Fibrosis-related gene expression in the prostate is modulated by doxazosin treatment

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ABSTRACT

Aims: To gain new insights into the molecular mechanisms of action of doxazosin, we investigated the prostatic stroma ultrastructure and the expression of genes involved with fibrosis, such as collagen type I and III (COL1A1 and COL3A1, respectively) and TGF-beta 1, in the rat ventral prostate.

Main methods: Adult Wistar rats were treated with doxazosin (25 mg/kg/day), and the ventral prostates were excised at 7 and 30 days after treatment. Untreated rats were controls. Ventral prostates were subjected to ultrastructural, immunohistochemical, biochemical and molecular analyses.

Key findings: Doxazosin-treated prostates showed thickened bundles of collagen fibrils, activated fibroblasts, enlarged neurotransmitter vesicles and increased tissue immunostaining for collagen type I and type III when compared to untreated prostates. After 7 and 30 days of doxazosin treatment mRNA expression of COL1A1 and COL3A1 was significantly increased and reduced, respectively, compared to the control group. TGF-beta 1 mRNA and protein levels were increased after 7 days of doxazosin treatment, whereas only mRNA levels remained increased after 30 days of treatment.

Significance: Our data suggest that relaxation of smooth muscle cells by alpha-blockers interferes with the mechanical dynamics of the prostatic stroma extracellular matrix components, generating a pro-fibrotic effect probably via the TGF-beta 1 signaling pathway. Long term treatment with doxazosin may also lead to a reduced turnover of extracellular matrix components. Our results add to a better understanding of the molecular mechanisms behind the effects of alpha-blockade on prosthetic histoarchitecture and the response to treatment for benign prostatic hyperplasia.

Introduction

Although the major function of the prostate gland is performed by the secretory epithelium, it is well known that the stromal compartment plays a crucial role in the structural support and in the maintenance of the differentiated state of epithelial cells (Marker et al., 2003). Fibroblasts and smooth muscle cells (SMC) synthesize, organize and maintain a complex extracellular network in the prostate stroma (Knox et al., 1994; Nagle, 2004). The collagen proteins are some of the most important extracellular matrix (ECM) macromolecules (Hynes, 2009), and their rates of synthesis, accumulation and degradation are crucial for prostate structure and function (Myllyharju and Kivirikko, 2001).

The main collagen proteins found in the prostatic stroma are types I, III, IV, VI and VII (Sinha et al., 1991; Knox et al., 1994; de Carvalho et al., 1997a; Vilamaior et al., 2000; Taboga and Vidal, 2003). Fibers of the elastic system, hyaluronan, proteoglycans and other glycoproteins are also present (Knox et al., 1994; de Carvalho et al., 1997b) and contribute to the dynamic epithelium-stroma cross-talk via paracrine signals that regulate the behavior of both epithelial and stromal cells (Marker et al., 2003). Similarly, epithelial and stromal cells dynamically interact with ECM molecules so that changes in the ECM microenvironment can lead to alterations in the physiology of both epithelial and stromal cells (Nelson and Bissell, 2006).

Stromal cells and extracellular matrix components are altered in prostatic diseases (Knox et al., 1994; Lee and Peehl, 2004). It is known that the fibrotic process is regulated by transforming growth factor-beta 1 (TGF-beta 1), a growth factor that recruits proinflammatory cells and fibroblasts to the fibrotic area, stimulating these cells to produce cytokines and extracellular matrix components, like type I collagen (Jimenez et al., 1994). However, the extent to which these alterations are causes or consequence of prostatic diseases is unknown.

Alpha-1 adrenergic receptor blockers, such as doxazosin, inhibit the binding of catecholamines (released from autonomous nerve varicosities) to alpha-1 receptors on the membrane of smooth muscle cells. Thus, they lead to relaxation of prostatic smooth muscle cells and relieve the symptoms associated with benign prostatic hyperplasia (BPH). Besides the direct action on the smooth muscle cells, doxazosin has been shown to induce apoptosis in stromal and epithelial prostatic cells, suggesting additional effects on the long-term
management of BPH (Anglin et al., 2002; Chiang et al., 2005; Kyprianou et al., 1998). Although the mechanisms by which doxazosin induces apoptosis are not clear, some studies suggest that apoptosis occurs as frequently in normal cells as it does in tumor cells (Kyprianou et al., 2009).

