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TNF- α knockout mice have increased corpora cavernosa relaxation

Abstract

Introduction—Erectile dysfunction (ED) is considered an early clinical manifestation of vascular disease and an independent risk factor for cardiovascular events associated with endothelial dysfunction and increased levels of pro-inflammatory cytokines. Tumor necrosis factor-alpha (TNF- α), a pro-inflammatory cytokine, suppresses endothelial nitric oxide synthase (eNOS) expression.

Aim—Considering that nitric oxide (NO) is of critical importance in penile erection, we hypothesized that blockade of TNF- α actions would increase cavernosal smooth muscle relaxation through an increase in NOS expression.

Methods—*In vitro* organ bath studies were used to measure cavernosal reactivity in wild type and TNF- α knockout (TNF- α KO) mice and NOS expression was evaluated by western blot. In addition, spontaneous erections (*in vivo*) were evaluated by videomonitoring the animals (30 min.). Collagen and elastin expression were evaluated by Masson trichrome and Verhoeff-van Gieson stain reaction, respectively.

Main Outcome Measures—Corpora cavernosa from TNF- α KO mice exhibited increased NO-dependent relaxation, which was associated with increased eNOS and neuronal NOS (nNOS) cavernosal expression.

Results—Cavernosal strips from TNF- α KO mice displayed increased endothelium-dependent [97.4 ± 5.3 vs Control: 76.3 ± 6.3 , %] and nonadrenergic-noncholinergic (NANC) [93.3 ± 3.0 vs Control: 67.5 ± 16.0 ; 16 Hz] relaxation compared to control animals. These responses were associated with increased protein expression of eNOS and nNOS ($p < 0.05$). Sympathetic-mediated [0.69 ± 0.16 vs Control: 1.22 ± 0.22 ; 16 Hz] as well as phenylephrine-induced contractile responses [1.6 ± 0.1 vs Control: 2.5 ± 0.1 , mN] were attenuated in cavernosal strips from TNF- α KO mice. Additionally, corpora cavernosa from TNF- α KO mice displayed increased collagen and elastin expression. *In vivo* experiments demonstrated that TNF- α KO mice display increased number of spontaneous erections.

Conclusion—Corpora cavernosa from TNF- α KO mice display alterations that favor penile tumescence, indicating that TNF- α plays a detrimental role in erectile function. A key role for TNF- α in mediating endothelial dysfunction in ED is markedly relevant since we now have access to anti-TNF- α therapies.

Keywords

tumor necrosis factor alpha; corpus cavernosum; endothelial nitric oxide synthase; neuronal nitric oxide synthase; mouse

INTRODUCTION

Erectile dysfunction (ED), defined as the consistent inability to achieve and maintain penile erection sufficient for adequate sexual relations, is a prevalent condition affecting more than 150 million men worldwide. ED, which is considered the early clinical manifestation of generalized vascular disease and an independent risk factor for cardiovascular events, is

associated with endothelial dysfunction and increased levels of pro-inflammatory cytokines¹⁻³.

Tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine originally defined by its antitumoral activity, is an important contributor to many cardiovascular diseases, such as myocardial ischemia-reperfusion injury, chronic heart failure, atherosclerosis, and sepsis-associated cardiovascular disorders⁴⁻⁶. The vascular endothelium is a major target for the actions of TNF- α in these diseases, in which TNF- α plasma levels are significantly elevated. Administration of TNF- α *in vivo* induces impairment of endothelium-dependent vasorelaxation in a variety of vascular beds and decreases the release of nitric oxide (NO)⁷. TNF- α has the ability to increase arterial reactive oxygen species (ROS) generation, which likely accounts for some of the reduction in NO levels. In addition, TNF- α suppresses endothelial NO synthase (eNOS) expression by inhibiting gene promoter activity^{8, 9}, predominantly through destabilization of eNOS mRNA in endothelial cells¹⁰.

