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L-Arginine and phosphodiesterase (PDE) inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures

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Abstract

Inducible nitric oxide synthase (iNOS) is expressed in both the fibrotic plaque of Peyronie's disease (PD) in the human, and in the PD-like plaque elicited by injection of TGFβ1 into the penile tunica albuginea (TA) of the rat. Long-term inhibition of iNOS activity, presumably by blocking nitric oxide (NO)- and cGMP-mediated effects triggered by iNOS expression, exacerbates tissue fibrosis through an increase in: (a) collagen synthesis, (b) levels of reactive oxygen species (ROS), and (c) the differentiation of fibroblasts into myofibroblasts. We have now investigated whether: (a) phosphodiesterase (PDE) isoforms, that regulate the interplay of cGMP and cAMP pathways, are expressed in both the human and rat TA; and (b) L-arginine, that stimulates NOS activity and hence NO synthesis, and PDE inhibitors, that increase the levels of cGMP and/or cAMP, can inhibit collagen synthesis and induce fibroblast/myofibroblast apoptosis, thus acting as antifibrotic agents. We have found by immunohistochemistry, RT/PCR, and Western blot that PDE5A-3 and PDE4A, B, and D variants are indeed expressed in human and rat normal TA and PD plaque tissue, as well as in their respective fibroblast cultures. As expected, in the PD fibroblast cultures, pentoxifylline (non-specific cAMP-PDE inhibitor) increased cAMP levels without affecting cGMP levels, whereas sildenafil (PDE5A inhibitor) raised cGMP levels. Both agents and L-arginine reduced the expression of collagen I (but not collagen III) and the myofibroblast marker, α-smooth muscle actin, as determined by immunocytochemistry and quantitative image analysis. These effects were mimicked by incubation with 8-Br-cGMP, which in addition increased apoptosis, as measured by TUNEL. When L-arginine (2.25 g/kg/day), pentoxifylline (10 mg/kg/day), or sildenafil (10 mg/kg/day) was given individually in the drinking water for 45 days to rats with a PD-like plaque induced by TGF β1, each treatment resulted in a 80–95% reduction in both plaque size and in the collagen/fibroblast ratio, as determined by Masson trichrome staining. Both sildenafil and pentoxiphylline stimulated fibroblast apoptosis within the TA. Our results support the hypothesis that the increase in NO and/or cGMP/cAMP levels by long-term administration of nitrergic agents or inhibitors of PDE, may be effective in reversing the fibrosis of PD, and more speculatively, other fibrotic conditions.

Keywords: Fibrosis; Peyronie's disease; Penis; Sildenafil; Pentoxifylline; PDE4; PDE5; Nitric oxide; Myofibroblast; Collagen; ROS; TGF-β1; L-Arginine; Inducible nitric oxide synthase; Apoptosis; cGMP; cAMP; L-NIL

Fibroblasts and myofibroblasts are the main cell types involved in extracellular matrix deposition during wound healing and remodeling after trauma [1,2]. The myofibroblast shares features of fibroblasts and smooth muscle cells, participates in the generation of traction forces required to close a wound, and is removed by apoptosis after this process is completed [3,4]. The persistence of myofibroblasts in this setting leads to abnormal wound healing, tissue fibrosis, and contracture, as observed in several localized fibrotic conditions [5], including Peyronie's disease

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(PD)[2] in the tunica albuginea (TA) of the penis [2,6–8]. Myofibroblasts are also observed in diffuse fibrotic diseases that may affect virtually every organ, such as kidney, heart, lung, and liver [4].

We have postulated that PD may constitute an excellent model for studying tissue fibrosis and the pathways of gene expression that are involved in this process [2,6,8–10]. The PD plaque affects 3–5% of the male population and may lead to penile curvature during tumescence [10,11]. PD is assumed to result from an abnormal healing process subsequent to trauma to the erect penis [12–14], that causes fibrin extravasation into the TA, leading to inflammation and release of fibrotic effectors, mainly transforming growth factor β1 (TGFβ1) and reactive oxygen species (ROS), which then trigger excessive deposition and disorganization of the collagen fibers. Animal models for PD have been developed based on the injection of TGFβ1 or fibrin to the TA of the rat, causing a lesion that histologically and biochemically resembles the human PD plaque [2,6,14–17]. In addition, some features of the fibrotic process can be studied in vitro utilizing primary cultures of fibroblasts from both the normal human TA and the PD plaque, that partially differentiate into myofibroblasts [2,7].

Studies on the corresponding human tissues and cells combined with data from the rat models have shown that the development of the PD plaque is associated with expression of the inducible nitric oxide synthase (iNOS) and stimulation of nitric oxide (NO) synthesis, in conjunction with an increase in oxidative stress and ROS levels [2,6,10,11,18]. Since the specific inhibition of iNOS activity with L-iminoethyl-L-lysine (L-NIL) exacerbates fibrosis in the TGFβ1 rat model, our group proposed that NO produced by iNOS plays an antifibrotic role in PD by at least three mechanisms [2,6,10,11,14]: (a) the quenching of the pro-fibrotic ROS by a reaction leading to the formation of peroxynitrite; (b) the down-regulation of fibroblast replication and myofibroblast differentiation; and (c) the consequent or independent reduction in the transcriptional expression of collagen I. It was also speculated that an additional mechanism for NO to counteract fibrosis may involve stimulation of myofibroblast and/or fibroblast programmed cell death. The induction of apoptosis by NO is well documented, either in vitro by NO donors, such as *S*-nitroso-*N*-acetyl penicillamine (SNAP) [18,19] or inducible nitric oxide synthase (iNOS) expression [19,20], or in vivo by neuronal NOS (nNOS) activation [21], iNOS induction [21,22], or administration of the NOS substrate L-arginine [23,24]. The proposed antifibrotic role of iNOS is in agreement with indirect results obtained in animal models of kidney and cardiac fibrosis, where general NOS inhibitors (not isoform-specific), such as L-N_ω-Nitro-L-arginine methyl esther (L-NAME), cause or exacerbate fibrosis [25–27]. L-arginine supplementation has been shown to be anti-fibrotic in vascular and renal disease [28], but has not been tested on the PD plaque.

Since many of the effects of NO are mediated via the stimulation of guanylyl cyclase to produce cGMP [29], it could be possible that part of the antifibrotic action of NO may occur through the elevation of cGMP levels in the PD plaque, and the subsequent activation of protein kinase G (PKG) [30]. An exogenous cGMP agonist, 8Br-cGMP [31], or recombinant PKG [30,32] can inhibit collagen synthesis and fibrosis. In addition, these compounds induce apoptosis [33,34] and 8Br-cGMP inhibits fibroblast differentiation into myofibroblasts [35,36]. Similarly, inhibition of phosphodiesterase-5 (PDE5), the PDE isoenzyme that catalyzes cGMP breakdown, leads to elevation of cGMP levels and activation of PKG, while simultaneously causing apoptosis and reducing collagen synthesis [37,38]. A general inhibitor of cAMP-dependent PDEs, pentoxifylline, has been shown to act as an antifibrotic agent and has been proposed for the treatment of liver, lung, skin, radiation, and other types of fibroses [39,40]. In the case of the penis, aside from PDE5, only PDE1, 2, and 3 have been identified at the protein level in this organ [41–43]. PDE5 as well as PDE4 are assumed to be present in the penile cavernosal smooth muscle cells, in analogy to their localization in vascular tissue [42,44], but no immunohistochemical detection of these isoforms has been published in penile tissues.

In the present work, we have investigated whether PDE5 and PDE4 are present in the human and rat normal TA and PD plaque tissues, as well as in their respective fibroblast cultures, and whether pentoxifylline and sildenafil affect, respectively, cAMP and cGMP levels as expected, and 8 Br cGMP can inhibit collagen synthesis, myofibroblast differentiation, and induce apoptosis in cultures of fibroblasts from the human PD plaque. We have determined whether these in vitro effects can be replicated in vivo by long-term oral administration of PDE inhibitors or L-arginine to an animal model of PD, and this leads to a reduction in the development of the PD-like lesion.

