IMT), whereas in the aorta and femoral the IMT was higher (Table 1). The measurements of the luminal diameter (Table 1) confirmed the clinical observation that the dorsal and bulbourethral arteries, with a luminal diameter well below 350 μm, fall within the definition of resistance arteries [4,34].

3.2. iNOS induction and peroxynitrite deposition in the arterial media with aging

All the antibodies used in this work have been validated by our group [10,17,18,25,32], and in the case of the iNOS antibody it was additionally tested in the current work by immunocytochemistry against rat penile fibroblast cultures (tunica albuginea [17,18]) induced to express iNOS. These cells were intensively stained, in comparison to uninduced cells that were negative, and expressed the expected single 130-kDa band visible on western blots (not shown). This band was absent in aorta extracts from a young iNOS knockout mouse that had received LPS (4 mg/kg) to induce iNOS, and present in the respective extract from the similarly treated wild type animal (not shown).

This antibody showed that iNOS is increased with aging in parallel with collagen deposition in the arterial media throughout the vascular tree, confirmed by detection of nitrotyrosinylated proteins. The latter arise from peroxynitrite produced by the reaction between NO and ROS, and therefore are an indirect measure of NOS activity. Fig. 2 (micrographies) shows negligible iNOS expression and nitrotyrosine formation in the dorsal artery of the penis of the young animals, and a remarkable intensification of both processes with aging. The iNOS staining in these vessels was mainly confined to the media and intima. As anticipated from previous results [25–27], a similar finding was seen in the aorta, brachial and femoral arteries (not shown). When L-NIL, the inhibitor of iNOS activity, was given, there was a reduction in iNOS expression, which combined with the direct decrease of iNOS activity, led to a reduction in peroxynitrite formation. Quantitation by image analysis confirmed these changes in the resistant dorsal and bulbourethral arteries of the penis (Fig. 2, bottom), as well as in the aorta and femoral arteries (not shown). The brachial artery was not subjected to image analysis.

3.3. Effects of iNOS inhibition on ROS production, apoptosis and PAI in the arterial media

Utilizing the Cu/Zn SOD as an indirect marker of ROS, the production of ROS in the arterial media was found to be considerably increased with aging in the femoral, brachial and resistant arteries but not in the aorta, and this process was further increased with iNOS inhibition by L-NIL (Fig. 3, top). This was additionally confirmed by image analysis