While penile erection is a complex neurovascular process where nerves, endothelium of sinusoids and blood vessels, and smooth muscle cells produce transmitters and modulators that control the erect versus flaccid state of the penis, it is evident that the NO pathway is of critical physiological importance in the induction and maintenance of erections¹¹. The constitutive eNOS and neuronal NO synthase (nNOS) isoforms are tightly regulated and produce physiologically relevant levels of NO in endothelial cells and autonomic nerve endings of the penis, respectively. Recent advances in the field of penile vascular biology indicate that the regulation of eNOS in the penis involves multiple molecular mechanisms that act in concert to both positively and negatively affect the function of this enzyme¹¹.

Based on the above-mentioned observations and considering recent evidence that TNF- α levels are increased in serum of patients with moderate-to-severe ED^{12, 13}, we hypothesized that TNF- α KO mice have increased cavernosal smooth muscle relaxation through an increase in NOS expression. To test our hypothesis we have used various pharmacological tools to evaluate relaxant and contractile responses of cavernosal segments from wild-type (control) and TNF- α KO mice. In addition, corporal NOS expression, elastin and collagen deposition, as well as number of spontaneous erections were determined in these animals.

METHODS

Animals

Male control (B6; 129SF2/J) and TNF- α KO (B6;129S6-*Tnf^{tm1Gkl}*/J) (16 weeks-old, from Jackson Laboratories, Bar Harbor, ME) were used in these studies. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed four per cage on a 12-h light/dark cycle and fed a standard chow diet with water *ad libitum*.

Arterial blood pressure measurement by telemetry

Surgery was performed on control and TNF- α KO mice to implant blood pressure radiotelemetry transmitters (Data Sciences PA-C20, International, Roseville, MN). Briefly, after isoflurane anesthesia, the transmitter was implanted into the carotid artery via a mid-ventral neck incision under aseptic conditions, and the transmitter body was routed to a subcutaneous pocket in the midback region. The incision was infiltrated with bupivacaine and closed with sterile 6-0 Ethicon Ophthalmic suture. Following recovery, the mouse was housed in an individual cage in the laboratory animal facilities and provided with standard laboratory chow and water *ad libitum*.

Functional Studies in Cavernosal Strips

After carbon dioxide (CO₂) euthanasia, the penis was excised, transferred into ice-cold buffer, and dissected to remove the tunica albuginea. One crural strip preparation (1×1×10 mm) was obtained from each corpus cavernosum (two crural strips from each penis). Cavernosal strips were mounted in 4-mL myograph chambers (Danish Myo Technology, Aarhus, Denmark) containing physiological salt solution (PSS) at 37°C, pH 7.4, continuously bubbled with a mixture of 95% O₂ and 5% CO₂. The tissues were stretched to a resting force of 2.5 mN and allowed to equilibrate for 60 min. Changes in isometric force were recorded using a PowerLab/8SP data acquisition system (Chart software, version 5.0; ADInstruments, Colorado Springs, CO). To verify the contractile activity of the preparations, a high potassium chloride (KCl) solution (120 mM) was added to the organ baths at the end of the equilibration period. Cumulative concentration-response curves to acetylcholine (ACh; endothelium-dependent vasodilator; 10⁻⁹ to 3×10⁻⁶ M), sodium nitroprusside (SNP; NO donor; 10⁻⁸ to 10⁻³ M), isoproterenol (or isoprenaline, beta-adrenergic receptor agonist; 10⁻¹⁰ to 10⁻⁵ M), tadalafil [phosphodiesterase-5 (PDE-5) inhibitor; 10⁻¹⁰ to 10⁻⁴ M] and Y-27632 (Rho-kinase inhibitor; 10⁻⁹ to 10⁻⁴ M) were obtained in cavernosal strips contracted with phenylephrine (PE; 10⁻⁵ M). Tadalafil was chosen to cause relaxation because it does not affect Ca²⁺ handling, in addition to increasing cGMP levels through inhibition of PDE-5, as vardenafil does¹⁴. Cavernosum contractility was evaluated with cumulative concentration-response curves to PE (10⁻⁹ to 3×10⁻⁵ M) in the absence and presence of Nω-nitro-L-arginine methyl ester (L-NAME) (10⁻⁴ M). Electrical field stimulation (EFS) was applied to strips placed between platinum pin electrodes attached to a stimulus splitter unit (Stimu-Splitter II), which was connected to a Grass S88 stimulator (Astro-Med West Warwick, RI), and EFS was conducted at 20 V, 1-ms pulse width and trains of stimuli lasting 10 s at varying frequencies (1 to 32 Hz). To evaluate sympathetic nerve-mediated responses, cavernosal strips were stimulated on basal tone and after incubation for 45 min with L-NAME (10⁻⁴ M) plus atropine (10⁻⁶ M). To evaluate nonadrenergic-noncholinergic (NANC)-mediated relaxation, strips were incubated for 45 min with bretylium (3×10⁻⁵ M) plus atropine (10⁻⁶ M) (to eliminate noradrenergic and cholinergic nerves-mediated responses, respectively), contracted with PE (10⁻⁵ M) and frequency-response curves were performed.