Previous results from our laboratory demonstrated for the first time that doxazosin treatment increases deposition of collagen fibers in the rat prostate stroma (Justulin et al., 2008). Recently, Imamura et al. (2010) found that patients treated orally with α1-blockers had accumulation of collagen fibers in the prostatic stroma. The authors suggested that this structural change could be one of the factors responsible for the development of resistance to this treatment. We recently showed that fibroblasts become more active and that smooth muscle cells shift from a predominantly contractile to a more synthetic phenotype under α-adrenergic blockade by doxazosin (Delella and Felisbino, 2010). However, doxazosin treatment also reduced the weight and volume of the prostate gland. Thus, additional studies using molecular approaches are needed to evaluate if the increased area of collagen fibers observed after doxazosin treatment is due to rearrangement of pre-existing fibers in a smaller gland or de novo synthesis of collagen.

Because the epithelium-stroma cross-talk is vital to prostate homeostasis, and to gain new insights into the molecular mechanisms of action of doxazosin on the prostatic stroma, we investigated the stroma ultra-structural morphology and the expression of some genes involved in the fibrotic pathway, such as collagens type I and III and TGF-beta1 in the rat ventral prostate.

Material and methods

Animals and doxazosin administration

Adult male 3-month-old Wistar rats (n = 30) were maintained in a controlled environment with free access to food and water. The experiment was performed according to the Guide for Care and Use of Laboratory Animals. The animals were divided into two groups: control rats and the doxazosin-treated group. Doxazosin-treated animals received daily doses of doxazosin (Doxazosin mesylate, Pfizer, Calena Pharmaceutical and Chemistry, SP, Brazil) at 25 mg/kg of body weight dissolved in corn oil as vehicle by oral gavage. The dosage and duration of treatments were determined by consulting with Pfizer and have previously been published (Yono et al., 2007). The control animals received only the vehicle. After 7 and 30 days of treatment, 10 animals from each group were euthanized by an overdose of pentobarbital. The ventral prostates were excised and immediately weighed, and the left and right lobes were snap frozen or immersed in cold methanol for 10 minutes. The grids were examined under a Phillips transmission electron microscope operating at 80 kV. The ventral prostate frozen sections (7 μm) were collected on silanized glass slides and fixed in cold methanol for 10 minutes. Sections were then blocked with 3% hydrogen peroxide in methanol for 15 minutes (to block endogenous peroxidase activity) before blocking with 3% bovine serum albumin in PBS for 1 hour at room temperature. The sections were incubated with the following primary monoclonal antibodies for 2 hours at room temperature: Type I Collagen, 1:300, Genetex, GTX 26308; Type III Collagen, 1:300, Abcam, ab 6310; TGF-beta 1, 1:100, Santa Cruz, sc-146; Alpha Actin, 1:300, Santa Cruz, sc-32251. The primary antibody was detected using a secondary peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), except for TGF-beta 1, which was detected using the Mach 4 Universal HRP-Polymer Kit with DAB (Biocare Medical, Concord, CA, USA). Chromogen color development was accomplished with 3,3’-diaminobenzidine tetrahydrochloride (Sigma, USA). Slides were counterstained with Harris’s hematoxylin. The negative control was performed by excluding the primary antibody incubation step.

Protein extraction and western blotting analysis for TGF-beta 1

The ventral prostates frozen samples were mechanically homogenized in 50 mM Tris–HCl buffer pH 7.5, 0.25% Triton X-100 and EDTA by means of a Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 30 s at 4 °C. Following centrifugation of the homogenate, the protein was extracted from the supernatant and was quantified as described by Bradford (1976). Equal amounts of protein (75 μg) from the ventral prostate frozen samples were heated at 95 °C for 5 minutes in the sample-loading buffer and were then subjected to SDS–PAGE under reducing conditions and were transferred to nitrocellulose membranes (Sigma Chemical Co., St. Louis, MO). The blots were blocked with 3% bovine serum albumin in TBST (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 hour and probed overnight with the primary antibody, anti-TGF-beta 1 (1:1,000; MAB240; R&D Systems, Minneapo-lis, USA), Goat anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), served as loading control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies, the blots were detected by means of chemiluminescence (Pierce® ECL Western Blotting Substrate). TGF-beta 1 and β-actin protein expression was quantified by densitometric analysis of the bands and was expressed as integrated optical density (IOD). The TGF-beta 1 expression was normalized to the β-actin values. Normalized data are expressed as the means ± SD.