Materials and methods

Human tissues and human and rat cell cultures

Human TA was obtained from non-PD patients ($n = 4$), two undergoing partial penectomy due to penile

[2] *Abbreviations used:* PDE, phosphodiesterase; ASMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; NO, nitric oxide; ROS, reactive oxygen species; TA, tunica albuginea; PD, Peyronie's disease; iNOS, or NOS II, inducible NOS; L-NIL, L-iminoethyl-L-lysine; PKG, protein kinase G; QIA, quantitative image analysis; SNAP, *S*-Nitroso-*N*-acetyl penicillamine.

cancer and two undergoing penile prosthesis surgery. Plaque tissue was isolated from PD patients ($n = 8$) who underwent a surgical procedure to treat this condition [2,6,8,14]. All procedures were IRB-approved and written informed consent was obtained. Fragments of the tissue were collected in "RNA-later" (Ambion, Austin, TX), for RNA analysis, in 4% formalin, for histochemistry and immunohistochemistry, or in culture medium containing fibroblast growth medium (FGM-2) (Clonetics, Walkersville, MD) with 20% fetal bovine serum, for protein analysis or cell culture. Tissues were then frozen at −80 °C until further use, except for fixed portions that were stored at 4 °C in PBS for paraffin embedding or small pieces that were used fresh for cell culture.

Human fibroblast primary cultures were obtained from fragments of PD plaque or TA essentially as described [3], and their purity was established by immunohistochemistry, as detailed below. Cells were incubated in 8-well chamber slides and allowed to grow to 50–60% confluence. At this point, cells received in duplicate sildenafil, pentoxifylline, or 8-Br cGMP at the concentrations indicated for each experiment, and were allowed to propagate for 3 days without changing medium. In certain cases, SNAP was added and replaced daily after changing the medium [2]. All experiments were done in duplicate or triplicate. For the isolation of rat TA fibroblasts, the TA was carefully dissected from rat corpora cavernosa tissue, and cultures were developed and their purity was tested as in the case of the human tissues.

Animal treatments and tissues

Male Fisher 344 rats (9–11-month-old) were purchased from the NIH/NIA colony (Harlan Sprague–Dawley, San Diego, CA), maintained under controlled temperature and lighting, and treated according to NIH regulations with an IUCAC-approved protocol. Animals ($n = 5$/per group) were anesthetized and injected in the penile TA close to the middle of the penis with either saline (group 1) or 0.5 μg TGF-β1 (groups 2-5) (Biotech Diagnostic, Laguna Niguel, CA) as described [2,6]. After the injection, groups 1 and 2 were given drinking water while the other groups received water with L-arginine (2.25 g/kg/day, group 3), as in our previous study [45], or sildenafil (10 mg/kg/day, group 4) or pentoxifylline (10 mg/kg/day, group 5). Forty-five days later, the animals were sacrificed and perfused through the left ventricle with saline followed by 4% formalin, [2,6]. The penises were excised, the skin was denuded removing the glans and adhering non-crural tissue, the penile shaft was separated from the crura, and a 2–3 mm transversal slice was cut around the site of the saline or TGF-ß1 injection. All tissues were post-fixed overnight in 4% formalin, washed in PBS, and stored at 4 °C.

Detection of PDE mRNA expression in tissues and cells

Total RNA was isolated from the human TA and PD tissues, from their respective fibroblast cultures, and from rat TA and penile shaft tissues, and their respective fibroblast and smooth muscle cell cultures, by the Trizol procedure (Gibco-BRL, Gaithersburg, MD). RNA was then submitted (1 μg) to reverse transcription [2,8,21] using Superscript II RNase H^- reverse transcriptase (Gibco BRL) and random primers (0.25 μg), followed by PCR with the respective gene specific primers [46]: (a) for human PDE5A, on nt 1027–1049 (forward) and nt 1788–1764 (reverse) of the respective cDNA (GenBank #I58526); encompassing a 762 bp band common to the three variants 1–3; (b) for rat PDE5A, the primers on nt 1905–1924 (forward) and nt 2479–2460 (reverse) of the respective cDNA (GenBank #NM133584), generating a 575 bp band; (c, d) for human PDE4A and B, on nt 942–965 (forward) and 1824–1802 (reverse), and nt 1909–1931 (forward) and 2315–2292 (reverse), respectively, of the cDNAs (GenBank #NM006202 and NM 002600, respectively); as the source of the expected 883 bp (A) and 406 bp (B) bands; and (e) for rat PDE4, the primers on nt 241–260 (forward) and nt 656–637 (reverse) of the respective cDNA (GenBank #M25350), generating a band of 416 bp. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide. For densitometry, normalization was performed against the GAPDH housekeeping gene fragment generated in the same PCR.

Detection of PDE5 and 4 protein expression in tissue and cell extracts

Tissue extracts were obtained by homogenizing in a 1:6 wt/vol ratio in a buffer containing 0.32 M sucrose, 20 mM Hepes (pH 7.2), 0.5 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). In the case of cell extracts, 0.5 ml of this solution per 10 cm Petri dish was used. The particulate and cytosolic fractions were obtained by homogenization of the cells in a Polytron Homogenizer (Brinkmann, Switzerland) and centrifugation at 12,000*g* for 60 min. Equal amounts of protein (30 μg) were run on 7.5% polyacrylamide gels and submitted to Western blot immunodetection with polyclonal anti-mouse PDE5 (against cGMP binding region) IgG (1:1000) (Calbiochem, La Jolla, CA) and a secondary donkey anti-mouse IgG linked to horse radish peroxidase (Amersham–Pharmacia, Piscataway, NJ), followed by a luminol reaction [8,21,53]. Human PDE5 does not cross-react with other PDE5 isoforms. Negative controls were performed without primary antibody. For PDE4 immunodetection, the following affinity purified IgGs were used (FabGennix, Shreveport, LA):

(a) anti-PDE4A selective antibody (detecting variants identified by 1, 5, 8, *x*, and unassigned); (b) anti-PDE4B (detecting variants 1–4) and anti-PDE4D (detecting variants 1–5) [47].

The presence of PDEs in the PD fibroblasts in culture was confirmed by the ability of increasing concentrations of sildenafil and pentoxifylline to raise the basal cGMP and cAMP levels in triplicate wells, either in the absence or the presence of the NO donor, SNAP (*S*-nitroso-*N*-acetyl penicillamine) (Alexis Biochemicals, San Diego, CA), added daily at 100 μM, as measured by cGMP and cAMP EIA (enzyme immunoabsorption) kits (Cayman Chemical, Ann Arbor, MI). Experiments were performed in duplicate. Values were expressed as pmoles cGMP or cAMP/mg protein. To normalize for differences between experiments, the changes in cGMP and cAMP levels exerted by sildenafil and pentoxifylline were expressed as percentage of their respective control values in the absence of the PDE inhibitors.

Histochemical and immunohistochemical determinations

In the case of cell cultures, at completion of incubations, slides were removed from the chambers and cells were fixed for immunodetection for 20 min in 4% buffered formalin at room temperature for α-smooth muscle actin (ASMA) (as a myofibroblast marker), vimentin (as a general fibroblast marker), and in certain cases for PDE5 and PDE4, or in ethanol at −20 °C for collagens I and III [2]. The cells were quenched, blocked with normal goat serum, and incubated with monoclonal primary antibodies for ASMA and vimentin (Sigma Immunohistology Kits, Sigma Chemical, St. Louis, MO), collagen I, and collagen III (1:40) (Chemicon International, Temecula, CA), overnight at 4 °C [2,6]. Processing was according to manufacturer's instructions for ASMA, vimentin, and collagen, consisting in the respective monoclonal antibodies and an anti-mouse biotinylated secondary antibody, followed by avidin-biotinylated HRP and the 3-amino-9-ethylcarbazol (AEC) chromogen. For PDE5A, the antibodies were as described above. Negative controls omitted the first antibodies or were replaced by IgG isotype at the same concentration of the first antibodies. Counterstaining was done with Mayer's hematoxylin. All the slides were mounted with Aqua Mount (Lerner, Pittsburgh, PA). For PDE4, the anti-PDE4A and PDE4B affinity purified IgGs used for Western blot were employed, and in addition the anti-PDE4A4 and anti-PDE4D (detecting variants 1–5) from the same source (FabGennix) were used.

In the case of tissue sections, the determinations of the collagen/smooth muscle ratio were carried out with Masson trichrome as described [6,14] on adjacent 5 μm paraffin-embedded cross-sections from the human normal tunical or plaque tissues, or from a 2 mm area around the site of injection in the rat saline- and TGF-β1-injected shaft tissues. Other distal sections were obtained along the rat penile shaft.

TUNEL assay for apoptosis

TUNEL assay [21,48] was performed in the adjacent matched tissue sections used for collagen staining, applying the Apoptag Oncor kit (Oncor, Gaithersburg, MD). In brief, after deparaffinization and rehydration, sections were incubated with proteinase K (20 μg/ml) and endogenous peroxidase activity was quenched with 2% H_2O_2. Sections were incubated with digoxigenin-conjugated nucleotides and TdT, and subsequently treated with antidigoxigenin-peroxidase. To detect immunoreactive cells, sections were stained with 0.5% DAB/0.01% H_2O_2 and counterstained with 0.5% methyl green. As a negative control, buffer was substituted for the TdT enzyme. Testicular sections from old animals were used as positive control. For cell cultures, the cells were fixed in 4% formaldehyde for 30 min on ice and post-fixed with ethanol–acetic acid 2/1 for 5 min at −20 °C. Then the above procedure was applied, except that the proteinase K was omitted.