Spontaneous erections measurements

In order to verify the number of erections displayed by control and TNF-α KO animals, studies were performed as previously described¹⁵. Briefly, mice were habituated to the testing room and test chamber during one week. The chamber was a glass (25 × 20 × 20 cm) with a video recorder underneath it to facilitate penis visualization. Two males, each in a separate test chamber without visual or physical contact, were usually observed at the same time. Observations were commenced after placing the male in the chamber and images were captured for 30 minutes. We reliably observed, distinguished and separately counted erection when the male stood on its hindlimbs, bent its body forward, bent its head down to reach the genital area, licked and held the penis with its forepaws and displayed hip movements. The erect penis was always visible, but often only briefly. Erection-like responses in mice, (when the male stood on its hindlimbs, bent its body forward, bent its head down, licked the abdomen but not the penis, and displayed hip movements), as described by Rampin and co-workers¹⁵ were also observed. Two criteria therefore distinguished erections from erection-like responses: in the case of erection, the male licked and held the penis. In the present study only erections responses were scored.

Histological and immunohistochemistry analysis of corpora cavernosa from control and TNF- α KO mice

Tissues were fixed in methacarn (60% methanol; 30% chloroform; 10% acetic acid) for 3 h at 4 °C. After washing, tissues were dehydrated and embedded in paraffin (Oxford, St. Louis, MO, USA) at 60 °C. Transversal sections (5- μ m thick) of penis from control and TNF- α KO mice were cut to obtain the midline plane of corpus cavernosum. Samples were then stained with Masson trichrome (Sigma Diagnostics, St. Louis, MO, USA). This identifies collagen as blue and smooth muscle as red. Verhoeff-van Gieson staining reaction was used to establish elastin expression. The corpora cavernosa of each penis were viewed using a Zeiss Axioskop 2 microscope from 2x to 40x objectives. Images of this region were captured using a digital camera Canon Power Shot G5 (Canon USA, Lake Success, NY, USA) and KS 100 3.0 software (Carl Zeiss). The entire area of corpus cavernosum, vascular spaces plus smooth muscle and collagen were measured using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). For collagen/smooth muscle staining, the ratio of the area stained for collagen (blue) to the total area of the lacunar spaces plus smooth muscle (white and red) was used. Other serial sections were stained immunohistochemically using standard protocols. Briefly, sections were treated with primary mouse monoclonal anti- α -actin antibody (1: 200 dilution) (Sigma, following manufacturer's instructions) and incubated overnight at 4° C. Then, sections were washed twice in phosphate buffer saline (PBS) and incubated with the secondary antibody, a biotin-conjugated immunoglobulin G (IgG) antibody, for 30 minutes. Bound antibodies were detected with streptavidin-peroxidase complex using 0.2 mg/ml 3, 3'-diaminobenzidine tetrahydrochloride in PBS containing 0.003% hydrogen peroxide. Sections were counterstained with haematoxylin. The number of positive cells were determined and expressed as the number of positive cells per field at 20x magnification. At least three fields per slide were analyzed and the mean value was used. Negative sections were incubated only with secondary antibody. In all cases, three sections per tissue specimen from groups of 4 animals were used to calculate means $\pm$ SEM.