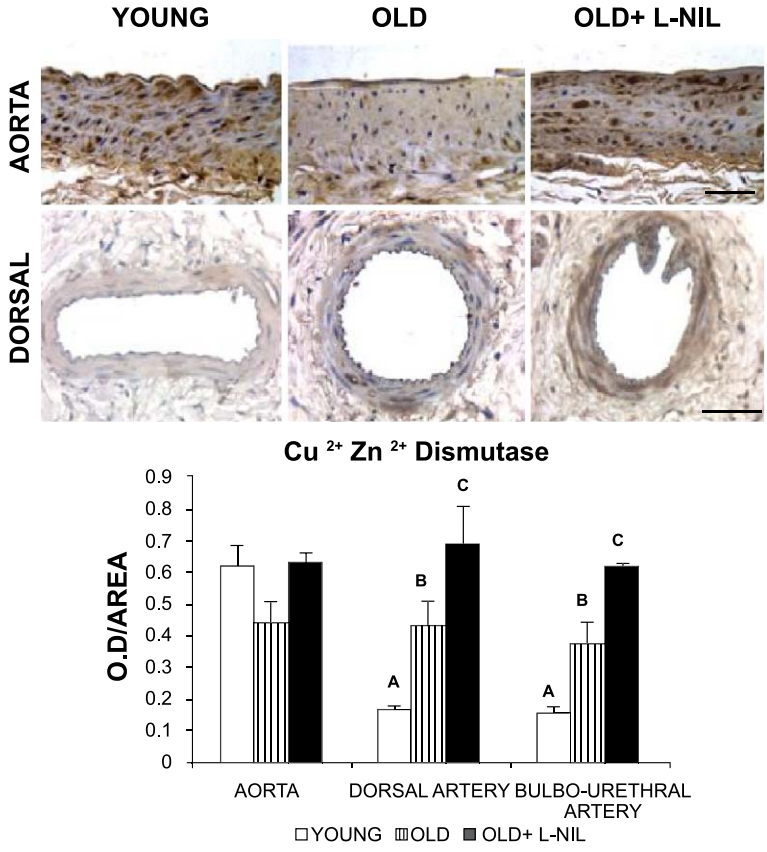

Fig. 3. Intensification by iNOS blockade of aging-related oxidative stress in the arterial media. Tissue sections were immunostained for $Cu^{2+}Zn^{2+}$ SOD and for Mn^{2+} SOD. Top: micrographs from selected arteries and tissue sections only for $Cu^{2+}Zn^{2+}$ SOD, as indicated. Bar=50 μm. Bottom: QIA as on Fig. 2, expressed as intensity of immunostaining per area, as means±S.E.M. Dorsal: A vs. C, $p<0.05$; A vs. NS; B vs. NS; bulbourethral: A vs. C, $p<0.001$; A vs. B, $p<0.05$; B vs. $p<0.01$; A vs. B, C, $p<0.001$; B vs. C, NS. Mn^{2+} SOD gave essentially similar results (not shown).

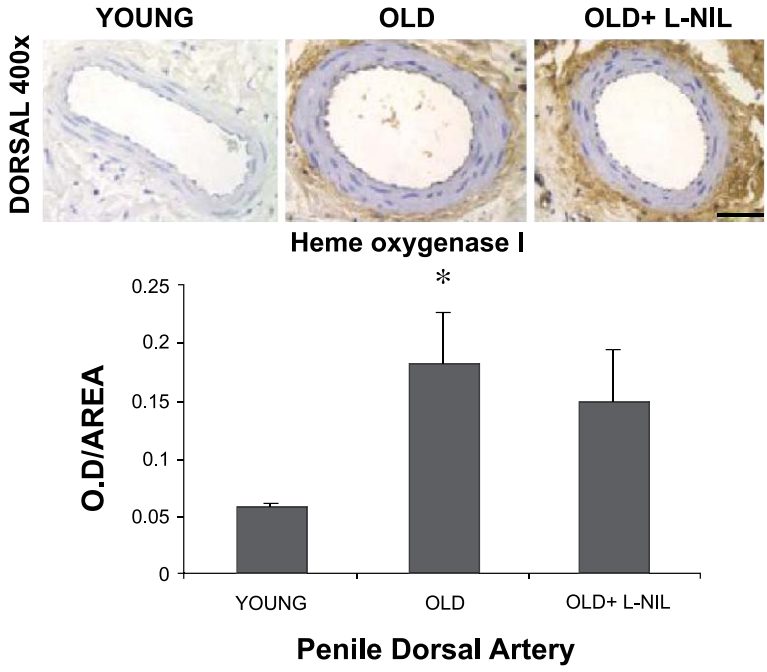

Fig. 4. Differential expression of another marker of oxidative stress, heme-oxygenase 1, in the adventitia of the arterial wall. Sections were immunostained with an antibody against heme-oxygenase I and counterstained with hematoxylin. Top: micrographs from the dorsal artery. Bar=50 μm. Bottom: QIA as on previous figures. Values expressed as means±S.E.M. *$p<0.05$: young vs. old (*t*-test).

(Fig. 3, bottom), that indicated that L-NIL blockade of iNOS activity raised Cu/Zn SOD by 40–50%. The Mn SOD gave similar results from the aorta to the resistant arteries (not shown). Another antioxidant enzyme, heme-oxygenase I, demonstrated the same aging related changes in the penile dorsal artery, as observed for both SOD enzymes, but remarkably, L-NIL did not induce a further significant change in the expression of this enzyme (Fig. 4). Interestingly, the localization of virtually all the expression of heme-oxygenase-1 was in the arterial adventitia, rather than in the media as seen for the SOD enzymes.