Quantitative image analysis

The quantitation of the staining obtained by either histochemical or immunohisto/cytochemical techniques was performed by computerized densitometry using the ImagePro 4.01 program (Media Cybernetics, Silver Spring, MD), coupled to an Olympus BHS microscope equipped with a Spot RT digital camera [2,6,14,41,48]. The number of positive cells was counted in a computerized grid against the total number of cells determined by counterstaining, and results were expressed as a percentage of positive cells over total cells. In addition, the integrated optical density (IOD) was obtained by measuring the density per object and multiplying it by the respective area. The sum of all the individual values in the field was then divided by the number of positive cells, to obtain the mean IOD/positive cell, as a measure of average immunoreactivity/cell. In certain cases, results were expressed as the unweighted average optical density per area (OD/AREA), to determine the relative concentration of immunoreactive antigen. For collagen/smooth muscle staining, the ratio between the width of the area stained positive for collagen (blue) divided by the total area of the lacunar spaces plus smooth muscle (white + red) was employed. The apoptotic index (rate of programmed cell death) was calculated as the percentage of apoptotic cells within the total number of cells in a given area (non-apoptotic nuclei plus apoptotic cells). In all cases, five non-overlapping fields were screened per tissue section or per well. Three sections per tissue specimen from groups of five animals, or two wells per

experimental point in cell incubations, were then used to calculate means ± SEM.

Statistical analysis

Values were expressed as means (M) ± standard error of the mean (SEM). The normality distribution of the data was established using the Wilk–Shapiro test, and the outcome measures between two groups were compared by the *t* 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–Neuman–Keuls test, according to the Graph Pad prism V. 30. Differences among groups were considered significant at $P < 0.05$.

Results

Presence of PDE5 in the human penile tunica albuginea and PD plaque, in the rat tunica albuginea, and in fibroblasts cultured from these tissues

Because inhibitors of cAMP-dependent PDEs (pentoxifylline) and the stimulation of cGMP levels have previously been shown to reduce collagen synthesis and induce apoptosis in other tissues [37–40], and the cGMP-dependent PDE5A is present in the penile corpora cavernosa smooth muscle [41–44], we have determined whether these PDE isoforms are expressed in the rat and human TA. As an example of cAMP-dependent PDE we chose PDE4. The RNA was subjected first to RT/PCR with primers common for the three splicing variants of the PDE5A coding region [46]. Fig. 1 top left shows the ethidium bromide staining of PCR DNA fragments from reactions carried out in duplicate and fractionated by agarose gel electrophoresis. The 575 bp PDE5A DNA band was generated as expected from the rat penile shaft (PS) and the 762 bp from the human corpora cavernosa (CC) RNAs, and amplified to a similar level in total RNA from the human TA and PD. No RNA was extracted from the normal TA and the TGFβ1-induced PD-like plaque in the rat, due to the difficulty in dissecting large amounts of tissue to avoid contamination by cavernosal smooth muscle.

PDE5A expression was confirmed at the protein level by Western blot assays of tissue extracts, as shown by the luminol-stained protein bands (Fig. 1 top right), that can discriminate the three splicing variant proteins of PDE5A designated as 1, 2, and 3 with respective apparent sizes of 100, 92, and 83 kDa, respectively [43,49]. The three variants were detected as expected in the rat cerebellum (CER), our control tissue, whereas in the rat penile crura (CRU) and shaft (PS), the predominant forms were the 1 and 3, respectively, with only traces of variant 2 in the crura, and a band smaller than the 3 variant in the penile shaft. This PDE5A-3 variant, accompanied by smaller amounts of the 2 variant, was also

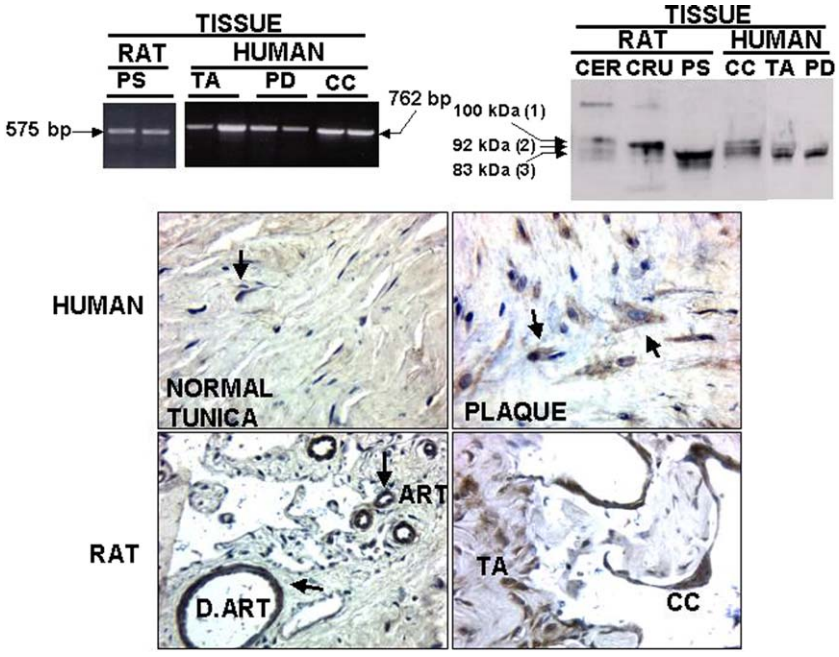

Fig. 1. Expression of PDE-5 mRNA and protein in the human PD plaque and normal tunica albuginea, and their homologous tissues in the TGFβ1 rat model of PD. Top left: ethidium bromide-stained DNA bands obtained by RT/PCR from RNAs isolated from the respective tissues and fractionated on agarose gels; top right: luminol-stained protein bands obtained by Western blot on polyacrylamide gels. PS, penile shaft; TA, tunica albuginea; PD, Peyronie's disease; CC, corpora cavernosa; CER, cerebellum; and CRU, penile crura. Middle and bottom: microphotographs (200×) of sections from human and untreated rat tissues. D.ART, dorsal artery; ART, artery. Arrows show positive cells for PDE-5.

expressed in the human corpora cavernosa (CC) and in the TA and PD plaque. Some PDE5A-1 variant was also detected in the human CC.

Immunocytochemical detection with an antibody detecting all three variants of PDE5A revealed that it was expressed in discrete cells interspersed among collagen fibers in the normal human TA and the PD plaque (Fig. 1 middle panels). PDE5A was also detected in the media of the dorsal artery and those within the corpora cavernosa, and in both the corporal smooth muscle and TA of the penis (Fig. 1, bottom panels)

PDE5 mRNA was also identified by RT/PCR in the fibroblasts cultured from the human normal TA and PD plaque, and from the rat TA (Fig. 2 top left), and the respective protein was detected by Western blot in the human cells as a single PDE5A-3 variant, which agrees with what was observed in vivo in the TA and PD plaque, (top right). The rat TA fibroblasts also express the 3 variant, accompanied by equal amounts of the 1 variant, despite the latter larger variant not being detected in the rat penile shaft. Immunocytochemical detection (middle panel micrographs) confirmed the expression of PDE5A in the three types of cells, namely fibroblasts from the human normal TA and PD plaque, and from the rat normal TA. However, in the latter case, as opposed to the human cell cultures derived from tissues reasonably free from contamination by cavernosal smooth muscle, the rat fibroblasts were obtained from whole corpora cavernosa including the smooth muscle. By successive passages in fibroblast culture medium [3], rather pure fibroblast cultures were selected, as evidenced from vimentin staining, and some were myofibroblasts, as seen with ASMA staining (bottom panel micrographs).