eNOS and nNOS protein expression

Cavernosal tissues were dissected and homogenized in protease inhibitor cocktail (Sigma) using a small tissue homogenizer. The Bradford assay was used to measure protein concentration. A total of 40 μ g of protein was loaded onto an SDS-PAGE 10% gel and transferred to a nitrocellulose membrane. Membranes were then probed with rabbit polyclonal anti-eNOS antibody (1:500; Cell signaling) or mouse monoclonal anti-nNOS (1:2000; BD Transduction Laboratories). The immunostaining was detected using horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; GE Healthcare) and anti-mouse IgG (1:2000; GE Healthcare) for 1 hour at room temperature. Bands were revealed by the SuperSignal Substrate (Pierce Biotechnology) and quantified by densitometry using a Scion Image analyzer software (Scion Image Corporation).

Drugs and Solutions

Physiological salt solution of the following composition was used: 130 mM NaCl, 14.9 mM NaHCO₃, 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄·7H₂O, 1.6 mM CaCl₂·2H₂O, and 0.026 mM EDTA. Rabbit polyclonal anti-eNOS antibody was purchased from Cell signaling and mouse monoclonal anti-nNOS from BD Transduction Laboratories. The kit biotin-streptavidin system used for immunohistochemistry studies was obtained from Vector Laboratories. Acetylcholine chloride, atropine, isoproterenol, sodium nitroprusside, N ω -nitro-L-arginine methyl ester (L-NAME), phenylephrine hydrochloride, Y-27632 dihydrochloride [(R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride] were purchased from Sigma Chemical Co. (St. Louis, MO). Tadalafil (Cialis, tablets containing 20 mg of tadalafil) and bupivacaine (Marcaine, 0.5% without epinephrine)

were obtained from CVS (Augusta, GA). 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

Contractions were recorded as changes in the displacement (mN) from baseline and are represented as mN for n experiments. Relaxation was expressed as percentage change from the PE contracted levels. Agonist concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 4.0; GraphPad Software Inc., San Diego, CA, USA). Agonist potencies and maximum response are expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximum response) and Emax (maximum effect elicited by the agonist), respectively. Constraining curve-fit parameters were used to fit a sigmoidal curve and to determine pD_2 values for the drugs tadalafil and Y-27632. Statistically significant differences were calculated by one-way analysis of variance (ANOVA) plus Bonferroni's post hoc test or Student's t -test. $P < 0.05$ was considered as statistically significant.

RESULTS

The body weight of the $\text{TNF-}\alpha$ KO mice used in these studies was statistically lower (28.0 ± 0.8 ; $n=22$) compared to the control group (31.3 ± 0.6 , $n=22$). However, no differences were observed in the mean arterial blood pressure [(mmHg) control, 102 ± 3 , $n=12$ vs. $\text{TNF-}\alpha$ KO, 98 ± 2 , $n=12$] or in the ratio of organ weight to body weight [(heart, kidney, penis)/body weight (not shown)] between the groups.

Functional responses of corpora cavernosa

The addition of PE (10^{-5} M) to the bathing medium caused a submaximal contraction of mouse cavernosal segments, which consisted of a rapid rise in force followed by a slower rise to a sustained level within 10 min. The cumulative addition of ACh (10^{-9} to 10^{-6} M) produced concentration-dependent relaxations of PE-contracted tissues in both groups (Figure 1A). Although there was no difference in pD_2 value between the groups, the maximal response elicited by ACh in cavernosal strips from $\text{TNF-}\alpha$ KO mice ($97.4 \pm 5.3\%$) were significantly augmented ($p < 0.05$) in comparison to that elicited in strips from control mice ($76.3 \pm 6.3\%$), (Figure 1B).