Collagen I, collagen III and TGF-beta 1 mRNA expression

Total RNA was isolated from ventral prostate samples using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s specifications. The RNA concentration (μg/μl) was determined in each sample using a NanoDrop® ND-1000 UV–vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA purity was ensured by obtaining a 260/280 nm OD ratio equal to 2.0. Total RNA (2 μg) was treated with Amplification Grade Deoxyribonuclease I according to the protocol provided by Invitrogen (Life Technologies Corporation, Carlsbad, CA, USA). The purified total RNA was reverse transcribed with random hexamer primers using a High-Capacity cDNA Archive Kit (Life Technologies Corporation, Carlsbad, CA, USA). Type I collagen, type III collagen and TGF-beta 1 gene expression levels were detected for each
One or two layers of smooth muscle cells surrounded the acini. Delicate collagen fibers and bundles of collagen fibrils filled the stroma between fibroblasts and smooth muscle cells. The smooth muscle cells showed a regular and smooth outline (Fig. 1b).

Seven days of doxazosin treatment promoted activation of fibroblasts (Fig. 1b), which contained large nuclei with loose chromatin and evident nucleoli. The bundles of collagen fibrils were denser between the cells (Fig. 1b). At day 30 of dox treatment, fibroblasts and smooth muscle cells showed many cytoplasm extensions closely associated with thickened bundles of collagen fibrils (Fig. 1c).

**Neurotransmitter vesicle diameter determination**

The ultrastructural analysis showed many axonal varicosities filled with neurotransmitter vesicles and mitochondria dispersed among smooth muscle cells in the ventral prostate from control rats (Fig. 2a), and from ventral prostate of rats treated with doxazosin for 7 (Fig. 2b) and 30 days (Fig. 2c). Morphometrical analysis showed that 30 days of doxazosin treatment significantly increased the mean diameter of the neurotransmitter vesicles (Fig. 3). At day 7, there was a trend towards an increase in the diameter of neurotransmitter vesicles, but without statistically significant difference.

**Type I and type III collagen analyses**

To further examine the effects of doxazosin on major components of the extracellular matrix, we assessed the mRNA and protein expressions of collagens type I and III. Type I collagen was found to be dispersed throughout the stroma, surrounding glandular acini and ducts in the ventral prostate from control rats (Fig. 4a). Dioxazosin treatment increased the density of type I collagen at day 7 (Fig. 4d) and at day 30 of treatment (Fig. 4f), mainly around acini. Expression of the mRNA for collagen type I (COL1A1) was transiently increased at day 7, and decreased significantly at 30 days of dioxazosin treatment (Fig. 7). Type III collagen was found distributed in the interstitial stroma, filling epithelial infoldings and around blood vessels (Fig. 4c) of the ventral prostate stroma from control rats. The α-1 adrenergic blockade also increased the distribution of collagen type III at day 7 (Fig. 4e) and day 30 (Fig. 4g) of the treatment, mainly below the epithelium. Expression of the mRNA for collagen type III (COL3A1) was also transiently increased at day 7, and was significantly decreased at day 30 (Fig. 7).

**TGF-beta 1 expression**

To evaluate TGF-beta 1 localization, mRNA and protein expression in the ventral prostate from control and doxazosin-treated rats, we used immunohistochemistry, real time quantitative PCR and western

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**Fig. 1.** (a) Ultrastructure of the ventral prostate from control rats showing few and thin bundles of collagen fibrils (black arrows) above the epithelium (ep) and around smooth muscle cells (smc) and fibroblast cytoplasmic extensions (f). Elaunic fibers (white arrows). (b) Ventral prostate from a rat treated with doxazosin for 7 days. (c) Ventral prostate from a rat treated with doxazosin for 30 days. Note the dense bundles of collagen fibrils (black arrows) around smooth muscle cells (smc) and active fibroblasts (f). Scale bars = 1 μm.
have been a safe and efficacious pharmacological treatment option for patients suffering from BPH (AUA, 2003; Caine, 1986).