Presence of PDE4 variants in the human penile tunica albuginea and PD plaque, in the rat tunica albuginea, and in fibroblasts cultured from these tissues

Since the cAMP-dependent PDE inhibitor, pentoxifylline, has been used previously as an antifibrotic compound [36,39,40], we investigated by RT/PCR whether it is expressed in the TA and PD tissues and the respective cell cultures, utilizing primers for two (A and B) of the three variants. Fig. 3 top shows that PDE4A and B mRNAs are expressed in the human normal TA and in the PD tissues. Both variants were also detected in human corpora cavernosa tissue containing mainly smooth muscle (not shown). Confirming these results, PDE4A and B mRNAs were also found in the fibroblasts cultured from human TA and PD (bottom right). In the case of the rat, PDE4 mRNA (without variant discrimination) was detected in the TA cells, and to a lesser degree in the penile shaft tissue, thus suggesting that PDE4 in the rat TA fibroblast cultures does not arise from contamination with smooth muscle, which in any

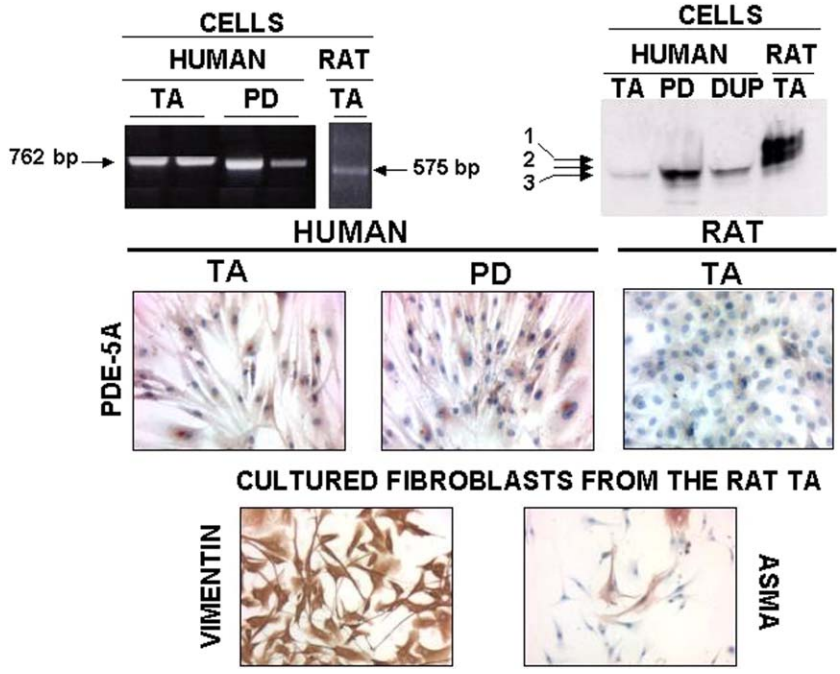

Fig. 2. Expression of PDE-5 mRNA and protein in fibroblast cultures from human PD plaque and human and rat normal tunica albuginea. Top left: ethidium bromide staining of PDE-5A cDNA bands generated from RNA by RT-PCR and fractionated on agarose gels. Top right: luminol detection of PDE5 protein bands obtained by Western blot of cell extracts on PAGE; arrows indicate the three PDE5A variants. DUP, cells from Dupuytren's nodules; Middle and bottom: microphotographs (200×) of cell cultures stained with the indicated antibodies. Counterstain with Mayer's hematoxylin.

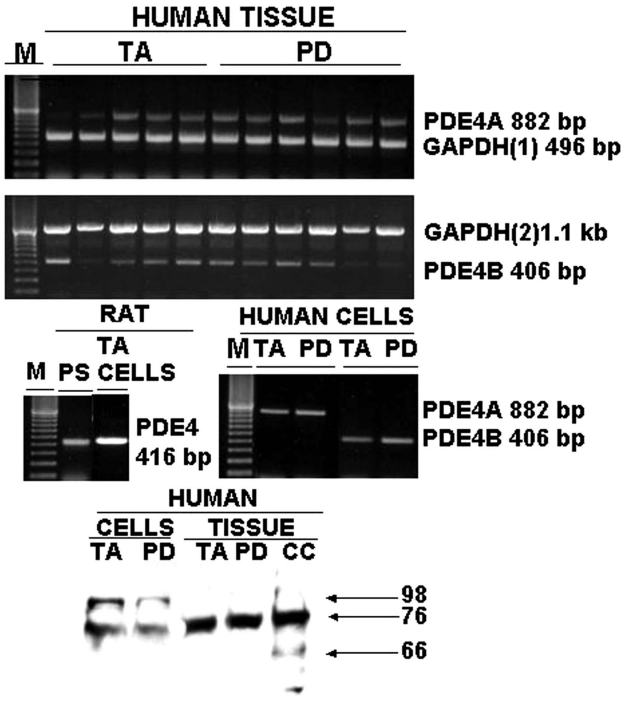

Fig. 3. Expression of PDE-4 mRNA and protein in the human PD plaque and normal tunica albuginea, and their homologous tissues in the TGFβ1 rat model of PD, and the fibroblasts cultured from these tissues. Top panels: ethidium bromide staining of DNA generated from tissue or cell RNA by RT/PCR with primers for PDE4A, PDE4B, and GAPDH (reference gene), and separated by agarose gel electrophoresis. TA, tunica albuginea; PD, Peyronie's disease; CC, corpora cavernosa smooth muscle; and PS, penile shaft. Bottom panels: luminol-stained protein bands on Western blots of human tissue and cell extracts with the antibody against PDE4A.

case had been reasonably excluded above by immunocytochemistry.

Confirmation of the expression of PDE4A at the protein level was obtained by Western blot with an antibody for the different variants, in extracts from the human cultured cells used for the identification of PDE5A in TA and PD plaque. Fig. 3 bottom shows an intense 76 kDa band that would correspond to a variant identified in testis [47], as well as a minor 102 kDa band for the so-called PDE4Ax, also seen in the testis. The 76 kDa protein is very intense in the three human tissues: TA, PD plaque, and corpora cavernosa, but the 102 kDa band was virtually not detected. No PDE4B could be visualized when the Western blot membranes were stripped and reacted with an antibody specific for this isoform.

Immunodetection with the same antibody identified cells all along the internal side of the TA, as well as in the corpora cavernosa smooth muscle, expressing PDE4A (Fig. 4, top). An antibody specific for PDE4D showed also cells reactive for this isoform, evidencing that both PDE4 genes are expressed in the TA and corpora cavernosa. A similar situation is seen in the rat TA fibroblasts in culture, with considerable expression of PDE4A in most cells, whereas only a fraction of the cells express PDE4D (middle). In contrast, most of the human TA fibroblasts were intensively stained with antibodies against the A and D isoforms (bottom). Despite the fact that the PDE4B mRNA was identified by RT/PCR (see Fig. 3), virtually no protein reactivity for this isoform was observed by immunodetection in tissue sections or cell cultures (not shown). A similar situation occurred with one of the variants of PDE4A (PDE4A4), utilizing a specific antibody, different from the general used above for PDE4A that according to the supplier does not detect variant 4.

Incubation of PD fibroblast cultures with PDE inhibitors or a cGMP analog reduces collagen I synthesis and myofibroblast differentiation, and increases apoptosis

Verification that the PDE5A and PDE4 proteins detected in the TA and PD cells and tissues are enzymatically active was obtained by measuring the levels of cGMP in cell extracts of the PD fibroblast cultures, with a basal mean value ± SEM of 5.0 ± 0.4 pmol/mg protein ($n = 5$) in the absence of additions, and increased 5.0-fold with 100 μM SNAP (an NO donor) for 3 days, with fresh daily replacement of medium with SNAP. A cGMP analog able to enter the cell, 8 Br-cGMP, at 10 μM, was also able to increase cGMP levels by 6.4-fold, and with 100 μM 8 Br-cGMP, the levels of cGMP were dramatically elevated by 38.7-fold. The basal levels of cAMP were 42.6 ± 12.7 pmol/mg protein in the absence of SNAP and did not vary significantly when measured after 3 days with SNAP. The cGMP-dependent PDE5 inhibitor sildenafil did not stimulate significantly cGMP levels in the absence of SNAP (not shown). However, in the presence of the NO donor, the cGMP levels expressed as percentage of the basal control levels in the absence of sildenafil were increased dose-dependently by sildenafil after the 3-day incubation, as expected (Fig. 5 top). When cells were incubated with this NO donor, increasing concentrations of pentoxifylline, also as expected, did not increase significantly cGMP levels expressed as % of control levels (middle), but were very effective in increasing cAMP levels (bottom), thus confirming its role as a cAMP-dependent PDE inhibitor with little or no effect on cGMP-dependent PDE.