Whereas eNOS activation seems to account for ACh-induced relaxation, NANC-induced relaxation of cavernosal segments is mediated via NO from nNOS. Accordingly, whereas ACh-induced relaxation is completely blocked in the presence of 10^{-6} M atropine (ACh: 76 ± 6 vs atropine: 8 ± 2 ; % of relaxation) or 10^{-4} M L-NAME (L-NAME: 4 ± 1 ; % of relaxation), relaxation induced by EFS is minimally altered in the presence of atropine (vehicle: 66 ± 5 vs atropine: 70 ± 4 ; % of relaxation at 32 Hz), but it is completely blocked by incubation with L-NAME 10^{-4} M (L-NAME: 1 ± 0.5 ; % of relaxation at 32 Hz). To evaluate the effects of NANC nerve stimulation, cavernosal smooth muscle strips were incubated with bretylium tosylate (3×10^{-5} M; sympathetic nerve blocking agent) and atropine 10^{-6} M. After 45 min of incubation, strips were contracted with PE (10^{-5} M) and frequency-dependent responses to EFS (1–32 Hz) were evaluated. EFS-induced relaxations were significantly augmented in cavernosal segments from $\text{TNF-}\alpha$ KO mice in comparison to strips from control mice (Figure 1C).

On the other hand, no differences were observed in the relaxant responses induced by sodium nitroprusside (NO donor), isoproterenol (beta adrenergic receptor agonist), tadalafil (PDE-5

inhibitor) or Y-27632 (Rho kinase inhibitor) between the groups (Figure 2). Emax and pD₂ values for the agonists are shown at Table 1.

In order to determine the presence of changes in contractile responses of cavernosal strips from TNF- α KO mice, frequency-dependent EFS curves and concentration-response curves to PE (10^{-9} to 10^{-4} M) were performed. In cavernosal smooth muscle strips under resting conditions and after incubation with atropine 10^{-6} M plus L-NAME 10^{-4} M, EFS (1–32 Hz) produced decreased frequency-dependent contractions in the TNF- α KO group compared to control (Figure 3A). These responses were completely abolished by the sympathetic nerve blocking agent bretylium tosylate (3×10^{-5} μ M; n=4) or by the alpha-adrenergic antagonist terazosin (10^{-6} M; n= 4) (vehicle: 2.4 ± 0.3 vs bretylium tosylate: 0.1 ± 0.1 vs terazosin: 0.1 ± 0.2 ; mN contraction at 32 Hz), confirming that these responses are neuronal in origin and adrenergic in nature and that there was not direct cavernosal smooth muscle activation by EFS.

Cavernosal segments from TNF- α KO mice also exhibited decreased responses to PE, both in the absence and in the presence of L-NAME 10^{-4} M, compared to strips from control mice (Figures 3B, 3C). Nevertheless, pD₂ values for PE were increased in cavernosal strips from TNF- α KO mice compared to control group (Table 1).

Corporal NOS isoforms expression and collagen and elastin content

To address if increased relaxant responses to ACh and NANC nerve stimulation are associated with increased expression of NOS isoforms, expression of eNOS and nNOS was evaluated by Western blot in corpora cavernosa from control and TNF- α KO mice. As shown at Figure 4, increased eNOS and nNOS expression was detected in tissues from TNF- α KO animals.

We further evaluated whether structural changes would occur in corpora cavernosa from TNF- α KO animals. Histological analysis showed that cavernosal tissue from TNF- α KO mice displays increased collagen/smooth muscle and elastin/smooth muscle ratio (Figures 5A and 5B, respectively). The number of corporal smooth muscle cells was not different between the groups (Control: 146 ± 11 vs TNF KO: 124 ± 17 ; number of positive cells at 20x magnification).

Spontaneous erections

In order to verify if the increased relaxation and decreased contractile responses observed in corpora cavernosa from TNF- α KO mice *in vitro* are translated into facilitated erectile responses in the intact animal, we determined the number of spontaneous erections displayed by these animals during 30 min intervals. TNF- α KO animals displayed an increased number of spontaneous erections compared to the control group (Figure 6).

DISCUSSION

The present study shows that isolated corpora cavernosa from TNF- α KO mice display increased endothelium-dependent relaxation and altered neural-mediated responses that would favor penile tumescence to occur: decreased contractile response to adrenergic nerve stimulation and increased relaxant responses upon activation of NANC nerves. This study also shows that the expression of key enzymes involved in the erectile responses, such as eNOS and nNOS, are increased in cavernosal strips from TNF- α KO mice.

