Elevated expression of orexin receptor 2 (*HCRTR2*) in benign prostatic hyperplasia is accompanied by lowered serum orexin A concentrations

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Abstract. In search for the new polypeptides responsible for energy homeostasis which are also involved in regulating the growth and function of the human prostate, we assessed the expression of orexins (OXs) and of orexin receptors (OXRs) in human normal prostate and in benign prostatic hyperplasia (BPH). Conventional RT-PCR revealed the expression of *OXR2* in all studied samples obtained either from normal prostates or BPH ones while neither preproorexin (ppOX)nor OXR1 mRNA were detected. In adenomatous prostates, expression levels of *OXR2* were 30- to 40-fold higher compared to controls. Western blot analysis demonstrated the presence of OXR2 protein in the studied samples and its expression levels were 4-fold higher in tissue samples from BPH. In normal glands, presence of OXR2-like immunoreactivity was found in the apical parts of epithelial cells as well as in smooth muscle cells of the stroma. Immunostaining for OXR2 was more intense in sections obtained from BPH. Immunohistochemistry did not detect the expression of OXR1-like protein. OXA serum concentrations were lowered in BPH patients (mean ± SE 56±4 ng/ml, n=12; P<0.01) and unaltered in prostate cancer (79±7 ng/ml, n=18) compared to the controls (69±2 ng/ml, n=16). On the contrary, serum OXB levels were similar in all studied groups of patients. We thus have demonstrated the mRNA and protein expression of OXR2, but not of ppOX and OXR1 in both normal and BPH human prostate glands. We also demonstrated notable up-regulation of OXR2 in benign prostatic hyperplasia, an alteration accompanied by lowered serum OXA concentrations. These findings suggest that both OXA and OXR2 may be involved in the pathogenesis and/or maintenance of BPH.

Introduction

Several lines of evidence show that some neuropeptides which control energy homeostasis, may also either directly or indirectly be involved in the development of benign prostatic hyperplasia (BPH) and prostate carcinoma (PC). In this regard, special attention is being paid on obesity and prostate cancer incidence and accumulating data suggest the higher risk of aggressive cancer development in obese patients (1-5). Leptin seems to be one of the most important factors related to obesity and PC, and numerous rapports suggest leptin association with an increased risk of prostate cancer (1,3,4,6-22). However, in human and animal prostates expression of the leptin-leptin receptor system is disputable and this system is probably not directly involved in BPH or PC development and maintenance (23-25).

Orexins [OXs; orexin-A (OXA) and orexin-B (OXB)] belong to a group of endogenous polypeptides involved in energy homeostasis. They originate from the proteolytic cleavage of preproorexin (ppOX, ppHCRT) and act through two subtypes of receptors, named OXR1 (HCRTR1) and OXR2 (HCRTR2). OXR1 almost exclusively binds OXA, whereas OXR2 is non-selective for both orexins (26-29). In search for the new polypeptides responsible for energy homeostasis which are also involved in regulating the growth and function of the human prostate, we performed studies on the expression of OXs and OXRs in human normal prostate and in BPH. To our knowledge, this is the first report demonstrating a very high expression of *OXR2* in BPH compared to its very low expression in normal prostate.

Materials and methods

Studies were performed on patients from the Department of Urology and Urooncology, Poznan University of Medical Sciences. The Bioethics Committee of the University provided

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Abbreviations: OX (HCRT), orexin; ppOX (ppHCRT), preproorexin; OXA, orexin A; OXB, orexin B; OXR1 (HCRTR1), orexin receptor 1; OXR2 (HCRTR2), orexin receptor 2; BPH, benign prostatic hyperplasia; PC, prostate cancer

Key words: orexin receptor 2, benign prostatic hyperplasia, QPCR, immunohistochemistry, orexin A blood levels, human

Table I. Basic clinical data of patients with benign prostatic hyperplasia (BPH) and of the control group.

Group	Age (years)	Body weight (kg)	Height (cm)	BMI (kg/m²)	BF (%)	PSA (ng/ml)
Control (n=15)	63±2	84±2	176±1	27.0±0.7	30.74±0.69	1.3±0.0
BPH (n=15)	72±2[a]	86±4	172±1	28.9±1.2	35.13±1.45[b]	11.6±3.5[a]

Unchanged prostate glands were removed from patients who underwent total cystectomy because of bladder carcinoma (ca urotheliale, n=14 and sarcomatoid urothelial carcinoma, n=1) and were subjected to molecular biology studies. BMI, body mass index (kg/m²); BF%, body fat percentage; PSA, prostate specific antigen (ng/ml). Results are means ± SE. The unpaired Student's t-test was used for the statistical evaluation of the differences in relation to the control group; [a]P<0.01, [b]P<0.02.