To determine whether the PDE inhibitors may reduce collagen synthesis, the PD cells were incubated with or without the drugs at lower concentrations: pentoxifylline at 200 nM and sildenafil at 50 and 200 nM. After 3 days, cells were fixed and the intracellular deposition of collagen I and III was determined by immunocytochemistry with specific antibodies against the two isoforms. The antibody against collagen I elicited an

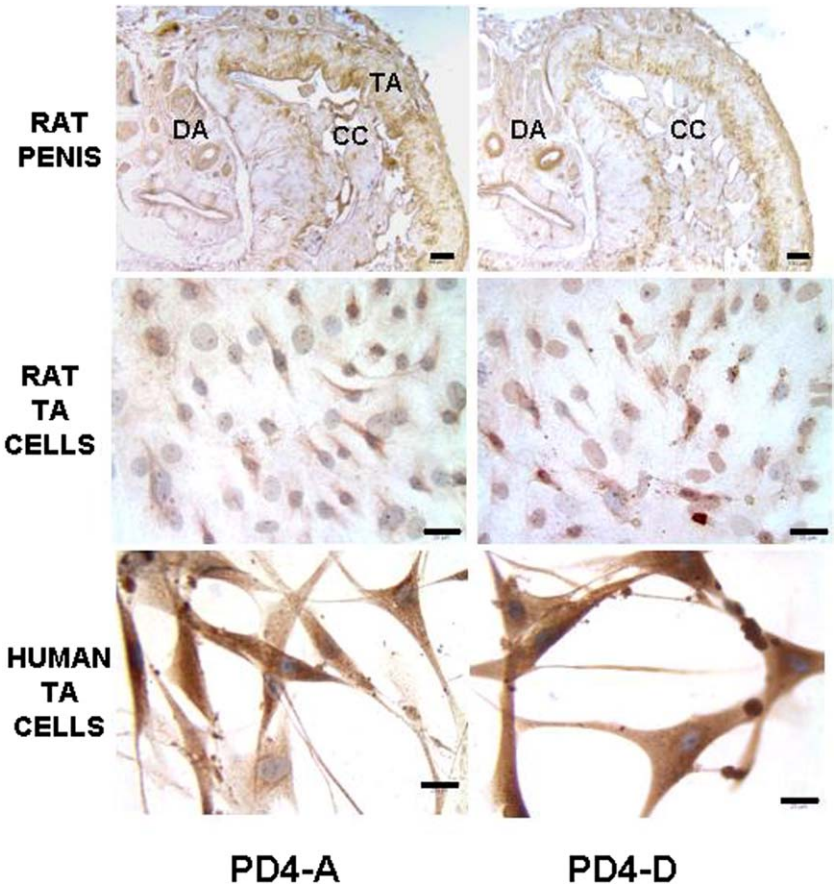

Fig. 4. Immunodetection of PDE-4 protein in the rat penis and cultures of rat and human tunica albuginea fibroblasts. Microphotographs of tissue sections (top panels) or cell cultures (middle and botom panels), as indicated, submitted to immunodetection with antibodies against PDE4A or PDE4D, and counterstained with Mayer's hematoxylin.

intense granular and perinuclear staining (not shown), similar to the one previously reported by us [2]. In contrast, collagen III was detected in only in about 30% of the cells and stained more diffusely and rather lightly, even when cells were treated with TGFβ1 (10 ng/ml), a known stimulator of collagen III synthesis (Fig. 6 bottom).

Quantitation by image analysis (Fig. 6 top) in the cultured human PD fibroblasts indicated that in the absence of additions, most of the cells (100%) expressed collagen I, and that both pentoxifylline and sildenafil at 200 nM completely inhibited collagen synthesis in a small number of cells (5–15% of the total), and significantly reduced (30–40% decrease) the average intensity of expression per cell. In contrast, the PDE inhibitors did not decrease, but even increased, the synthesis of collagen III (not shown). More drastically than in the case of collagen I, both of the PDE inhibitors (pentoxifylline and sildenafil) significantly reduced the number of ASMA positive cells (myofibroblasts) from 37% in the control to about 24%. The average ASMA expression per cell was significantly reduced by the PDE inhibitors by more than 90% in all cases.

To show that in the case of sildenafil some of the effects are mediated by the elevation of cGMP, the PD fibroblasts were then incubated for 3 days with 8-Br-cGMP, and a significant (30%) reduction in the number of cells expressing collagen I was obtained at 10 μM 8 Br-cGMP, although a higher concentration (400 μM) did not induce further decrease (Fig. 7). In contrast to the effects of the PDE inhibitors observed in the previous experiments, collagen I expression per cell was reduced only moderately and non-significantly by the cGMP analog. The differentiation of fibroblasts into myofibroblasts measured by the level of ASMA expression per cell was decreased significantly by 400 μM 8 Br-cGMP, as in the case of the PDE inhibitors, but there was no effect on the relative number of positive cells.

The increase of cGMP in the PD cells incubated with 8 Br-cGMP leads to a stimulation of apoptosis, as shown by an increase in apoptotic bodies detected with the TUNEL technique (Fig. 8 top). However, because of variability between experiments, the considerable 2.3-fold-increase measured by image analysis did not achieve statistical significance (bottom).

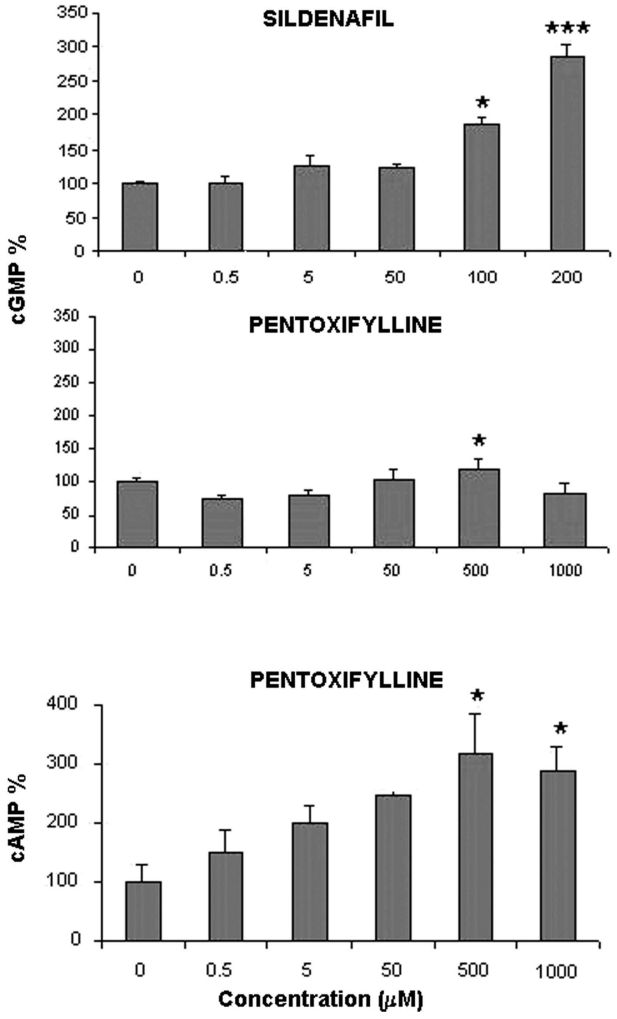

Fig. 5. Effect of pentoxifylline and sildenafil on cAMP and cGMP levels in fibroblast cultures from human PD plaque, estimated by enzyme immunoassays. Cells were incubated for 3 days in fibroblast growth medium (FGM)/10% fetal bovine serum, in the presence of SNAP (100 μM; medium changed daily) added 4 h prior to the PDE inhibitors, and increasing concentrations of sildenafil or pentoxifylline. cGMP and cAMP levels were measured in cell homogenates. Results are means of two separate experiments conducted in triplicate.