These immunohistochemical data were confirmed by the estimation of the GSH/GSSG ratio in tissue homogenates, that is inversely related to ROS levels, e.g., the higher this value, the lower oxidative stress is. The GSH/GSSG ratio in the penis of young rats was reduced by 55% in the old rats and by another 45% in the old rats treated with L-NIL, indicating the respective increases in ROS levels (Table 2). An even further reduction (77%) of this ratio by aging was seen in the aorta, although the very low GSH/GSSG value in the old rats was not further reduced by L-NIL. A comparison of the ratios obtained in the old rats between both tissues, indicate a 10-fold higher level of oxidative stress in the aorta as compared to the penis, even in the presence of a considerable high level of circulating ROS markers (very low GSH/GSSG) in the blood.

The NO/ROS balance was significantly altered throughout the entire arterial media by iNOS inhibition with L-NIL via a reduction in NO synthesis (denoted by peroxynitrite) and a stimulation of ROS formation (denoted by the antioxidant enzymes). Apoptosis of the SMC within the media of the penile resistance arteries increased with aging, and decreased subsequently in the old animals receiving L-NIL treatment (Fig. 5, micrographies). The apoptotic index was calculated for both the dorsal and bulbourethral penile arteries by image analysis, and was higher in aged compared to young rats but L-NIL treatment resulted in a reduction in this index (Fig. 5, bottom). The apoptotic index was also measured in the endothelium of the dorsal penile artery and it was considerably and significantly increased by aging, from 6.1±2.7 in the young rats to 40.6±2.8 in the old rats. However, inhibition of iNOS activity by L-NIL in the old rats did not significantly reduce this index (30.2±4.8).

Aging alone or in combination with iNOS inhibition affected the expression of PAI-1, a well characterized inhibitor of metalloproteinases [20,32]. Inhibition of PAI is associated with an increase in collagen fibers due to its interference with metalloproteinases that are involved with the breakdown of collagen. Compared to young animals, PAI expression was considerably increased in the arterial media with aging, but was not further stimulated by iNOS inhibition, as seen in the dorsal penile artery (Fig. 6, micrographies). Quantitative image analysis for both the mean intensity of expression (bottom left) and the number of PAI positive cells (bottom right) indicated that the increase in PAI by aging alone was between two- and five-fold, respectively. However, the effect of L-NIL on PAI expression in the aged media was negligible (bottom).

Table 2
Determination of ROS in tissue homogenates and whole blood by the reduced/oxidized glutathione ratios

	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.
Blood	GSH (μM)	GSSG (μM)	GSH/GSSG ratio
Young	521 ± 38	58 ± 15	16 ± 4
Old	883 ± 165	88 ± 14[a]	5 ± 0.5[a]
Old + L-NIL	872 ± 92	165 ± 20[a,b]	3 ± 0.5[a,b]
Penis	(μmoles/mg prot.)	(μmoles/mg prot.)	GSH/GSSG ratio
Young	51.2 ± 5.1	0.07 ± 0.01	975 ± 197
Old	43.3 ± 4.7	0.14 ± 0.02[a]	402 ± 34[a]
Old + L-NIL	40.3 ± 8.7	0.21 ± 0.02[a,b]	237 ± 50[a,b]
Aorta	(μmoles/mg prot.)	(μmoles/mg prot.)	GSH/GSSG ratio
Young	59.6 ± 10.6	0.3 ± 0.04	179.7 ± 26.2
Old	48.4 ± 6.1	1.2 ± 0.34[a]	42.2 ± 10.3[a]
Old + L-NIL	35.1 ± 5.1	1.5 ± 0.99[a]	40.0 ± 3.8[a]

Fresh tissue and blood were assayed for GSH and GSSG with or without the addition of M2VP. Ratios were calculated as: (GSH-2*GSSG)/GSSG.
[a] Significant compared to young, $p < 0.01$.
[b] Significant compared to old, $p < 0.05$.