The quinolizone-based class of α-adrenoceptor blockers, such as doxazosin, has the additional effect of inducing apoptosis of prostate cells, which has been demonstrated to be involved in the long-term efficacy of treatments for BPH (Anglin et al., 2002; Chiang et al., 2005; Kyprianou, 2003; Kyprianou et al., 1998; Yang et al., 1997). The induction of apoptosis in prostate cells by doxazosin has been attributed to an increased expression of TGF beta-1 (Glassman et al., 2001; Ilio et al., 2001; Partin et al., 2003; Yang et al., 1997). However, Zhao et al. (2005), using a molecular approach, showed an involvement of TNF-alpha in the doxazosin molecular mechanism of action and disproved the participation of the TGF-beta 1 pathway in doxazosin-induced apoptosis in stromal cells. Thus, more studies are needed to better understand the direct and indirect effects of doxazosin treatment on prostatic tissue cells apoptosis.

Although the involvement of TGF-beta 1 in doxazosin-induced apoptosis is still controversial, there is no doubt that this multifunctional cytokine regulates extracellular matrix production and degradation (Fleisch et al., 2006; Mauviel, 2005; Roberts et al., 1990). Our laboratory has been focused on the changes in the prostatic stroma induced by doxazosin treatment, with special attention to the extracellular matrix components. We have previously demonstrated that doxazosin treatment increases both collagen and elastic system fiber volume fractions in the stroma of the rat prostatic complex (Delella and Felisbino, 2010; Justulin et al., 2008).

In the present study, we showed that 7 days of doxazosin treatment significantly increased the mRNA and protein expressions of collagen type I and III and TGF beta-1 in the rat ventral prostate. Our results are in agreement with previous results of total collagen fibers quantification by picrosirius staining coupled to morphometric analyses (Justulin et al., 2008). Moreover, the ultrastructural findings clearly showed thickened bundles of collagen fibrils around stromal cells in the ventral prostate from doxazosin-treated rats. Together, these data suggest an early proinflamatory effect of doxazosin on prostate stroma via TGF-beta-1 signaling. Although rodent models present some limitations and no entirely results can be applied for studying human BPH, they provide interesting information about the behavior of the prostatic tissue under several different experimental conditions and shed light on the molecular mechanisms behind it. In this sense, our results using rats corroborate the findings of Inamura et al. (2010) that have addressed the effect of doxazosin treatment on collagen fibers deposition in the human hyperplastic prostate gland. As observed in our study, these authors have found an increased amount of collagen fibers in a group of patients treated by doxazosin and suggested that this stromal alteration may be related to reduction in the long-term effectiveness of the treatment.
Notably, mRNA expression for both collagens I and III was significantly decreased after 30 days of doxazosin treatment, whereas TGF-beta 1 mRNA, but not the protein, remained increased. This late effect of doxazosin on collagen mRNA expression suggests a reduction in the turnover of these proteins by stromal cells. These results reinforce the importance of evaluating different times of exposure to a better understanding of doxazosin effects on the prostate gland. The consequence of this late down regulation of collagen mRNA expression by doxazosin for the maintenance of normal prostatic stroma homeostasis and for the epithelium-stroma interactions still needs to be determined and is currently under investigation by our laboratory.

The morphology of stromal cells was also affected by alpha1 blockade. We showed previously that prostatic smooth muscle cells exhibited a synthetic phenotype (Delella and Felisbino, 2010) after doxazosin treatment. Smooth muscle cells are known to reversibly switch phenotypes between a synthetic and a contractile state (Halayko et al., 2008; Zhou et al., 2009). Smith et al. (1999) showed that doxazosin decreased the contractility and expression of actin and myosin proteins in prostatic smooth muscle cells in vitro. This suggests that alpha1-blockers may induce dedifferentiation of smooth muscle cells into fibroblasts and myofibroblasts. However, if this possible dedifferentiation occurs in vivo remains to be determined. In the present study, we observed activated fibroblasts with enlarged cytoplasm and loose chromatin after 7 days of doxazosin treatment. Activated fibroblasts are in agreement with the up regulation of collagen mRNA and TGF-beta 1 protein observed at this period of the treatment. Furthermore, fibroblast phenotype at 30 days of doxazosin treatment is also in agreement with the down regulation of collagen mRNA and the normalization of TGF-beta 1 proteins. Together, these results reinforce our previous hypothesis (Delella and Felisbino, 2010; Justulin et al., 2008) that the reduction of smooth muscle cell tonus induced by doxazosin treatment promotes changes in the prostatic tissue mechanical properties, leading to extracellular...
matrix remodeling by fibroblasts and smooth muscle cells. We propose that this stromal remodeling following doxazosin treatment may be detrimental for BPH therapy, since it increases the static component of the prostatic tissue that does not respond to alpha blockers (Imamura et al., 2010). Since quinazoline derivatives has alpha-1 adrenoceptor independent effects, future studies from our laboratory will evaluate our hypothesis using Tamsulosin, a non-quinazoline selective α-adrenergic antagonist that has more specificity for α-1A and α-1D receptor subtypes.