Table II. Conventional RT-PCR and QPCR analyses of preproorexin (*ppOX*), orexin receptor 1 and 2 (*OXR1* and *OXR2*) gene expression in studied prostates.

cDNA	GenBank Accession No.	Primer	Primer sequence (5'-3')	Position	PCR product size (bp)
ppOX	NM_001524	S	CATCTCCTTTCCCGGCTACC	47-66	73
		A	GCCCAGGAGACCTTTGTGGA	100-119	
OXR1	NM_001525	S	CAACAGGTTCTTGGTGAAG	177-195	82
		A	TCAGCCTCAAACTTCCTTA	240-258	
OXR2	NM_001526	S	CCAATAGTGCTGCGAATCC	1379-1397	177
		A	AGTTGCTGATTTGAGTGGTC	1536-1555	
HPRT	NM_000194	S	AATCCAAAGATGGTCAAGGTC	627-647	256
		A	TTTACTGGCGATGTCAATAGG	862-882	

HPRT (hypoxanthine-guanine phosphoribosyltransferase) is the reference gene. Oligonucleotide sequences for sense (S) and antisense (A) primers are shown.

consent for the study protocol. Before the study, every participant signed an informed consent form.

Prostates. PCR, Western blotting and immunohistochemical studies were performed on 15 normal prostates obtained from patients who underwent total cystectomy because of bladder carcinoma, and on the same number of glands obtained by adenectomy of BPH patients. Histopathologically, no significant alterations were found in normal glands, while BPH prostates had typical features of hyperplasia. Basic clinical data of studied patients are presented in Table I.

QPCR analysis. Tissue samples from normal prostates were taken from the regions adjacent to the colliculus seminalis (subsequently called the central part) and the connective tissue capsule (subsequently called the peripheral region). Tissue samples from BPH patients (adenectomy) were taken from the anterior periurethral area. Total RNA was extracted by means of the TRI reagent and the RNeasy Mini kit (Qiagen, Hilden, Germany) using the standard procedure. The concentration and purity of the RNA were determined spectrophotometrically (NanoDrop, Thermo Scientific, Waltham, USA). From every sample, 1 μg of total RNA was reversely transcribed using the

MMLV reverse transcriptase kit (Novazym, Poznan, Poland) using Oligo dT (PE Biosystems, Warrington, UK) as primers. The reaction was performed at 42.8˚C for 60 min (UNO II Thermocycler, Biometra, Göttingen, Germany). All primer sets were designed to span at least one intron (Table II). They were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). Real-time PCR was carried out in the Roche LightCycler 2.0 (Roche) with software version 4.05. The SYBR-Green detection system was used with the above-mentioned primers. PCR reactions were carried out in 20 μl mixtures, containing 4 μl template cDNA, 0.5 μM of each gene-specific primer and 3.5 mM of Mg^{2+} ions. LightCycler FastStart DNA Master SYBR-Green I mix (Roche) was used. The real-time PCR program included a 10 min denaturation step to activate the Taq DNA polymerase, followed by a three-step amplification program: denaturation at 95.0˚C for 10 sec, annealing at 58.0˚C for 5 sec and extension at 72.0˚C for 5 sec. Specificity of the reaction products was routinely checked by determination of melting points (0.1˚C/sec transition rate) and the sample was separated in a 2.5% ethidium bromide/agarose gel. All PCR reactions were performed in triplicates, and the hypoxanthine

phosphoribosyltransferase (*HPRT*) gene was used as a reference to normalize the data. Templates not submitted to the RT reaction served as negative controls. PCR efficiency was assessed by a serial dilution method. Briefly, the products of the RT-PCR reactions were separated in a 2.5% agarose gel and specific bands were extracted using a DNA gel extraction kit (Millipore, Billerica, USA). The amount of extracted DNA was estimated spectrophotometrically. Extracted DNA was diluted (10-fold serial dilutions) in order to generate a standard curve for efficiency calculation. The employed version of the LightCycler software (version 4.05) allowed evaluation of the amplification efficiency plots.