4. Discussion

This study to our knowledge is the first to suggest that the arterial media from the aorta to the small resistant arteries undergoes many of the changes that occur within the corporal tissue with aging, namely: (a) a reduction in the SMC/collagen ratio; (b) an increase in markers of oxidative stress and of inhibitors of collagen degradation, such as PAI, which are known pro-fibrotic factors; and (c) the spontaneous induction of iNOS, which is believed to act as an anti-fibrotic agent [17,18,31,32]. However, the increase in SMC apoptosis in the media of the resistant arteries of the penis, presumably leading to a reduction in the absolute SMC content, contrasts with what has been reported for large vessels such as the aorta and the femoral artery [35,36], but does agree with the process described in the corporal SMC [9,10].

Our results also confirm the putative role for NO derived from iNOS produced by the SMCs of the media in combating aging-related fibrosis within the media, as evidenced both by the increase in ROS and an intensification of fibrosis when iNOS activity is inhibited. The deposition of collagen fibers observed in the arterial media of the aged rats is thought to lead to arterial stiffness or arteriosclerosis in the vascular system. Because of the apoptosis occurring in the SMC, the relative reduction in the SMC/collagen ratio is intensified in the resistant arteries of the penis, in comparison to the larger arteries, e.g. the aorta. We hypothesize that this process is a major factor, together with the endothelial

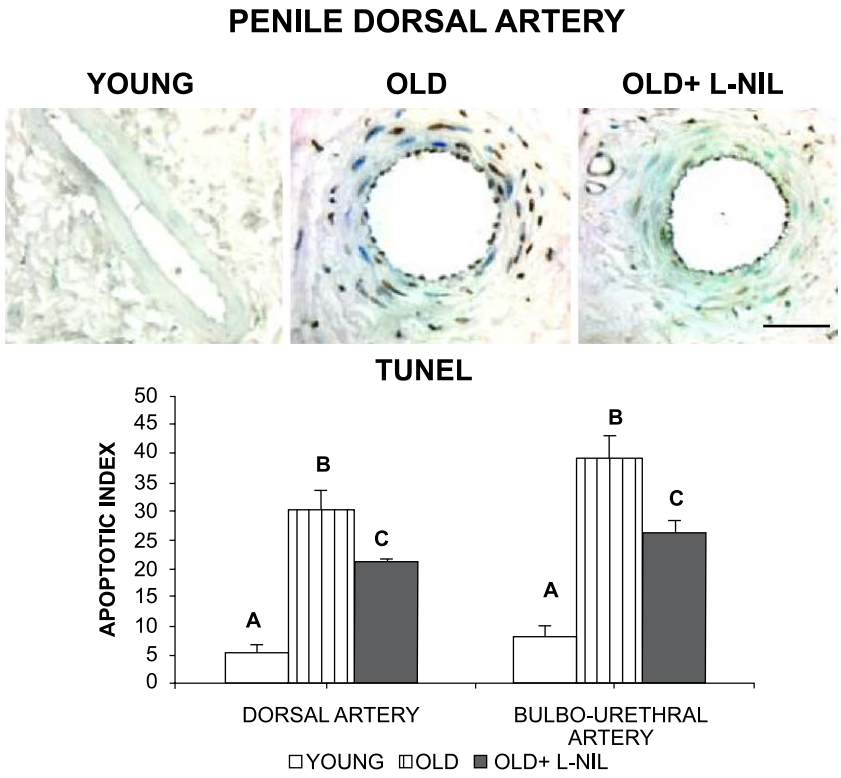

Fig. 5. Reduction by iNOS blockade of the aging-related stimulation of apoptosis in the media of the penile arteries. Sections were immunostained with the TUNEL procedure and counterstained with methyl green. Top: micrographs from selected arteries and tissue sections, as indicated. Bar = 50 μm Bottom: QIA as on previous figures, expressed as apoptotic index (percent number of apoptotic cells/total number of cells), as means ± S.E.M. Dorsal: A vs. B, C, $p < 0.001$; B vs. C, $p < 0.05$; bulbourethral: A vs. C, $p < 0.001$; A vs. $p < 0.05$; B vs. C, $p < 0.01$.