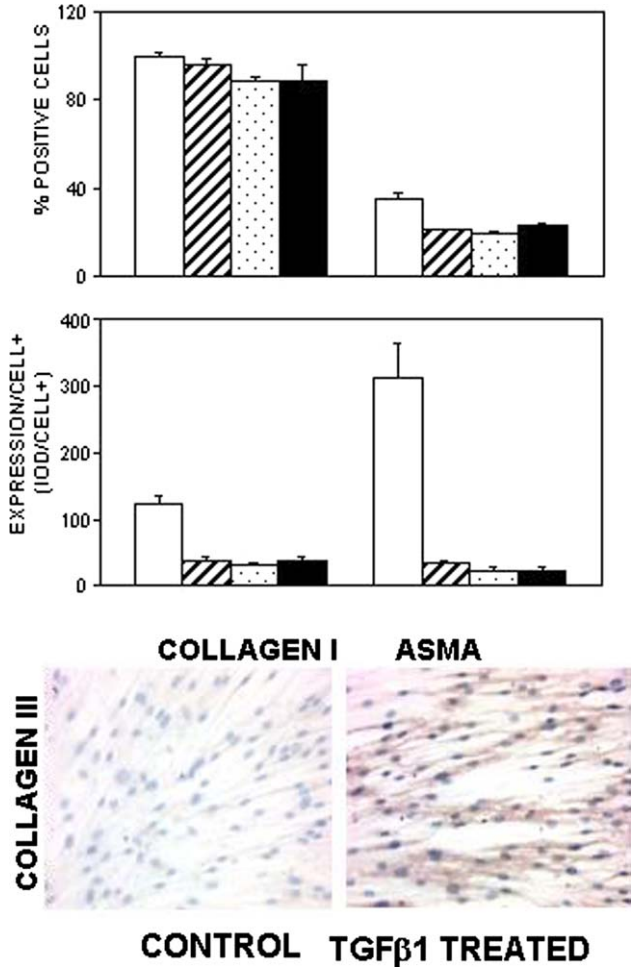

Fig. 6. Inhibition of collagen I synthesis and myofibroblast differentiation by PDE inhibitors in fibroblast cultures from human PD plaque, estimated by quantitative immunocytochemistry. Top: cells were incubated for 3 days in FGM/10% serum. QIA evaluation of collagen I and ASMA expression as means ± SEM for three separate incubations. Blank bars: control (no addition); hatched bars: sildenafil (50 nM); dotted bars: sildenafil (200 nM); and solid bars: pentoxifylline (200 nM). Bottom: AEC-stained immunocytochemical detection of collagen III in PD cells incubated for 3 days in FGM-serum free medium in the presence or absence of 5 ng/ml TGFβ1 (200×). $p < 0.001$ in all treatments vs control, except positive cells for collagen I, where no statistical difference was observed.

Administration of PDE inhibitors and the NOS substrate L-*arginine to the rat model of Peyronie's disease reduces collagen deposition and increases apoptosis*

The effect of sildenafil and pentoxifylline in inhibiting collagen I synthesis and myofibroblast differentiation in the cultured PD fibroblasts, while stimulating apoptosis, would suggest that counteracting PDE activity may exert a similar in vivo effect and prevent the development of the PD fibrotic plaque in the rat model. To test this hypothesis, we administered sildenafil and pentoxifylline in the drinking water to rats where a PD-like lesion was initiated by the injection of TGFβ1 into the penile TA, and maintained this treatment for the 45 days required to obtain a typical lesion [2,6,14]. In addition, since we previously showed that reducing NO release by inhibiting iNOS activity favored the development of the fibrotic PD-like plaque, we have tested whether the NOS substrate, L-arginine, also in the drinking water, would exert the opposite effect, namely to inhibit the development of the plaque. The amount of ingested water was measured daily, and no significant variation was observed in any of the treatments as compared with controls, throughout the experiment.

Fig. 9 top (micrographs) shows as a representative example the considerable decrease of the deposition and disorganization of collagen fibers in the PD-like

plaque of one individual animal in each case, induced by L-arginine, sildenafil, and pentoxifylline, in comparison to controls receiving regular drinking water, as assessed by Masson staining. This is illustrated by the reduction in the area of fibrosis, and the recovery of the intense blue staining that denotes normal collagen fibers in lieu of the pale blue identifying disorganized fibers. Quantitative image analysis for five animals per group confirmed the visual inspection (bottom). The ratio between the area occupied by collagen fibers versus the area of smooth muscle and cisternae in the half section of the penis corresponding to the site of injection was considerably increased in the TA injected with TGFβ1, as compared with saline. Treatment of animals injected with TGFβ1 with either L-arginine, sildenafil, or pentoxifylline virtually normalized this ratio to the one observed in the control animals injected with saline. The thickness of the TA (not shown) was also significantly decreased by L-arginine and sildenafil, but not by pentoxifylline.

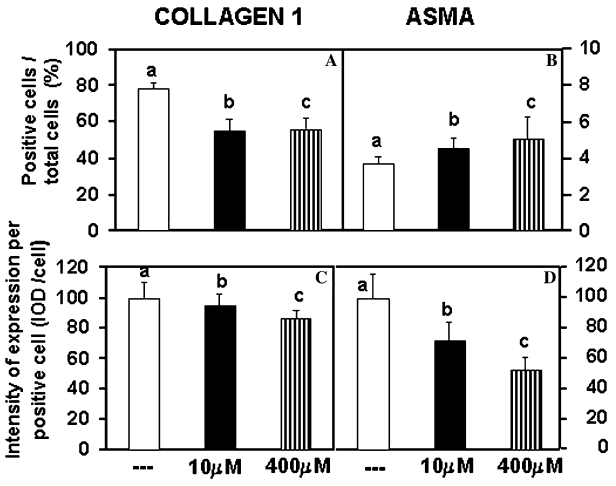

Fig. 7. Effects of 8 Br-cGMP on collagen I synthesis and myofibroblast differentiation in fibroblast cultures from the human PD plaque, estimated by quantitative immunocytochemistry. Cells were incubated for 3 days as in Fig. 6 with the indicated concentrations and collagen I and ASMA were immunocytochenmically detected. For typical staining see Smith and Liu [3]. Values are means ± SEM for three separate incubations. $p < 0.05$ were as follows: (A) a vs b, c; (D) a vs c; all others were non-significant.

From the experiments in vitro where the PD cells were incubated with 8-Br-cGMP and the apoptotic index was determined, it was assumed that one of the mechanisms by which PDE inhibitors, and presumably L-arginine, reduce collagen deposition in vivo would be the intensification of fibroblast or myofibroblast apoptosis. This would lead to a decrease in the PD-like plaque of the relative number of these cells, known to be

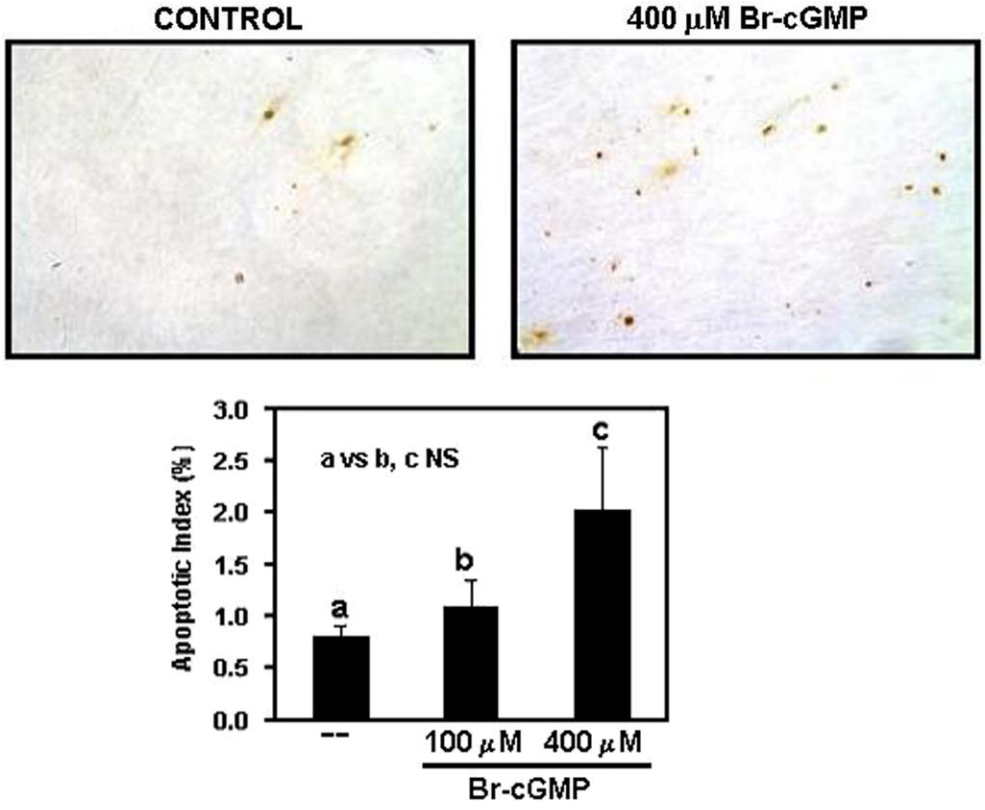

Fig. 8. Effect of 8 Br-cGMP on apoptosis in fibroblasts from human cultured PD plaque, estimated by TUNEL. Top: Microphotographs (200×) of apoptotic cells in incubations receiving no addition and 400 μM 8 Br-cGMP for 3 days. Bottom: apoptotic index, as means ± SEM for three separate incubations. $p > 0.05$: a vs b, c.