Western blot analyses. Tissue samples were homogenized in RIPA buffer with the addition of protease inhibitor buffer, and were centrifuged at 600 x g for 30 min at 4°C to remove cell debris. The protein concentration was determined by the Bradford method. For every sample, 20 μg of protein were separated on a 4-20% gradient SDS-polyacrylamide electrophoretic gel and transferred onto a nitrocellulose membrane. The transferred proteins were stained with Ponceau S. Blocking of non-specific binding and incubation with antibodies was carried out with the SNAP i.d. Protein Detection System (Millipore) according to the manufacturer's guidelines. Membranes were incubated with primary goat anti-OXR2 serum (1:100) (Santa Cruz Biotechnology). Afterwards, the membranes were thoroughly washed and incubated with a secondary horseradish peroxidase-conjugated anti-goat (Santa Cruz Biotechnology) serum. For reference purposes, GAPDH detection was performed using primary mouse monoclonal GAPDH-specific antibody (Abcam Inc., Cambridge, USA) and secondary anti-mouse antibody (Amersham). The signal was detected by the ECL Advanced Western blotting detection kit (Amersham) and visualized on the GelDoc-It® imaging system (UVP, Upland, USA) with use of the VisionWorks® LS software. Expression levels were normalized to the GAPDH densitometry values.

Immunohistochemistry (ICH). Human prostate samples were Bouin's-fixed (24 h) and paraffin-embedded. After deparaffinization and rehydration, sections were quenched by incubation in 1% H_2O_2 (30 min) and blocked with goat normal serum (30 min) (Dako X0907). Subsequently, sections were incubated with primary antibodies against ppOX, OXR1 or OXR2 (rabbit anti-preproorexin polyclonal antibody, Milipore AB3096; OXR-1 (C-19, sc-8072) and OXR-2 (C-20, sc-8074), Santa Cruz Biotechnology) for 2 h at room temperature or overnight at 4°C (dilution range 1:50-1:1000). PBS was used as the negative control. Sections were then processed with peroxidase-conjugated secondary antibodies and finally developed with DAB (Dako Real EnVision detection system, peroxidase/DAB+, rabbit/mouse, DAKO K5007). Except for pre-incubation with goat normal serum, after every step the sections were rinsed in PBS (3 times for 5 min). Specimens were counterstained with hematoxylin, dehydrated, and mounted.

Serum concentrations of OXA and OXB. Estimations were performed on serum samples obtained from patients characterized in our earlier study (30). Serum concentrations of OXA and OXB were determined in patients with BPH (n=12), PC

Figure 1. Ethidium bromide-stained 2% agarose gel showing prostatic cDNA amplified with human orexin receptor 2 (*OXR2*)-specific primers. Note the presence of the reaction products with the expected size of 177 bp. 1, DNA size marker; 2-9, control prostates (even numbers - peripheral region; odd numbers - central part); 10-19, benign prostatic hyperplasia. Negative controls (no RT of the RNA) are not shown.
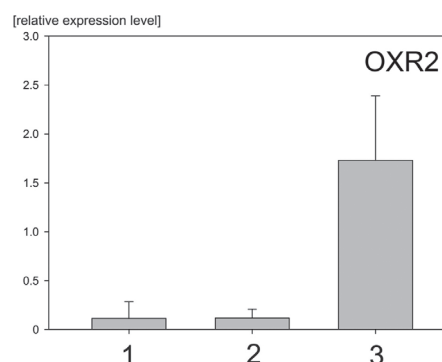

Figure 2. QPCR analyses of *OXR2* expression in control and BPH prostates. 1, peripheral and 2, central regions of the control; 3, benign prostatic hyperplasia. Bars present the relative expression of the studied gene in relation to hypoxanthine-guanine phosphoribosyltransferase gene expression). Results are expressed as the means ± SE; n>10.

(n=18) and in the control group (n=16) using commercial EIA kits, following appropriate protocols (Peninsula Laboratories, OXA, cat. no. S-1374, OXB, cat. no. S-1147). Briefly, wells of the immunoplate were filled in with standard, blank (diluent only) or appropriate samples and incubated with antisera against OXA or OXB followed by addition of a biotinylated tracer. Before the next step, wells were washed 5 times, using the Labsystems Wellwash 4 and filled with a streptavidin-HRP complex. Immunoplates were then washed once more (as described earlier) and TMB solution was added to each well. Finally, the reaction was terminated by HCl addition and within 10 min the absorbance was read out at 450 nm. All steps were performed at room temperature. OXA and OXB concentrations were specified by comparing absorbance values of samples with a pre-determined standard curve.