In the first set of experiments, we observed that cavernosal strips from TNF- α KO mice display increased endothelium-dependent and NANC relaxation compared to control animals. In order to verify if the increased relaxation was a specific phenomenon, concentration-response curves were performed to different relaxant compounds, such as, sodium nitroprusside, isoproterenol, tadalafil and Y-27632. However, no differences were observed between responses from cavernosal strips from control and TNF- α KO mice, suggesting specific functional changes in

endothelium-dependent and NANC-induced relaxation. Our results are consistent with previous studies showing that administration of TNF- α *in vivo* induces impairment of endothelium-dependent vasorelaxation and decreases the release of NO, not only in animals, but also in humans patients ⁷. The constitutive eNOS and nNOS are tightly regulated and produce physiologically relevant levels of NO in endothelial cells and autonomic nerve endings of the penis, respectively ¹¹. Despite its ability to induce the expression of inducible NO synthase (iNOS) and increase NO production by the endothelium, *in vitro* administration of TNF- α inhibits endothelium-dependent relaxation in cat carotid and rat pulmonary arteries, consistent with TNF- α -induced impairment of NO production by the endothelium. Accordingly, it has been demonstrated that TNF- α suppresses eNOS expression by inhibiting gene promoter activity ^{9, 10}, predominantly through destabilization of eNOS mRNA in endothelial cells ¹⁰. In fact, these studies corroborate with our present finding that cavernosal strips from TNF- α KO animals show increased expression of eNOS.

Vlachopoulos and collaborators ¹² have demonstrated the association between low grade inflammation and altered endothelial-prothrombotic state on one side and ED on the other, in patients with and without coronary artery disease (CAD) by analyzing a wide spectrum of circulating markers and mediators. According to their findings, the level of TNF- α and endothelial-prothrombotic parameters are inversely associated with sexual performance. Additionally, this study corroborates recent findings that endothelial dysfunction in ED is a systemic arterial defect not only confined to the penile vasculature, and offers a humoral basis for this association. In fact, several lines of evidence suggest that the pro-inflammatory cytokine TNF- α plays a key role in inducing endothelial dysfunction. First, in clinically stable heart transplant patients a strong positive relation between plasma TNF- α and the vascular response to acetylcholine has been documented ¹⁶. Second, Baghat and Vallance ¹⁷ have shown that whereas infusion of TNF- α alone impaired endothelial function in health subjects, infusion of IL-6 did not. Finally, a key role for TNF- α in mediating endothelial dysfunction is of interest not only because markedly elevated serum levels of TNF- α have been documented in patients with ED, but also because we now have access to targeted anti-TNF- α therapies, such as etanercept.

While eNOS is mainly involved in endothelium-dependent relaxation, nNOS is considered the major mediator of NANC relaxation in the penis ¹¹. Although there is no report about TNF- α actions on nNOS expression in corpora cavernosa, it has been demonstrated that TNF- α infusion significantly induced an increase in MAP and reduction in renal nNOS protein expression, within both the cortex and medulla from pregnant rats ¹⁸. In addition, these animals also showed reduced endothelium-dependent relaxation. In accordance with our results, and further supporting increased NANC-mediated relaxation, penes from TNF- α KO mice were shown to have increased nNOS expression. Taken together, these findings suggest that TNF- α impairs cavernosum relaxation not only by decreasing eNOS expression on endothelial cells, but also by negatively modulating nNOS present in nerve terminals in the penis.

Traditionally, ED is thought of as an outcome of occlusive systemic vascular disease that occurs as a late consequence of atherosclerosis. Current and emerging clinical research studies are showing that the penile vascular bed may be a sensitive indicator of early systemic endothelial cell and/or smooth muscle dysfunction. Numerous clinical epidemiology studies have demonstrated that ED is highly prevalent in men with vascular risk factors for diabetes ^{19, 20}, hypertension ²¹, dyslipidemia ²², cigarette smoking, obesity, and sedentary lifestyles ²³⁻²⁵. These conditions are all associated with an increased incidence of ED and increased levels of cytokines. Recently, Zhang and co-workers ²⁶ have demonstrated a substantial proatherogenic role for TNF- α . Furthermore, the presence of tumor necrosis factor receptor-1 (TNFR1) in the vascular wall causes atherosclerosis independently of macrophage infiltration ²⁶.

