The Prostate 44:111–117 (2000)

Modulating Effect of Estrogen and Testosterone on Prostatic Stromal Cell Phenotype Differentiation Induced by Noradrenaline and Doxazosin

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BACKGROUND. Noradrenaline (NA) has been shown to enhance expression of the contractile phenotype of human prostatic stromal cells in tissue culture. This study examined the possibility that changing levels of sex hormones in elderly men with BPH may modulate the differentiating effect of NA and hence the efficacy of α_1-adrenoceptor-blocking drugs.
METHODS. Confluent, quiescent stromal cell cultures from 6 different patients were treated with combinations of 20 μM NA, 1 μM doxazosin, 0.1 μM β-estradiol, and 0.1 μM testosterone, over a period of 10 days. Harvested cells were labelled with fluorescein-conjugated antisera to α-smooth muscle actin and myosin to identify cells of contractile phenotype which were thereafter analyzed flow-cytometrically.
RESULTS. NA increased mean immunoexpression of both actin and myosin. Enhancement of myosin expression was highly significant ($P \leq 0.02$). This effect was incompletely opposed by doxazosin. Neither estradiol nor testosterone influenced mean expression of contractile filaments and did not significantly enhance or inhibit the effects of NA or doxazosin. However, both sex hormones exhibited a differentially powerful effect on cell lines from individual patients. The expression of myosin increased by NA was further elevated by addition of estradiol in four of the cell lines and by testosterone in three.
CONCLUSIONS. The data suggest that levels of estrogens and androgens, either alone or in combination, are unlikely to predict the development of obstructive symptoms in patients with BPH or their response to doxazosin. Nevertheless, prostatic stromal cells from individual patients may be exceptionally sensitive to both sex hormones, with enhanced modulation towards a contractile phenotype. Since α- and β-subtypes of the estrogen receptor are differentially expressed between the stroma and epithelium of the early fetal prostate, it is likely that interaction between sex hormones and noradrenaline is an important factor in determining the phenotypic composition of prostatic stroma at this early stage of development, and possibly predisposition to BPH during later adult life. *Prostate 44:111–117, 2000.*
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KEY WORDS: **human prostate; smooth muscle cells; flow cytometry; doxazosin; estrogen; androgen; noradrenaline; differentiation**

INTRODUCTION

Benign prostatic hyperplasia (BPH) is frequently associated with symptomatic urinary outflow obstruction accompanied by urinary retention. Although much of this obstruction may be due to physical occlusion of the urethra by the enlarged prostate gland,

Grant sponsor: Pfizer Research International; Grant sponsor: Engineering and Physical Science Research Council.

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Received 12 May 1999; Accepted 10 March 2000

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it is also exacerbated by contraction of stromal smooth muscle cells surrounding the urethra. This contraction is mediated, in part, by noradrenaline which binds $\alpha1_A$-adrenoceptors expressed on plasma membranes of stromal smooth muscle cells and myofibroblasts [1–6]. In recent years, α_1-adrenoceptor antagonists have been employed clinically to successfully oppose the influence of noradrenaline (NA) and hence improve urinary flow [7–9].

Recently, we showed by in vitro studies that noradrenaline not only induces contraction of prostatic stromal cells but also induces expression of a more contractile cell-type [10]. Such phenotypic modulation is most pronounced when cells are plated out sparsely and are in a logarithmic phase of active proliferation. Furthermore, addition of the α_1-adrenoceptor antagonist, doxazosin, to the medium largely reverses this phenotypic modulation [11], particularly when the cells are confluent and proliferatively quiescent, as in the intact hyperplastic gland. These findings suggest that α_1-adrenoceptor antagonists may not only inhibit contraction of prostatic stroma, but may also reduce its initial capacity to contract. However, despite giving valuable insight into one important aspect of the interaction between noradrenaline and prostatic stromal cells, these particular experiments did not address the possible modulating role of sex hormones on the response of hyperplastic prostatic tissues to NA. It is generally acknowledged that an imbalance between the relative levels of estrogens and androgens, consequent upon a declining secretion of testosterone from middle age onwards, is an important factor in the etiology of BPH [12–15].