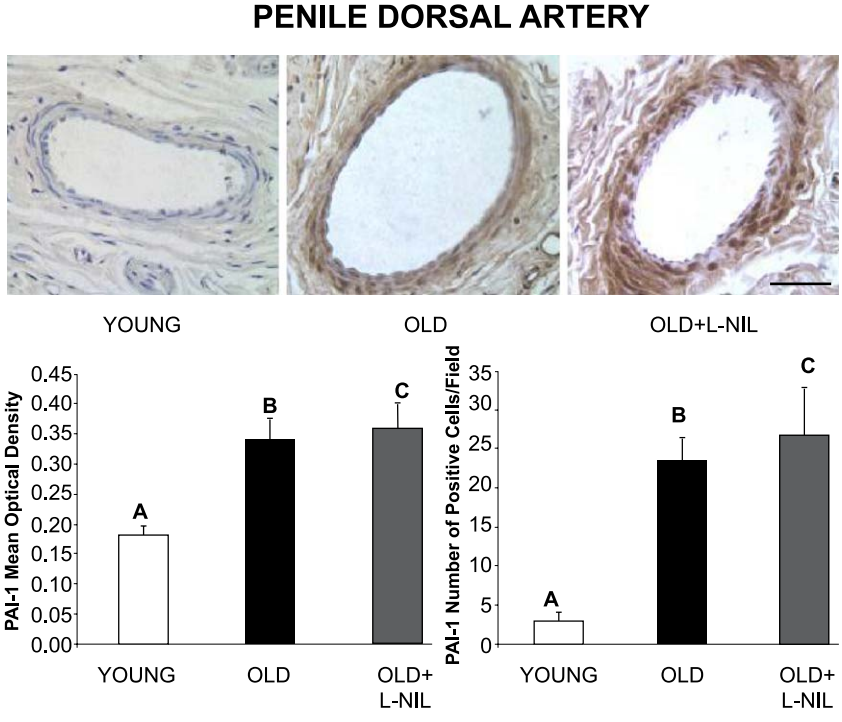

Fig. 6. Intensification by iNOS blockade of the aging-related stimulation of PAI expression in the media of the penile arteries. Sections were immunostained with an antibody against PAI and counterstained with hematoxylin. Top: micrographs from the dorsal artery. Bottom: QIA for both the dorsal penile and bulbourethral arteries, as on previous figures. Bar = 50 μm Values expressed as means ± S.E.M. A vs. B, C, $p < 0.001$.

damage (apoptosis) seen in the resistant vessels, e.g. the penile dorsal artery, in the primary etiology of essential hypertension that is so prevalent with aging.

In the case of the penile arteries, these data also suggest that a reduction in the ability of penile vessels to relax normally during cavernosal nerve stimulation leading to an erection may contribute in part to the high prevalence of ED associated with aging. In addition to this aging-related fibrosis of the arterial media [3–6], it is well documented that similar fibrotic changes occur within the penile corporal sinusoids [37]. The corporal tissue comprises primarily of a syncytium of vascular SMC with an endothelium lining, which is biologically and physiologically indistinguishable from the one present in the media and intima of the vascular tree and may be considered a highly evolved extension of these arterial tissues. Therefore, insults that afflict the arterial media may also afflict the corporal SMCs, resulting in defective vasorelaxation in both the corporal tissue (ED) and the arterial tree (hypertension). Indeed, the prevalence of ED and hypertension in man seems to parallel each other as a function of age [1,2], and many disorders that damage one of these vascular tissues also seem to impact the other, e.g. diabetes, chronic renal failure, etc. In all these disorders, vascular oxidative stress and fibrosis, leading to arteriosclerosis, are common denominators at the histological and molecular and levels.