Excessive deposition of extracellular matrix components produced by the synthetic phenotype of smooth muscle cells in the intima is postulated to be one of the most important pathological changes in atherosclerosis. In the present study, it was demonstrated that corpora cavernosa from TNF- α KO mice have increased collagen expression. In addition, this result is further supported by Hiraga and co-workers²⁷, which have shown that TNF- α decreases the synthesis of collagen in smooth muscle cells, especially collagen types IV and V. Although smooth muscle cells are postulated to be one of the cell types in which collagen synthesis is strongly suppressed by TNF- α ²⁷, indirect effects produced by TNF- α or compensatory mechanisms brought on by TNF- α knock out cannot be ruled out. Considering that changes of elastic fibers or collagen types can alter mechanical properties of the penis, the increased collagen content in TNF- α KO corpora cavernosa could indicate a fibrotic state with reduced elasticity and compliance, finally leading to the impairment of erectile responses. However, collagen types I, III and IV are the predominant isoforms in the corporal tissue. Type I collagen, which forms stiff bands of fibrils, has been shown to be less compliant than type III collagen, which is found predominantly in distensible elastic tissue and is essential for normal tensile strength²⁸. Based on that, we speculate that the discrepancy observed may be due to changes in the ratio of collagen I/collagen III, which would diminish the impact caused by increased collagen expression on cavernosal contractility. Furthermore, cavernosal tissue of TNF- α KO mice displayed increased elastin content, which may contribute to increased cavernosal elasticity. In addition, elastin expression has been demonstrated to be decreased in patients with erectile dysfunction²⁹. Finally, cavernosal responses *in vitro* indicating that TNF- α KO mice have facilitated penile tumescence, were confirmed by the *in vivo* experiments. Taken together, our results present the novelty that TNF- α can negatively modulate erectile responses, at physiological conditions, partially via inhibition of NOS expression. Thus, deleterious actions of TNF- α in cavernosal function, may be particularly important in conditions where its levels are increased. We are currently addressing whether TNF- α contributes to hypertensive or diabetes-associated ED.

In summary, we have shown that TNF- α KO mice exhibit changes in cavernosal reactivity that would facilitate erectile responses: decreased responses to adrenergic nerve stimulation and increased NANC and endothelium-dependent relaxation that are associated with increased corporal eNOS and nNOS protein levels. A key role for TNF- α in mediating endothelial dysfunction in ED is markedly relevant since we now have access to anti-TNF- α therapies. Blockade of TNF- α actions may represent an alternative therapeutic approach for erectile dysfunction, especially in pathological conditions associated with increased levels of this cytokine.

Acknowledgments

ABBREVIATIONS

ACh	acetylcholine
ANOVA	one-way analysis of variance
CO ₂	carbon dioxide
ED	erectile dysfunction
EFS	electrical field stimulation
E _{max}	maximum effect elicited by the agonist
eNOS	endothelial nitric oxide synthase
IgG	immunoglobulin G

KCl	potassium chloride
L-NAME	N ω -nitro-L-arginine methyl ester
MAP	mean arterial pressure
mN	millinewton
NANC	nonadrenergic-noncholinergic
nNOS	neuronal NO synthase
NO	nitric oxide
PBS	phosphate buffer saline
pD ₂	negative logarithm of the molar concentration of agonist producing 50% of the maximum response
PDE-5	phosphodiesterase-5
PE	phenylephrine
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF- α KO	TNF- α null mice
TNF- α	tumor necrosis factor-alpha
Y-27632	R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride; Rho-kinase inhibitor

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Figure 1. Endothelium-dependent relaxation and NANC nerves stimulation in cavernosal segments from control and TNF- α KO mice

(A) Representative traces showing ACh-induced relaxation in cavernosal segments from control (top) and TNF- α KO (bottom) mice; (B) acetylcholine concentration-effect curves and (C) frequency-response curves elicited by EFS (1-32 Hz) were performed in cavernosal strips, contracted with PE (10 $^{-5}$ M), from control (●) and TNF- α KO (■) mice. Experimental values of the relaxations induced by EFS (n=4 and 6, respectively), and acetylcholine (n=5 in each group) were calculated relatively to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Data represent the mean $\pm$ SEM of *n* experiments. *, P<0.05 vs. control (WT).