Studies of rat early fetal prostate have shown that estrogen receptor (ER) subtypes are differentially expressed between the epithelium and stroma [16], such that ERα is expressed by stromal mesenchymal cells, while ERβ is differentially expressed by epithelial cells within different lobes of the gland. In the nonneoplastic mature adult prostate, estrogen receptors are located almost exclusively in stromal cells which are, therefore, able to respond to any relative estrogen excess [13,17–20]. One such response in vitro is an increased density of intracellular desmin and myosin filaments [21]. Stromal estrogen receptors also modulate the mitogenic effects of estrogen on prostatic epithelium [22]. Neonatal exposure to low-dose estrogen regulates estrogen receptor expression in the developing and adult rat prostate stroma and epithelium [15], thus providing mechanisms for genetic imprinting or for possible development of hyperplastic/proliferative diseases in later life. In fibroblasts known to express ER, exposure to estrogen increases levels of the androgen receptor. Furthermore, functional interaction with androgen receptor-expressing stromal cells is required for successful functional development of prostatic epithelium [23]. However, the role of estrogenic stimulation is complex with respect to proliferation and to expression of other differentiation proteins, including the androgen receptor. Depending on the stage of intrauterine growth and relative levels of hormone achieved, estrogen exhibits a biphasic response on prostatic enlargement in mice. While low concentrations modulate the actions of androgen to stimulate a 40% growth in epithelium through a twofold induction of androgen receptors, high concentrations of estrogen cause a reduction in prostatic size relative to body mass [24].

Since estrogen and noradrenaline, independently, appear to influence the phenotype of prostatic stromal cells in a similar manner, this current study was performed to test the hypothesis that a synergistic relationship between these two humoral agents may result in enhanced differentiation of a population of stromal cells. Following our previous observation that the selective α_1 adrenoceptor antagonist, doxazosin, inhibits, or partially reverses, the differentiating effect of noradrenaline, we predicted that any synergistic estrogen effect should be diminished in its presence. Although androgen receptors are found in the stroma as well as the glandular epithelium [25,26], little is currently known about the effect of testosterone on the phenotype of stromal cells. Therefore, the final aim of this study was to analyze the interactive effect of testosterone with noradrenaline and doxazosin on the phenotypic differentiation of cultured human prostatic stromal cells, as assessed by flow cytometry. The data from this study confirm the initial hypothesis that a synergistic effect between estrogen and noradrenaline modulates the differentiated phenotype of stromal cells derived from adult human prostate. However, this interaction is not one of simple synergy but is complex and likely to be influenced by other factors.

MATERIALS AND METHODS

Prostatic tissues were obtained from 6 patients who had undergone transurethral prostatic resection for BPH. Stromal cells were isolated from the tissues by collagenase digestion, as previously described [10]. Cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, Life Technologies, Paisley, Scotland, UK) in a humidified atmosphere of 5% CO_2 and air at 37°C. Cells were passaged 4–5 times to provide six different cell lines for study. Cells from each line were seeded into 16 tissue culture dishes (9-cm diameter) at a density of approximately 6×10^3 cells/cm^2 and maintained in the above growth medium until

TABLE I. Mean Fluorescence Intensities of Alpha-Smooth Muscle Actin and Myosin Expressed by Six Prostatic Stromal Cell Lines†