Our results on the abdominal aorta and the rest of the smaller arteries and arterioles are in agreement with previous studies from other groups showing in the aging rat both an intensification of oxidative stress and collagen deposition [25,26,38,39], that is most likely the cause of the reduction of the SMC/collagen ratio within the media. This alteration, that in the large vessels does not appear to be caused by SMC apoptosis [35,36], would explain the clinical observation in humans of diminished arterial elasticity associated with aging, which in some instances is compounded by a reduction of the arterial lumen due to media/intimal thickening [34]. The fact that different vessels in the arterial tree, regardless of size or location, seem to experience fibrosis of the media may explain dysfunctional vasorelaxation or impaired perfusion of many organs that occurs with aging [3–6,9–12]. The ability of the resistant arteries to relax normally is fundamental for the control of the systemic blood pressure, and as exemplified by the dorsal penile and bulbourethral arteries in this study, they showed an intensification of SMC loss due to apoptosis without a change in IMT, which agrees with has been previously reported for the mesenteric small resistant arteries in hypertension [40].

Although NO has been shown in animal models to be protective against atherosclerosis and restenosis in the vascular system [19–21], and fibrosis throughout the vascular tree and other organs [17–19], the concept that NO may prevent aging-related arteriosclerosis is novel. In fact, the pro-apoptotic action of NO [19,23] would suggest that it decreases the SMC/collagen balance through increased cell death. We have found in the aged animals treated with L-NIL an association between NOS inhibition and subsequent reduction of nitrotyrosine formation, with a decrease of apoptosis, which would suggest that NO does cause some SMC loss in the penile resistant arteries similar to what has been previously assumed to occur in the corpora cavernosa [10]. However, an increased apoptotic index may be balanced by a stimulation of cell replication [5], and what really matters physiologically is the net balance between both processes. In our data, the relative number of SMC in the arterial media (represented by the SMC/collagen ratio), was severely reduced when NO synthesis was diminished by L-NIL. In addition, although endothelial damage was increased with aging as expected in the penile dorsal artery, L-NIL did not increase the apoptotic index, thus suggesting that aging-related iNOS induction does not cause endothelial dysfunction. In fact, it has recently been claimed that iNOS may protect the endothelium in atherosclerosis [41]. This, together with the well known effects of NO in scavenging the profibrotic compound, ROS, thereby decreasing collagen synthesis and down-regulating its breakdown (see Refs. [17,18]), would support the view of an overall beneficial role of NO in preventing arterial stiffness and loss of compliance of the corpora cavernosa.

A final question is whether collagen accumulation with aging is at least partially mediated via the regulation of PAI-1, TIMP1 and other metalloproteinase inhibitors [20,32], that increase in different types of fibrosis. Our current results with PAI, combined with our previous data where we observed considerable metalloproteinase and PAI mRNA expression in the fibrotic plaque of Peyronie's disease [32], would suggest that although the increase in the pro-fibrotic PAI may induce a compensatory elevation of metalloproteinase levels, the enzyme would remain inhibited and the net result would be an impaired collagen breakdown.

In conclusion, our results suggest that within the arterial system and the cavernosal tissue it may be possible to pharmacologically modulate (a) the NO/ROS balance with NO donors or other NO generators together with antioxidants and (b) the PAI/MMP balance with agents modifying their relative expression. Such novel therapies may constitute viable approaches for the prevention and/or therapy of vascular disorders that involve the arterial media and the corpora.

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References

[1] Kloner RA, Speakman M. Erectile dysfunction and atherosclerosis. Curr Atheroscler Rep 2002;4:397–401.