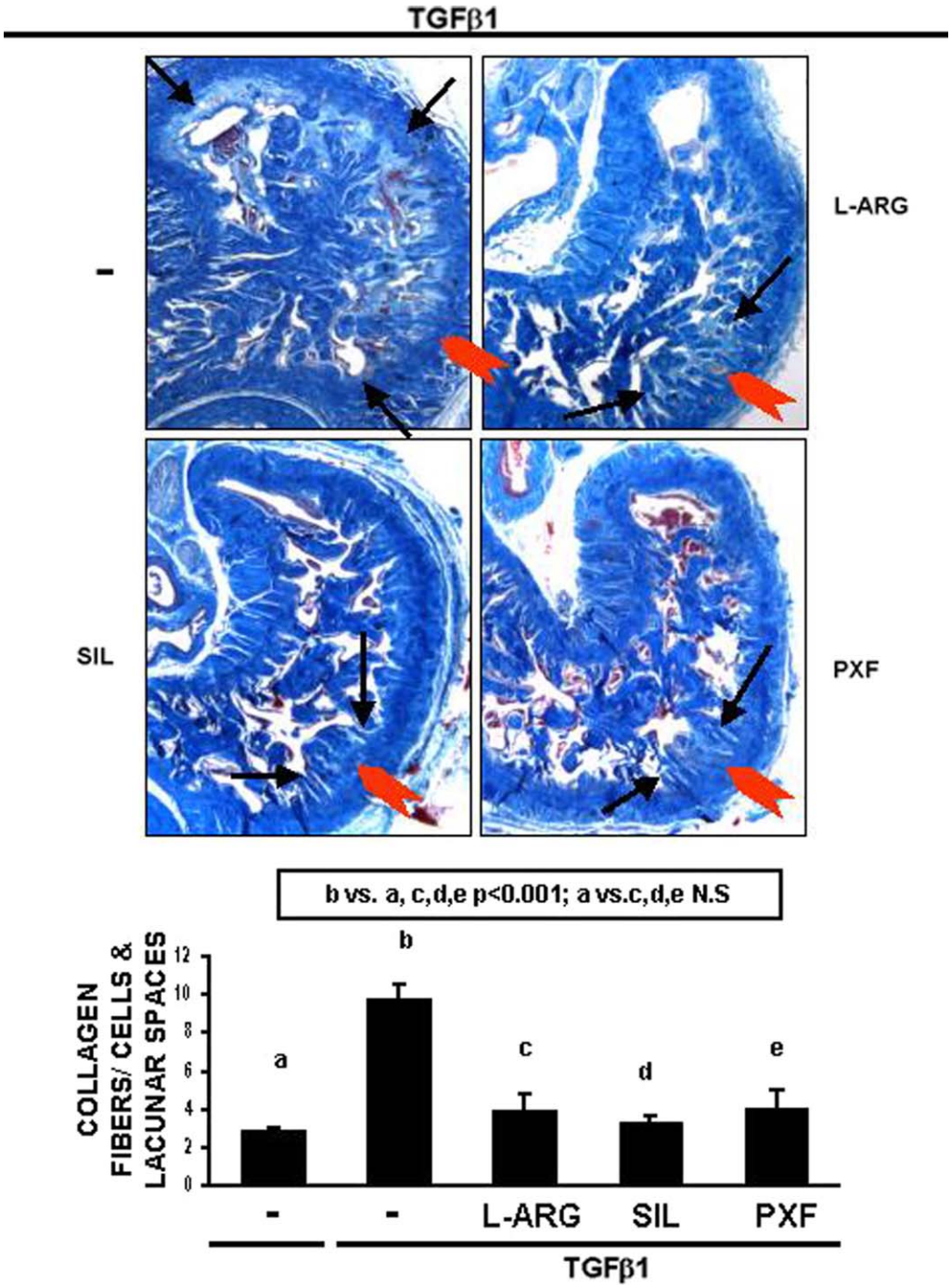

Fig. 9. Inhibition of collagen deposition in the fibrotic plaque induced by TGFβ1 in the rat TA, by long-term oral treatment with L-arginine and PDE inhibitors, estimated by quantitative Masson staining. Top: Microphotographs (40×) of cross-sections of half of the rat penis corresponding to the site of TGFβ1 injection in the TA, from rats treated with compounds added to the drinking water and sacrificed 45 days later. Site of injection is indicated by red arrows. Black arrows indicate the location of the PD-like plaque and site of tunical thickening. −: control; no treatment, L-ARG, L-arginine; SIL, sildenafil; and PXF, pentoxifylline. Bottom: QIA evaluation as means ± SEM ($n = 5$ per group). Left bar represents animals injected with vehicle alone (saline), not included on microphotographs above. $p < 0.001$: b vs a, c, d, e; non-significant: all others.

very active in synthesizing collagen. Fig. 10 top shows the typical appearance of apoptotic bodies visualized by TUNEL in fibroblasts in the TGFβ1-injected TA and subjected or not to treatment with L-arginine, sildenafil, and pentoxifylline. Quantitative image analysis (bottom) indicated a significant increase in the apoptotic bodies and the corresponding apoptotic index, in animals treated with sildenafil (2-fold) and pentoxifylline (5-fold), but not with L-arginine, in comparison to no treatment.

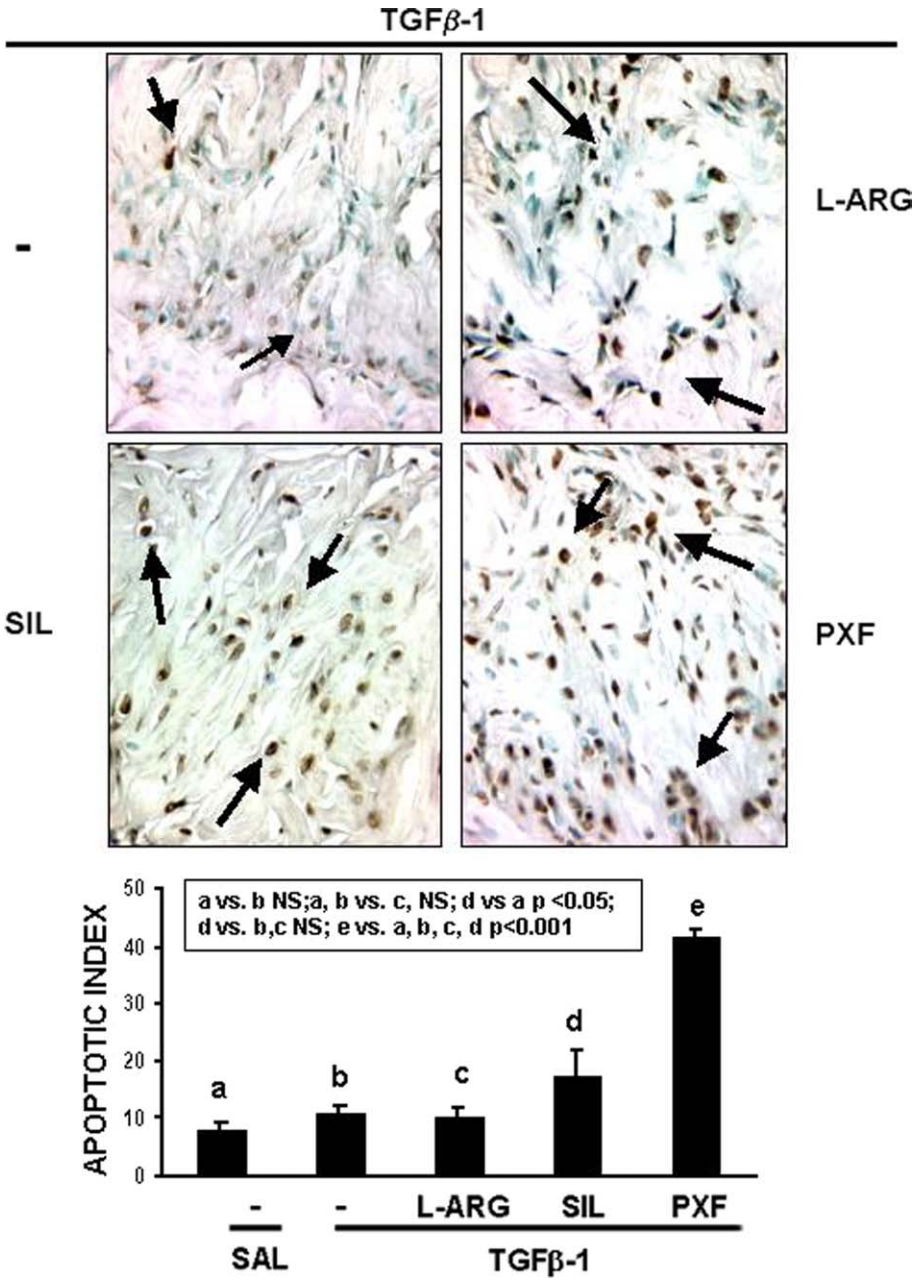

Fig. 10. Stimulation of apoptosis in the fibrotic plaque induced by TGFβ1 in the rat TA, by long-term oral treatment with L-arginine and PDE inhibitors, estimated by TUNEL. Top: Microphotographs (400×) of tissue sections adjacent to those depicted in Fig. 7. For symbols see Fig. 7. Bottom: QIA evaluation as means ± SEM ($n = 5$ per group). Left bar represents animals injected with vehicle alone (saline), not included on microphotographs above. $p < 0.001$: e vs a, b, c, d; $p < 0.05$: d vs a; non-significant: all others.