		Smooth muscle actin		Myosin	
Group	Treatment	Fluorescence intensity	Fluorescence as ratio of control	Fluorescence intensity	Fluorescence as ratio of control
1	C	64.4 (46.1)	1.00	56.7 (29.3)	1.00
	N	75.2* (55.2)	1.14* (0.14)	72.7* (35.9)	1.32* (0.18)
	D	61.6 (42.5)	0.97* (0.06)	52.0* (28.5)	0.92* (0.05)
	ND	70.4* (50.2)	1.08*,** (0.11)	71.6* (36.7)	1.28* (0.13)
2	E	67.2 (42.1)	1.07 (0.17)	57.3 (30.5)	1.01 (0.10)
	EN	71.6 (42.3)	1.15 (0.22)	75.7* (33.8)	1.47 (0.48)
	ED	63.5 (42.7)	1.02 (0.12)	53.3 (29.3)	0.94* (0.07)
	NED	69.8* (42.5)	1.07* (0.13)	71.1* (32.8)	1.40 (0.49)
3	T	63.6 (51.0)	0.96* (0.10)	56.6 (33.8)	0.98 (0.10)
	TN	73.0 (61.9)	1.09 (0.21)	75.1* (36.7)	1.43* (0.41)
	TD	61.0 (45.6)	0.94* (0.08)	54.1* (30.3)	0.94* (0.05)
	NTD	77.2 (58.9)	1.18 (0.39)	73.3* (36.8)	1.39 (0.40)
4	TE	62.1 (47.0)	0.96 (0.10)	56.2 (31.1)	0.97 (0.08)
	TEN	74.5 (63.6)	1.10 (0.18)	72.4* (36.3)	1.37* (0.39)
	TED	66.8 (50.6)	1.01 (0.13)	56.1 (31.6)	0.97 (0.07)
	TEND	75.2 (62.9)	1.11 (0.16)	70.2*,** (33.6)	1.31* (0.26)

†C, untreated control; D, doxazosin at 1 μM; T, testosterone at 0.1 μM; N, noradrenaline at 20 μM; E, estradiol at 0.1 μM. Numbers in parentheses are ± one standard deviation from the mean.
*Significantly different from control (C).
**Significantly different from noradrenaline (N); $P < 0.05$.

they had proliferated to confluence. The medium was then replaced with 10 ml RPMI-1640 containing 2 mM L-glutamine, antibiotics, and 1% (v/v) dialyzed and carbon-stripped FCS (to remove exogenous steroid hormones) to render cells quiescent. The dishes were then divided into four groups of four dishes each, to which noradrenaline bitartrate (Arterenol, Sigma Chemical Co., Poole, Dorset, UK), β-estradiol, testosterone (Sigma), and doxazosin mesylate (Cardura®, Pfizer, Ltd., Sandwich, Kent, UK) were added with a micropipette, according to the scheme summarized in Table I. The final concentrations of each substance in the medium in each case were as follows: noradrenaline, 20 μM; β-estradiol, 0.1 μM; testosterone, 0.1 μM; and doxazosin, 1.0 μM. This is higher than the plasma concentration of approximately 0.075 μM of doxazosin in a man taking 4 mg of the drug daily [27].

An extra dish of untreated cells was reserved as a negative control for subsequent immunolabeling. The medium, hormones, and doxazosin were replaced on alternate days for a total of 10 days.

Cells were released from each of the 102 dishes by exposure to trypsin/EDTA for 5 min, followed by agitation until a monodisperse suspension of cells was obtained. Trypsin was inactivated by addition of 1 ml FCS, and the cells washed with phosphate-buffered saline (PBS) and centrifuged, and the pelleted cells resuspended in 1 ml of 70% (v/v) chilled methanol. Cells from each treatment were then divided equally into two microcentrifuge tubes and rehydrated with PBS for 30 min, and the supernatants were replaced with 200 μl of blocking buffer, comprising PBS containing 1% (w/v) bovine serum albumin, and left overnight. After centrifugation, supernatants were replaced with 100 μl of monoclonal antibodies to either α-smooth muscle actin (Sigma clone 1A4, dilution 1:640) or to smooth muscle myosin (Sigma clone hSM-V, dilution 1:200). Cells were incubated at 37°C for 40 min with periodic agitation to maintain cells in a dispersed state. The antisera were then withdrawn and the cells were washed in 1 ml of blocking buffer for 10 min, followed by the addition of 100 μl of sheep anti-(mouse IgG) conjugated to fluorescein isothiocyanate, diluted 1:250 in blocking buffer. Incubation was for a further 30 min at 37°C, after which supernatants were replaced with 200 μl PBS. Negative control cells from each cell line were treated with an antibody of irrelevant specificity but of the same isotype as the primary antibody, followed by the fluorescent secondary antibody.