Statistics. Data are expressed as the mean ± SE. Statistical comparisons were done by the unpaired Student's t-test.

Results

As demonstrated in Fig. 1, conventional RT-PCR revealed the expression of *OXR2* in all studied samples obtained either from normal or BPH prostates. On the contrary, in all studied cases presence of neither ppOX nor OXR1 mRNA could be detected (data not shown). In all assays reaction products were of the expected length. Subsequently, we performed QPCR studies. Expression levels of *OXR2* were low and similar in samples taken from the central part and the peripheral region of control prostates (Fig. 2). Unexpectedly, in adenomatous
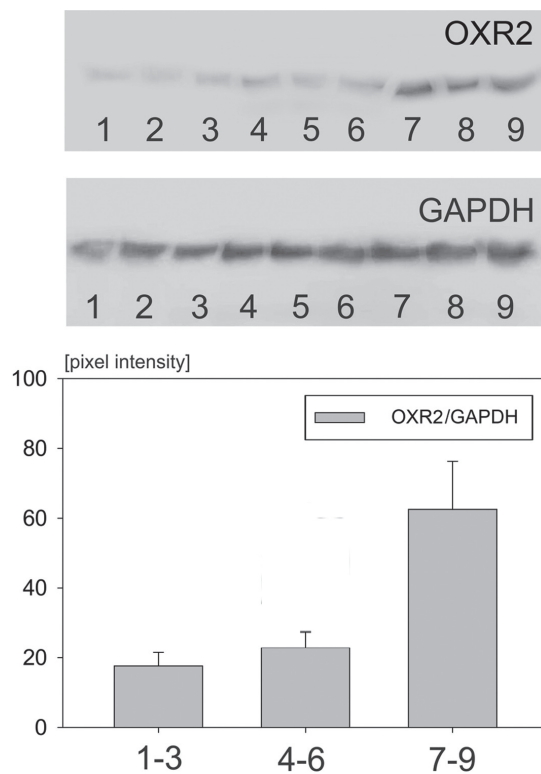
Figure 3. Semi-quantitative evaluation of OXR2 protein expression in control and BPH prostates. Western blotting. Upper part: chemiluminescence signals of OXR2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Lanes 1-3, peripheral and 4-6, central regions of the control; 7-9, benign prostatic hyperplasia. Graph: densitometric evaluation relative to GAPDH of the HCRTR2 protein expression intensity. Results are expressed as the means ± SE; n=3.

prostates, the expression levels of the studied gene were 30- to 40-fold higher compared to controls. Western blot analyses clearly demonstrated the presence of OXR2 protein in the studied samples (Fig. 3). In samples taken from the central part and peripheral region of control prostates the expression levels of the studied protein were similar, while in the samples from BPH they were approximately 4-fold higher.

In normal glands ICH revealed the presence of OXR2-like immunoreactivity in apical parts of epithelial cells as well as in smooth muscle cells of the stroma (Fig. 4A-C). Both in the stroma and in the epithelium numerous intensively stained cells were also present. They possessed lobated nuclei characteristic of neutrophils. Such cells seemed to migrate from the stroma, via the epithelium into the lumen of the glands. Immunostaining for OXR2 seemed to be more intense in sections obtained from BPH (Fig. 4D-F). The reaction product was visible mainly in the epithelial cells, and to a lesser degree in smooth muscle cells of the stroma. Intensively stained neutrophils were rarely seen in cases of BPH. In all studied prostates (control and BPH ones) ICH did not reveal the expression of OXR1-like protein. There was no immunostaining in control reactions (omission of antibodies specific for OXR1 or OXR2).