Curiously, phenylephrine treatment, a selective α1-adrenergic receptor agonist, did not exhibit opposite effect than doxazosin with regards to fibrosis. Previous studies showed that phenylephrine treatment induced atypical prostatic hyperplasia and mild fibrosis in the rat ventral prostate (Golomb et al., 1998; Rosenzweig et al., 2004; Rosenzweig-Bublil and Abramovici, 2006). These effects have been related to a tissue repair process occurring subsequent to the inflammatory exudates that takes place during early period of phenylephrine treatment.

Conclusion

In summary, our data suggest that relaxation of smooth muscle cells by treatment with an alpha-blocker modulates collagen mRNA expression in the prostatic stroma via the TGF-beta 1 signaling pathway, leading to a marked remodeling of cellular and extracellular elements of the prostate stroma. Our results contribute to a better understanding as the human prostate, the rat ventral prostate is largely innervated by both the sympathetic and parasympathetic nervous system (Vaalasti and Hervonen, 1979, 1980). The contraction of the smooth muscle cells that surround acini and ducts is under sympathetic control, and its activation initiates the contractile function of the genital duct system and the prostatic muscular element (Bruschini et al., 1978). It is well known that doxazosin is a selective alpha 1 blocker that occupies the alpha 1 adrenoceptor site for norepinephrine, thereby inhibiting smooth muscle contraction (Lepor, 2007). Our study showed for the first time that alpha blockade increases the size of neurotransmitter vesicles inside the axonal varicosities dispersed along smooth muscle cells. It is known that after exocytosis, norepinephrine may be transported back into synaptic vesicles by the norepinephrine transporter and the vesicular monoamine transporter (Eisenhofer, 2001). However, how doxazosin is involved in this process remains to be determined. Our results encourage future studies to investigate if doxazosin and other alpha-blockers may increase norepinephrine reuptake into axonal varicosities and vesicles.

![Fig. 5. Immunohistochemistry for TGF-beta 1 protein in the rat ventral prostate. (A) Control; (B) doxazosin day 7; (C) doxazosin day 30. Note the positive immunostaining for TGF-beta 1 in the epithelial and stromal compartments (arrows), which is increased in the doxazosin day 7 group. Bars = 10 μm.](image)

![Fig. 6. (A) Representative western blot analyses of TGF-beta 1 and β-actin (Actin) in homogenates from ventral prostates (lane 1 = control; lane 2 = doxazosin day 7; lane 3 = doxazosin day 30). Seventy five micrograms of protein was loaded into each lane (15 μg from five different individual homogenates). (B) Relative expression (IOD integrated optical density) of TGF-beta 1 and β-actin (loading control) (Fig. a). TGF-beta 1 was detected in the ventral prostate from all groups and was increased in the doxazosin day-7 group (Fig. B). Data are as mean±SD. *Statistically significant different from control with p<0.05.](image)

![Fig. 7. Collagens type I (COL1A1) and III (COL3A1) and TGF-beta 1 mRNA expression as quantified by qRT-PCR in the ventral prostate of rats treated with doxazosin for 7 and 30 days. Levels of COL1A1 and COL3A1 and TGF-beta 1 mRNA were significantly up regulated at day 7 of treatment and down regulated at day 30. The target genes were normalized to the level of the housekeeping gene GAPDH. Data are significantly different from control group at p<0.05 (*), p<0.01 (**) and p<0.001 (***)](image)
of the molecular mechanisms of action of alpha-blockade on prostate histoarchitecture and the response to BPH treatment.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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