The fluorescence intensity of each sample of cells was measured in a Becton Dickinson FACsort flow cytometer (San Jose, CA) in which the sensitivity was adjusted so that 95% of the negative controls fell within the first log decade. A total of 20,000 events was counted, with electronic noise and subcellular de-

TABLE II. Percentage Increases in Fluorescence Above Baseline for Actin and Myosin in Each Stromal Cell Line Treated With Noradrenaline, Estradiol, and Testosterone

Treatment	Percent increase in filament expression for cell lines:					
	A	B	C	D	E	F
Actin						
Noradrenaline	−4	17	28	−2	21	26
Estradiol + noradrenaline	7	3	34	13	−3	−1
Testosterone + noradrenaline	17	20	45	−3	−10	8
Testosterone + estradiol + noradrenaline	1	31	50	−2	3	9
Myosin						
Noradrenaline	42	33	35	29	0	53
Estradiol + noradrenaline	122	22	50	33	−6	60
Testosterone + noradrenaline	139	39	66	21	−1	29
Testosterone + estradiol + noradrenaline	143	12	61	33	−15	41

bris excluded by setting a threshold on forward light scatter. Data were recorded as frequency distribution histograms and as mean fluorescence intensity units derived from cells with a fluorescence greater than that of 95% of the negative control cells. Data were analyzed statistically by ANOVA and paired *t*-tests, and were considered to be statistically significantly different where $P < 0.05$.

RESULTS

Flow cytometric fluorescence intensities expressed as the mean of the six stromal cell lines are shown in Table I. To aid comparison, the data are also normalized by expressing each fluorescence as a ratio of the untreated control (C). Analysis of variance (ANOVA) was performed on the data derived from the four treatments collectively in each experimental group, as defined in Table I. For cells labelled for α-smooth muscle actin, differences in the variance of fluorescence intensity were not significant and, when expressed as a ratio of control, were significant only in group 1. Similarly, ANOVA of myosin fluorescence intensity was not significant but, when expressed as a ratio of control, all four of the experimental groups showed a significant difference (group 1, $P < 0.001$; group 2, $P < 0.05$; group 3, $P < 0.025$; group 4, $P < 0.01$). The data were also analyzed by paired *t*-tests which compare the arithmetic differences between related pairs of data. The results of this analysis are indicated in Table I. Addition of noradrenaline to the medium increased the mean fluorescence of α-smooth muscle actin above control values in each instance, although this was statistically significant only for group 1. For myosin the increases were more pronounced and significant in all four groups.

Addition of estradiol (E), testosterone (T), or both hormones (TE) to the medium induced negligible changes to mean fluorescence of either actin or myosin, as shown by ratios close to unity. Similarly, both hormones exhibited little effect on the ability of noradrenaline (N) to alter the expression of myofilaments. Thus, although EN, TN, and TEN were all higher than the control (significantly so for myosin), in no instance were they significantly different from N or from one another.