Subsequently we studied OXA and OXB levels in sera of control individuals, BPH or PC patients (Fig. 5). OXA serum concentrations were lowered in BPH (mean ± SE, 56±4 ng/ml, n=12; $P<0.01$) and unaltered in PC patients (79±7 ng/ml, n=18) compared to controls (69±2 ng/ml, n=16). On the contrary, serum OXB levels were similar in the control, BPH and PC patients (36±4, 38±5 and 35±4 ng/ml, respectively).
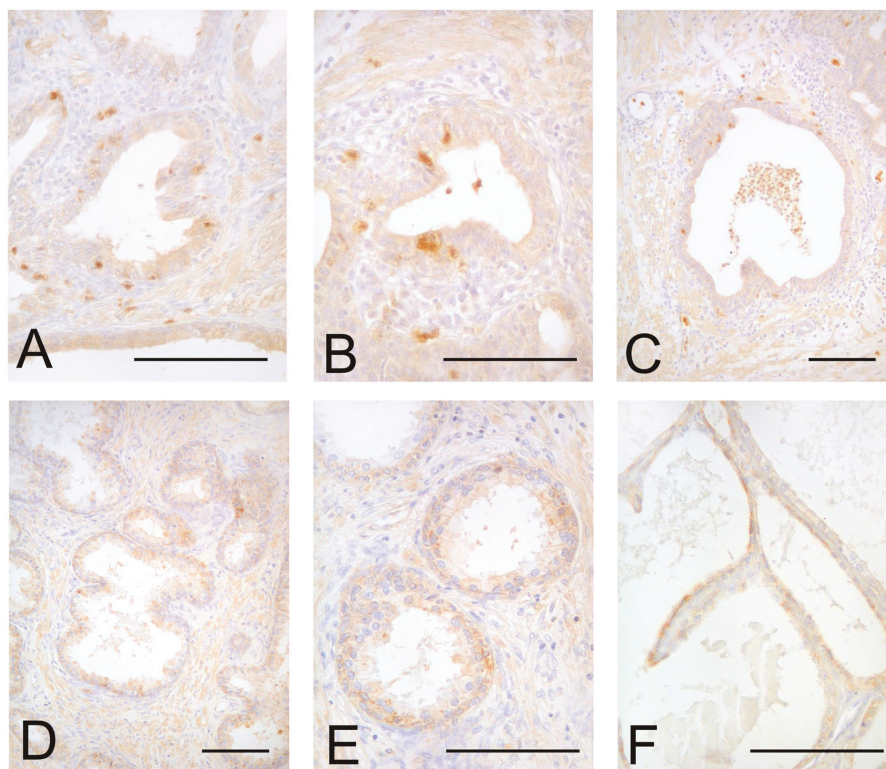

Figure 4. OXR2-like immunoreactivity in normal human prostate (A-C) and in benign prostatic hyperplasia (D-F). Immunoreactivity is seen in both epithelial cells and smooth muscle cells of the stroma. Intensely stained cells in the normal prostate possess lobated nuclei, characteristic of neutrophils. Such cells seem to migrate from the stroma (A), via the epithelium (B) into the lumen (C) of the glands. Benign prostatic hyperplasia (D-F). Note that immunostained neutrophils are not seen in BPH. Sections were counterstained with hematoxylin. Magnification is defined by bars equivalent to 100 μm.
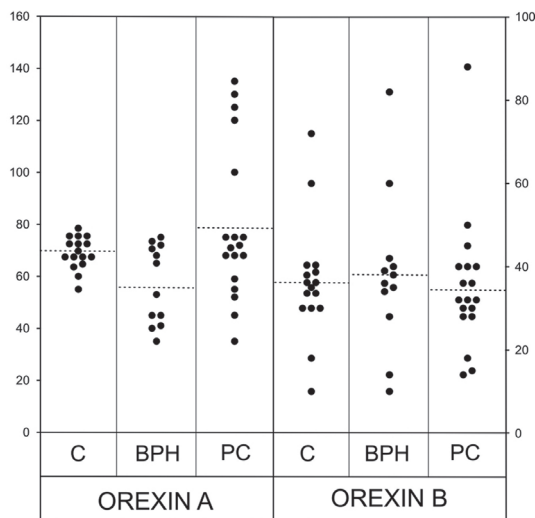
Figure 5. OXA and OXB concentrations (ng/ml) in the blood serum of the controls (C) and benign prostatic hyperplasia (BPH) and prostate cancer (PC) patients. Individual results (black dots) are presented. Broken lines correspond to the means.

Discussion

A growing body of evidence suggests that the OX-OXR system is involved in the regulation of sleep/awake conditions, reward processes as well as in feeding behavior and stress (31-35). This widely distributed system also plays an important role in regulating cell proliferation and apoptosis in both normal and cancer cells (36-44). In this regard, reports have indicated that OXs, via OXR1 or OXR2 induce apoptosis, resulting in a notable reduction in cell growth of various cancer cell lines (37,44).