[2] Melman A, Gingell JC. The epidemiology and pathophysiology of erectile dysfunction. J Urol 1999;161:5–11.
[3] Breithaupt-Grogler K, Belz GG. Epidemiology of the arterial stiffness. Pathol Biol (Paris) 1999;47:604–13.
[4] Intengan HD, Schiffrin EL. Structure and mechanical properties of resistance arteries in hypertension: role of adhesion molecules and extracellular matrix determinants. Hypertension 2000;36:312–8.
[5] Intengan HD, Schiffrin EL. Vascular remodeling in hypertension. Roles of apoptosis, inflammation, and fibrosis. Hypertension 2001; 38:581–7.
[6] Fornieri C, Quaglino Jr D, Mori G. Role of the extracellular matrix in age-related modifications of the rat aorta. Ultrastructural, morphometric, and enzymatic evaluations. Arterioscler Thromb 1992;12: 1008–16.
[7] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840–4.
[8] Melman A. Pathophysiologic basis of erectile dysfunction. What can we learn from animal models? Int J Impot Res 2001;13:140–2.
[9] Garban H, Vernet D, Freedman A, Rajfer J, Gonzalez-Cadavid NF. Effect of aging on nitric oxide-mediated penile erection in the rat. Am J Physiol 1995;268:H467–75.
[10] Ferrini M, Magee TR, Vernet D, Rajfer J, Gonzalez-Cadavid NF. Aging-related expression of inducible nitric oxide synthase (iNOS) and markers of tissue damage in the rat penis. Biol Reprod 2001;64: 974–82.
[11] Rogers RS, Graziottin TM, Lin C-S, Kan YW, Lue TF. Intracavernosal vascular endothelial growth factor (VEGF) injection and adeno-associated virus -mediated VEGF gene therapy prevent and reverse venogenic erectile dysfunction in rats. Int J Impot Res 2003;15:26–37.
[12] Zalba G, Beaumont J, San Jose G, Fortuno A, Fortuno MA, Diez J. Vascular oxidant stress: molecular mechanisms and pathophysiological implications. J Physiol Biochem 2000;56:57–64.
[13] Jones RW, Rees RW, Minhas S, Ralph D, Persad RA, Jeremy JY. Oxygen free radicals and the penis. Expert Opin Pharmacother 2002;3:889–97.
[14] Bivalacqua TJ, Armstrong JS, Biggerstaff J, et al. Gene transfer of extracellular SOD to the penis reduces O2-* and improves erectile function in aged rats. Am J Physiol Heart Circ Physiol 2003;284: H1408–21.
[15] Kitamoto S, Egashira K, Kataoka C, et al. Chronic inhibition of nitric oxide synthesis in rats increases aortic superoxide anion production via the action of angiotensin II. J Hypertens 2000;18:1795–800.
[16] Gonzalez W, Fontaine V, Pueyo ME, et al. Molecular plasticity of vascular wall during *N*(G)-nitro-L-arginine methylester-induced hypertension: modulation of proinflammatory signals. Hypertension 2000;36:103–9.
[17] Ferrini MG, Vernet D, Magee TR, et al. Antifibrotic role of inducible nitric oxide synthase (iNOS). Nitric Oxide 2002;6:1–12.
[18] Vernet D, Ferrini MG, Valente E, et al. Effect of nitric oxide on fibroblast differentiation into myofibroblasts in cell cultures from the Peyronie's fibrotic plaque and in its rat model in vivo. Nitric Oxide 2002;7:262–76.
[19] Gewaltig MT, Kojda G. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. Cardiovasc Res 2002;55:250–60.
[20] Kaikita K, Schoenhard JA, Painter CA, et al. Potential roles of plasminogen activator system in coronary vascular remodeling induced by long-term nitric oxide synthase inhibition. J Mol Cell Cardiol 2002; 34:617–27.
[21] Cheng JW, Baldwin SN, Balwin SN. L-Arginine in the management of cardiovascular diseases. Ann Pharmacother 2001;35:755–64.
[22] González-Cadavid NF, Ignarro L, Rajfer J. Nitric oxide and cyclic GMP in the penis. Mol Urol 1999;3:51–9.