Doxazosin alone (D) was associated with reduced expression of both myofilaments, which was significant for mysoin and also for the ratio of actin:control. Similar reductions occurred in groups 2 and 3 (ED and TD) and were significant in several instances (Table I). In group 4 (TED), there was little change from control values. Doxazosin in conjunction with noradrenaline (ND) induced a significantly higher immunofluorescence than control values (C) for both actin and myosin. Similar increases were obtained from groups 2 and 3 (NED and NTD). These values, particularly those for myosin, were lower than the corresponding values obtained from noradrenaline in the absence of doxazosin (EN and TN), indicating that the drug had incompletely reversed the influence of noradrenaline on myofilament expression. However, only in two instances (ND, actin ratio; and TEND, myosin fluorescence) were these values significantly lower than those for noradrenaline alone (N). Sex hormones did not significantly enhance or inhibit the influence of doxazosin plus noradrenaline, despite the fact that testosterone (NTD and TEND) appeared to prevent doxazosin from reducing the effect of noradrenaline on smooth muscle actin (Table I).

Although sex hormones had little effect on mean fluorescence values from the six cell lines, they had a considerable effect on myofilament expression by some individual cell lines. Table II shows the percent-

age increases above baseline (C, E, T, and TE) for each stromal cell line treated with noradrenaline in conjunction with estradiol, testosterone, and both hormones, respectively (N, EN, TN, and TEN). In cell line C there was a 28% increase in fluorescence of actin induced by noradrenaline alone, which was raised to 34% in the presence of estradiol, to 45% with testosterone, and further to 50% with both hormones. The increased expression of myosin was further raised by addition of estradiol in four cell lines (A, C, D, and F) and by testosterone in three cell lines (A, B, and C). Cell line A in particular showed a 122% increase in myosin expression with estradiol and a 139% increase with testosterone, as contrasted with a 42% increase with noradrenaline alone. In other cell lines, sex hormones failed to augment, or even reduced, the influence of noradrenaline (Table II).

DISCUSSION

The data from this study confirm our previous reports that noradrenaline promotes expression of contractile filament proteins in prostatic stromal smooth muscle cells [10,11]. The study further supports our observation that the α_1-adrenoceptor antagonist, doxazosin, reduces expression of myofilaments actin and myosin within these cells [11]. Doxazosin, in the absence of noradrenaline, induced a fall in the mean expression of actin and myosin, an effect which was greater and significant for the latter filament protein in experimental groups 1–3 (see Table I). This reduction in contractile filaments was greater than that recorded in our earlier study [11], in which neither actin nor myosin showed a significant fall. Doxazosin also partially reversed the increase in myofilament expression induced by noradrenaline in most of the experimental groups (the exceptions being NTD and TEND labeled for actin). However, this response was incomplete and varied greatly within the six cell lines examined, so that statistical significance was reached only in two instances (Table I). In a previous study of 10 stromal cell lines, we found a greater and more consistent response to doxazosin, such that the level of actin fluorescence and the ratios of actin and myosin to control values were significantly lower than their corresponding levels with noradrenaline alone [11]. These findings suggest that noradrenaline may enhance the contractile phenotype of prostatic stromal cells in BPH, with the potential effect of increasing their contractile force and thus exacerbating urethral obstruction. Moreover, doxazosin may not only inhibit noradrenergic contraction of stromal smooth muscle cells but, by reducing their quantity of myofilaments, may reduce their original capacity to contract. The effect of doxazosin on the expression of myofilaments shows a minimal dose dependency over the concentration range of 0.001–10.0 μM [11], suggesting that the drug may have an action other than that of a simple adrenoceptor antagonist. This possibility is supported by the observation that doxazosin, when administered to patients with BPH, enhances apoptosis of prostate cells, particularly those of the stroma [28,29]. Also, mitogenesis and migration of vascular smooth muscle in culture are inhibited by doxazosin, even when there is prior α_1-adrenoceptor blockade by phenoxybenzamine [30].