OXs and OXRs are also expressed in the male reproductive system (45-50). The expression pattern of this system or its elements suggests that OXs may be involved in the regulation of testosterone secretion by the Leydig cells, while their action on the remaining elements of the reproductive system may be organ-specific. In humans, RT-PCR analyses revealed that both *OXR1* and *OXR2* were expressed in the testis, epididymis, penis, and seminal vesicle, whereas *ppOX* was expressed only in the epididymis and penis (46). By means of ICH, OXA expression could not be detected in the human prostate (51). A study on the cattle urethroprostatic complex, evaluated the expression of the orexin system in the prostate (50). By means of RT-PCR, Western blot analysis and ICH, the expressions of ppOX in endocrine cells and of OXR1 in the exocrine cells of this complex were determined. Lack of reports on the expression of the orexin-orexin system in the human prostate prompted us to perform the present study. Our findings provide the first evidence for the expression of *OXR2* in the prostate and its up-regulation in BPH. Expression of both, OXR2 mRNA and protein suggested the local synthesis of OXR2 in the human prostate. On the contrary, we could not demonstrate *ppOX* and *OXR1* expression in either normal or adenomatous glands.

In our hands, ICH revealed the localization of OXR2-like immunoreactivity in the apical parts of the epithelial cells as well as in the smooth muscle cells of the stroma in both normal and BPH glands. In this regard it should be emphasized that in the cattle urethroprostatic complex, OXR1 was found to be expressed exclusively in the epithelial cells of the glands (50). Expression of OXRs in smooth muscle cells is well documented, especially in muscle cells of the gut. In rats, these cells mainly express OXR1, although the presence of OXR2 has also been reported, and presumably they are involved in the contractile activity of the gut (52-55). Our results suggest the expression of OXR2 in both smooth muscle and epithelial cells of the human prostate. This suggestion is supported by the presence of OXR2-like immunoreactivity in frequently migrating neutrophils of the normal prostate. As reported, these cells express OXR2 (56,57). Of interest is that OXR2 immunoreactive neutrophils were only sporadically observed in BPH. In contrast with an earlier report in cattle (50), in both normal and BPH prostates we observed neither OXR1- nor OXR2-immunoreactive neuroendocrine cells and this might depend on a distinct species-dependent structure of the gland.

The novel and original findings of our study involve the demonstration of a notably enhanced OXR2 expression in hyperplastic human prostate. In both normal and BPH prostates there is a rather close interrelationship between the stroma and glandular part of the glands (58). In human BPH a 4-fold absolute increase in the expression was found in the stromal part and a nearly 2-fold increase in the glandular part (58-60). In this regard, the nearly 4-fold increase in OXR2 protein expression in BPH is not unexpected. However, since *OXR2* gene expression levels are calculated in relation to the *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) gene, the obtained data have clearly shown a notable increase in *OXR2* gene expression. It may only be speculated that up-regulation of the *OXR2* gene in BPH may be connected to hyperplasia of the prostate or elimination of cells from adenomas. In this regard it is worth mentioning that in various cancer cell lines OX-stimulated apoptosis is linked to a notable reduction in cell growth (37,44).

Basic clinical data indicate that BPH patients were older and had a higher percentage of body fat and serum PSA concentrations when compared with donors of the normal gland. However the range of the age and of the body fat percentage seems to indicate that these factors are not responsible for the enhanced OXR2 mRNA and protein expression in BPH.

Since in the human prostate gland ppOX is not expressed, the function of the OXR2 may be dependent on circulating OX. Therefore, we measured OXA and OXB concentrations in the serum of controls and BPH or PC patients. Surprisingly, in BPH patients serum OXA concentrations were lower than in controls, and in PC they were similar to those of controls. On the contrary, serum OXB concentrations were similar in all three studied groups. This unexpected finding may suggest that up-regulation of *OXR2* gene expression in the prostate of BPH patients may be related to the lowered levels of serum OXA. Taken together, these findings suggest that both OXA and OXR2 may be involved in BPH pathogenesis. However, the role of this system in BPH development and maintenance remains unclear and requires further investigation.

Thus, the novel data presented in this study demonstrate the mRNA and protein expression of OX2R, but not of ppOX and OXR1 in human prostate glands. Furthermore, we have

demonstrated a notable up-regulation of OXR2 in BPH, an alteration accompanied by lowered serum OXA concentrations. These findings suggest that both OXA and OXR2 may be involved in the pathogenesis and/or maintenance of BPH.

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