Figure 2. Relaxation responses in cavernosal segments from control and TNF- α KO mice
Effects of (A) sodium nitroprusside, (B) isoproterenol, (C) tadalafil and (D) Y-27632 were evaluated in cavernosal strips, contracted with PE (10^{-5} M), from control (●) and TNF- α KO (■) mice. Experimental values of the relaxations induced by sodium nitroprusside (n=5 in each group), isoproterenol (n=4 and 6, respectively), tadalafil (n=5 in each group) and Y-27632 (n=5 in each group) were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Data represent the mean $\pm$ SEM of n experiments.

Figure 3. Contractile responses upon stimulation of adrenergic nerves or α_1 -adrenergic receptor in cavernosal segments from control and TNF- α KO mice

(A) Frequency-response curves elicited by EFS (1-32 Hz) were performed in the presence of L-NAME 10^{-4} M plus atropine 10^{-6} M (n=10 and 8, respectively). Phenylephrine concentration-response curves were performed in the absence (B) or presence (C) of L-NAME 10^{-4} M (n=5 in all groups). Experimental values of contraction of cavernosal strips from control (●) and TNF- α KO (■) mice are in mN and data represent the mean $\pm$ SEM of n experiments. *, $p < 0.05$ compared with values from control segments.

Figure 4. Protein NOS expression in corpora cavernosa from control and TNF- α KO mice
 Representative immunoblots for eNOS (A) and nNOS (B) (top panel). Corresponding bar graphs demonstrating increased NOS expression in the corporal tissue from TNF- α KO animals (bottom panel). Results are mean $\pm$ SEM of n=5-6 experiments. * p <0.05, TNF- α vs. control (WT).

Figure 5. Bar graphs represent collagen/smooth muscle ratio (A) and elastin/smooth muscle ratio (B)

Overall view of the corpus cavernosum, corpus spongiosum (CS), urethra (U), dorsal artery (DA), and central artery (CA) in penes from control (A) and $\text{TNF-}\alpha$ KO (B) mice (upper panel). Observe in corporal sections from $\text{TNF-}\alpha$ KO increased collagen and elastin deposition (dashed arrows). Original magnification 4 $\times$. * $p < 0.05$ vs control.

Figure 6. Spontaneous erections

Bar graphs demonstrating the number of erections in control and TNF- α KO animals during 30 min intervals. Only the numbers of erections, but not erections-like were scored. * $p < 0.05$, TNF- α vs. control (WT).

Table 1Emax and pD₂ values for agonists in cavernosal strips from control and TNF- α KO mice

Agonist	Control		TNF- α KO	
	Emax (%)	pD ₂	Emax (%)	pD ₂
acetylcholine (n=5)	76.3 $\pm$ 6.3	6.99 $\pm$ 0.13	97.4 $\pm$ 5.3 *	7.11 $\pm$ 0.07
sodium nitroprusside (n=5)	75.6 $\pm$ 4.2	6.13 $\pm$ 0.16	76.6 $\pm$ 2.4	5.84 $\pm$ 0.07
isoproterenol (n=5)	65.9 $\pm$ 4.4	7.47 $\pm$ 0.17	63.5 $\pm$ 3.9	7.12 $\pm$ 0.13
tadalafil (n=5)	99.2 $\pm$ 4.1	6.61 $\pm$ 0.16	96.4 $\pm$ 3.9	6.26 $\pm$ 0.24
Y-27632 (n=6)	94.4 $\pm$ 6.3	5.03 $\pm$ 0.07	95.7 $\pm$ 11.7	5.36 $\pm$ 0.10
phenylephrine (n=5)	2.5 $\pm$ 0.1	5.54 $\pm$ 0.08	1.6 $\pm$ 0.1 *	5.99 $\pm$ 0.17 *
phenylephrine + L-NAME (n=5)	2.2 $\pm$ 0.2	5.73 $\pm$ 0.12	1.5 $\pm$ 0.1 *	6.34 $\pm$ 0.19 *

Values are means $\pm$ SEM for n experiments in each group.*
p<0.05 vs control (*t* test).