Recently, Boesch et al. [31] demonstrated a change in the relative proportions of mRNA encoding the two isoforms of smooth muscle type myosin heavy chain (SM1 and SM2) in cultured prostatic stromal cells exposed to doxazosin for 6 days. Thus, the ratio of SM2:SM1 was increased 10-fold, suggesting a change from a secretory to a more contractile phenotype. This finding is at variance with our own observations and also with the authors' earlier paper [32], both of which imply that doxazosin reduces the contractility of stromal smooth muscle. The anti-smooth muscle myosin antibody which we employed does not distinguish between SM1 and SM2, but demonstrates instead a reduction in total myosin immunoexpression induced by doxazosin. Furthermore, Boesch et al. [31] demonstrated that stimulation of stromal adrenoceptors by phenylephrine for 6 days had a negligible effect on the myosin heavy-chain ratio, though we have shown repeatedly in this and previous papers [10,11] that noradrenaline consistently increases the quantity of myosin in stromal cells. Clearly, the SM2:SM1 ratio and the total myosin content are independently modulated by noradrenaline and doxazosin.

In the present study, additional modulating effects of estrogen and androgen were tested concurrently with noradrenaline and doxazosin. Since the concentrations of sex hormones change during the aging process [12–14], we reasoned that they may modify the influences of noradrenaline and doxazosin on the prostatic stromal cell phenotype. This effect appeared likely following the report by Zhang et al. that estradiol (and to a lesser extent testosterone) increases the density of myosin and desmin filaments in cultured human prostatic stromal smooth muscle cells [21]. We found no such enhanced fluorescence as assessed by flow cytometry in the mean values from six stromal cell lines, despite the use of identical concentrations (0.1 μM) of estradiol and testosterone. However, an important methodological difference may be responsible for this apparent discrepancy. Zhang et al. [21] added hormones while their cultures were in a proliferative phase and then fixed them at confluence. In our experimental model, cells were allowed to reach confluence before the addition of hormones, and were

then prevented from any further growth by replacing the serum in the medium with 1% dialyzed and carbon-stripped FCS. The purpose of this treatment was to more closely simulate the normal physiological state of stromal smooth muscle cells in the intact hyperplastic prostate in which the stromal cells are compact and largely nonproliferating. Under these conditions, neither estradiol nor testosterone exhibited any significant influence on the phenotype of prostatic stromal cells, either with or without the addition of noradrenaline. Furthermore, the two hormones failed to affect the efficacy of doxazosin to modulate the phenotype of stromal cells to a less contractile form. With respect to an individual patient with BPH, our results suggest that relative levels of androgens and estrogens are unlikely to be useful predictors of the development of obstructive symptoms, or for the response to doxazosin or other α_1-adrenoceptor blocking agents.

Although our data and conclusions are based on mean values obtained from six cell lines, there was considerable variation in response between individual cell lines, with some showing a dramatic increase in myofilament expression in response to estradiol and/or testosterone together with noradrenaline, while in others there was little change or even a negative response (Table II). Hence, although patients with BPH may not show a consistent relationship between levels of sex hormones and expression of prostatic stromal cell myofilaments, there may be some individuals in whom sex hormones are of profound importance. However, a similar or greater response to testosterone was obtained in those stromal cell cultures in which estrogen stimulated an enhanced response to noradrenaline. In such individuals, predisposition to certain responses may reflect cellular and molecular changes occurring during fetal development, including exposure to different levels of estrogen, resulting in differential genetic imprinting. Thus, prostatic stromal cells from some patients with BPH may be highly sensitive to sex hormones and may show enhanced modulation towards a contractile phenotype under noradrenergic stimulation. Our results suggest that a shift in hormone balance towards estrogen excess is unlikely to be a significant contribution to this phenotypic modulation, and that other humoral factors are probably of greater importance, such as those derived from the prostatic epithelium.

ACKNOWLEDGMENTS

C.S.F. thanks Pfizer Research International for continued generous support of this project. N.P.R. acknowledges the support of the Engineering and Physical Science Research Council through a rolling program grant on biocompatibility. We thank Mrs. Jill Gosney for typing the manuscript.

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