Discussion

To our knowledge this is the first demonstration that PDE5 and 4 are both expressed in the human and rat normal tunica albuginea, and the respective PD and PD-like fibrotic plaques, as well as in the cell cultures obtained from these tissues. Our results are also the first published report on the inhibition of a TGFβ1-induced fibrotic plaque in the rat model of PD, through the reduction of collagen deposition and possibly an increase in apoptosis of the resident fibroblasts and myofibroblasts, by long-term oral administration of the respective PDE5 and cAMP-dependent PDE inhibitors, sildenafil and pentoxifylline, and the NOS substrate, L-arginine. The in vitro effects of both PDE inhibitors and a cGMP analog, 8 Br-cGMP, on fibroblast cultures obtained from the human PD plaque, support the hypothesis that these agents may, at least in part, be effective against fibrosis by reducing the relative number of these fibroblasts/myofibroblasts through the induction of

apoptosis of these cells. We also found that these compounds: (a) interfere with fibroblast differentiation into myofibroblasts, the cells that are key players in tissue fibrosis, and (b) down-regulate the synthesis of collagen I but not collagen III. The effects of sildenafil are probably exerted through the inhibition of PDE-5, and in the case of pentoxifylline through a cAMP-dependent PDE, potentially PDE4, although we cannot discard other mechanisms. We believe that our results may open a new approach for the treatment of PD and, by extension, tissue fibrosis, based on the use of PDE inhibitors and other enhancers of PDK activity, and possibly of compounds and biologicals that enhance NO synthesis.

Our results with the PDE inhibitors, whose main effects are to increase cGMP or cAMP levels [50], are a natural extension of our previous work demonstrating the antifibrotic effects of NO [2,6,14], one of the main regulators of guanylyl cyclase, and hence of cGMP synthesis [29]. Both cGMP and cAMP participate to a certain extent in biochemical cross-talk, by mutually regulating their respective levels [51,52], so that even cAMP is in a way related to the NO-cGMP cascade. We have shown previously that iNOS is spontaneously induced in the human PD plaque and in the PD-like lesion in the rat model, increasing NO synthesis in these tissues, and that long-term blockade of iNOS by L-NIL intensified fibrosis in the rat lesion [2,6]. This led us to postulate that NO inhibits fibroblast differentiation into myofibroblasts and reduces collagen synthesis based on our own results in the animal model of PD and the fibroblasts cultured from human TA and PD plaque.

The reduction of the fibrotic plaque we have now observed in vivo in animals receiving L-arginine coincides with its effects in preventing experimental ethanol-induced inflammatory and fibrotic changes in liver, kidney, lung, and cardiovascular system [52–57]. The action of L-arginine is probably mediated by the stimulation of NOS activity, because although the endogenous levels of L-arginine in the rat penis appear to be above the Km of the pure recombinant enzyme, this does not take into account the interplay of factors that may increase the effective Km in vivo. This was previously shown by the increase of L-arginine levels in the penis and the improvement of erectile dysfunction in the aging rat by NOS stimulation achieved after a regimen of L-arginine administration similar to the one used in the current work, 2.2 g/kg/day [45]. This dose is within the range normally employed as vasculoprotective, for long-term studies in the rat [56,57].

As to the PDE inhibitors themselves, our in vivo and in vitro results showing an inhibition of collagen synthesis and stimulation of apoptosis in the PD-like plaque and in PD cells by both sildenafil and pentoxifylline are in good agreement with the extensive use pentoxifylline as an antifibrotic agent in liver and vascular fibrosis [39,40,58,59]. The fact that the cGMP analog 8-Br-cGMP inhibited collagen I synthesis and induced apoptosis in PD cells suggests that in the case of sildenafil the in vivo effects on the function of the fibroblasts/myofibroblasts in the TA may be mediated by the elevation of cGMP levels. In addition, cGMP analogs, PKG activators, and PDE inhibitors have been shown to inhibit collagen synthesis [31,32], and induce apoptosis [33], and some of the PDE inhibitors like sulindac sulfone (Exisulind) are effective as anticancer agents because of their intense pro-apoptotic action [37,38]. However, since pentoxifylline did not affect cGMP levels in the human PD fibroblasts, and the drug is considered to be a non-specific inhibitor of cAMP-PDE [60,61], and at least in some cell types does not affect cGMP levels [58], the increase in cAMP may also have played a role in the antifibrotic effects we observed with pentoxifylline. Whether this occurs via the inhibition of PDE4 present in TA and PD remains to be established with isoform-specific inhibitors like rolipram [50,61,62]. It should be noted that pentoxifylline may also act through its blockade of PDGF-induced activation of the mitogen activated protein kinase system [62] and of other cytokine-mediated fibrogenic mechanisms [40].

Our daily dose of pentoxifylline is 1/5 of the oral dose normally employed in rats for the long-term treatment of fibrosis [58,59], and in the case of sildenafil, it is 1/2 to 1/7 of the chronic dosage used in recent studies in rats [63,64]. When our 10 mg/kg/day dose is translated into the equivalent dose in humans by correcting for differences in the body weight/skin area [65], it is roughly 1.5 mg/kg which is about the dose ingested by men with an on demand single 100 mg tablet. Our selected dose was dispensed in 24 hours and not as a bolus administration, so that concentrations at a given time should be much lower, considering the short half-life (about 4-6 hours) of sildenafil. Therefore, the daily doses of the PDE inhibitors tested in the current work are not supra-pharmacological or associated with toxicity. In addition, it may be possible that local administration of either L-arginine or the PDE inhibitors, e.g., by injection into the plaque or in vehicles able to traverse the skin and TA may considerably reduce the effective dosage.

We do not know why administration of L-arginine, which should increase NO synthesis and hence cGMP levels and has been shown to be effective in arresting the growth of the TGFB-1 induced plaque in the rat model of PD, failed to stimulate apoptosis, as could be expected from its effects increasing it in vivo in the smooth muscle of the pulmonary arteries [23,24]. However, the absence of a stimulation of the apoptotic index in the PD plaque by L-arginine may agree with the decrease in apoptosis observed in liver transplants which is in line with the anti-apoptotic effects of NO in certain conditions and tissues [66]. In any case, not only cGMP but its down-stream compound in the NO-cGMP cascade, PKG, is also effective in preventing fibrosis and remodeling in balloon-injury and arterial restenosis [32,34],

as shown by gene transfer of the PKG cDNA in rats. However, despite this evidence for pentoxifylline and cGMP-PKG, we are not aware of reports on any anti-fibrotic action for the most widely used PDE inhibitor, sildenafil, that is specific for PDE5A, the main PDE protein detected in the corpora cavernosa [41,42].

Our results demonstrating the presence of PDE5A and PDE4 in the TA and PD plaque in the human and rat, and in their respective fibroblast cultures, provide a rationale for the anti-fibrotic effects of PDE inhibitors on the PD animal model and on the PD cell cultures. The PDE5A1 and PDE5A2 proteins have been previously localized in human penile corpora cavernosa [43]. The PDE5A3 variant was also found in corpora cavernosa and confined to tissues with a smooth muscle or cardiac muscle component, and is twice as sensitive as PDE5A1 to sildenafil, but, as with PDE5A1 and 2, is subjected to transcriptional up-regulation by both cAMP and cGMP [49,67]. This has been postulated to imply that long-term treatment with sildenafil may induce partial resistance. As to PDE4, it is interesting that cAMP can activate PKG nearly as effectively as cGMP, so that eventually, the inhibition of PDE4 may end up causing PKG effects (e.g., counteracting fibrosis) similar to those exerted by as the inhibition of PDE5A.

In summary, we propose that pharmacological interventions aimed at elevating NO, cGMP, or PKG levels, and possibly cAMP, in the penis are potentially useful for the treatment of PD, and more speculatively, for other fibrotic conditions. This work has not addressed the question on whether this intervention would induce regression of an already well-formed plaque, but comparison of multiple gene expression profiles in human PD and the related Dupuytren's disease suggests that both conditions are in a dynamic cell and protein turnover involving replication, differentiation, apoptosis, and collagen and extracellular matrix synthesis and breakdown [8–10]. Therefore, modulation of any of these processes may eventually involute the plaque, as has been observed in generalized fibrotic conditions [68,69].

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