[23] Kibbe M, Billiar T, Tzeng E. Inducible nitric oxide synthase and vascular injury. Cardiovasc Res 1999;43:650–7.
[24] Ferrini M, Wang C, Swerdloff R, Sinha Hikim AP, Gonzalez-Cadavid NF. Aging-related expression of inducible nitric oxide synthase (iNOS) and cytotoxicity markers in rat hypothalamic regions associated with male reproductive function. Neuroendocrinology 2001;74:1–11.
[25] Goettsch W, Lattmann T, Amann K, et al. Increased expression of endothelin-1 and inducible nitric oxide synthase isoform II in aging arteries in vivo: implications for atherosclerosis. Biochem Biophys Res Commun 2001;280:908–13.
[26] Chou TC, Yen MH, Li CY, Ding YA. Alterations of nitric oxide synthase expression with aging and hypertension in rats. Hypertension 1998;31:643–8.
[27] Cernadas MR, Sanchez de Miguel L, Garcia-Duran M, et al. Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. Circ Res 1998;83:279–86.
[28] Lee PC, Shears II LL , Billiar TR. Role of inducible nitric oxide synthase in transplant arteriosclerosis. Clin Exp Pharmacol Physiol 1999;26:1013–5.
[29] Niu XL, Yang X, Hoshiai K, et al. Inducible nitric oxide synthase deficiency does not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions. Circulation 2001;103: 1115–20.
[30] Behr-Roussel D, Rupin A, Simonet S, et al. Effect of chronic treatment with the inducible nitric oxide synthase inhibitor *N*-iminoethyl-L-lysine or with L-arginine on progression of coronary and aortic atherosclerosis in hypercholesterolemic rabbits. Circulation 2000;102: 1033–8.
[31] Hochberg D, Johnson CW, Chen J, et al. Interstitial fibrosis of unilateral ureteral obstruction is exacerbated in kidneys of mice lacking the gene for inducible nitric oxide synthase. Lab Invest 2000;80: 1721–8.
[32] Davila HH, Ferrini MG, Rajfer J, Gonzalez-Cadavid NF. Fibrin induction of a Peyronie's-like plaque in the rat penile tunica albuginea. A new model for Peyronie's disease. BJU Int 2003;91:830–8.
[33] Martin W, McAllister KH, Paisley K. NANC neurotransmission in the bovine retractor penis muscle is blocked by superoxide anion following inhibition of superoxide dismutase with diethyldithio carbamate. Neuropharmacology 1994;33:1293–301.
[34] Moore MA, Schiffrin EL. Consortium for Southeastern hypertension control. Small artery remodeling in hypertension: can it be corrected? Am J Med Sci 2001;322:7–11.
[35] Connat JL, Busseuil D, Gambert S, et al. Modification of the rat aortic wall during ageing; possible relation with decrease of peptidergic innervation. Anat Embryol 2001;204:455–68.
[36] Asai K, Kudej RK, Shen YT, et al. Peripheral vascular endothelial dysfunction and apoptosis in old monkeys. Arterioscler Thromb Vasc Biol 2000;20:1493–9.
[37] Grein U, Schubert GE. Arteriosclerosis of penile arteries: histological findings and their significance in the treatment of erectile dysfunction. Urol Int 2002;68:261–4.
[38] Demaree SR, Lawler JM, Linehan J, Delp MD. Ageing alters aortic antioxidant enzyme activities in Fischer-344 rats. Acta Physiol Scand 1999;166:203–8.
[39] Csiszar A, Ungvari Z, Edwards JG, et al. Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function. Circ Res 2002;90:1159–66.
[40] Rizzoni D, Rodella L, Porteri E, et al. Time course of apoptosis in small resistance arteries of spontaneously hypertensive rats. J Hypertens 2000;18:885–91.
[41] Hemmrich K, Suschek CV, Lerzynsli G, Kolb-Bachoven B. iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage. J Appl Physiol 2003;95:1937–46.