Endothelin-1 induces changes in the expression levels of steroidogenic enzymes and increases androgen receptor and testosterone production in the PC3 prostate cancer cell line

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Abstract. Endothelin-1 (ET-1) is involved in the regulation of steroidogenesis. Additionally, patients with castration-resistant prostate cancer (PCa) have a higher ET-1 plasma concentration than those with localized PCa and healthy individuals. The aim of the present study was to evaluate the effect of ET-1 on steroidogenesis enzymes, androgen receptor (AR) and testosterone (T) production in PCa cells. The expression levels of endothelin receptors in prostate tissue from patients with localized PCa by immunohistochemistry, and those in LNCaP and PC3 cells were determined reverse transcription-quantitative PCR (RT-qPCR) and western blotting. Furthermore, the expression levels of ET-1 were determined in LNCaP and PC3 cells by RT-qPCR and western blotting. The ET-1 receptor activation was evaluated by intracellular calcium measurement, the expression levels of AR and enzymes participating in steroidogenesis [cytochrome P450 family 11 subfamily A member 1 (CyP11A1), cytochrome P450 family 17 subfamily A member 1, aldo-keto reductase family member C2 and 3β-hydroxysteroid dehydrogenase/isomerase 2 (3β HSD2)] were determined by western blotting and T concentration was determined by ELISA using PC3 cells.

The present results revealed higher expression levels of endothelin A receptor (ET_AR) in tissues obtained from samples of patients with PCa with a low Gleason Score. No changes were identified for endothelin B receptor (ET_BR). PC3 cells expressed higher levels of ET-1 and ET_AR, while LNCaP cells exhibited higher expression levels of ET_BR. Blocking of ET_AR and endothelin B receptor decreased the expression levels of CyP11A1 and 3β HSD2 enzymes and AR in PC3 cells, as well as T secretion. These findings suggested that ET-1 has a potential role in modulating the intratumoral steroidogenesis pathway and might have relevance as a possible therapeutic target.

Introduction

Prostate cancer (PCa) is the fifth most common cause of cancer-associated mortality among men worldwide (1). Digital rectal examination and determination of prostate-specific antigen levels within the blood are techniques that are commonly used to screen for PCa (2). If these techniques suggest the presence of PCa, the diagnosis is confirmed using a transrectal biopsy analysis (2,3). Current treatments for clinically localized or advanced PCa include radical prostatectomy, cryoablation, radiation therapy, brachytherapy and androgen deprivation therapy (ADT) (2,4).

The androgens [testosterone (T) and dihydrotestosterone] are hormones, which are required for the development of the reproductive system and male secondary sex characteristics (4,5). These hormones exert their physiological actions via interaction with the androgen receptor (AR), which is a ligand-dependent transcriptional factor belonging to the superfamily of nuclear receptors (5). Androgens serve an important role in the development and growth of a normal prostate gland and in the proliferation of PCa cells (6,7). Therefore, ADT is often used as a first line treatment to control advanced PCa (8).

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However, after 2-3 years of treatment, PCa develops resistance to ADT, resulting in castration-resistant PCa (CRPC) (8). Hypersensitivity, mutations, splicing variants or amplification of the AR and intratumoral steroidogenesis have been indicated to be mechanisms that may lead to androgen resistance (6). Steroidogenesis begins with the translocation of cholesterol to the inner membrane of the mitochondria via steroidogenic acute regulatory protein (9). In addition, cytochrome P450 family 11 subfamily A member 1 (CyP11A1), cytochrome P450 family 17 subfamily A member 1 (CyP17A1), 3β hydroxysteroid dehydrogenase type 2 (3β HSD2), 17β-hydroxysteroid dehydrogenase type 3 and 5α reductase type 1 and 2 are the main enzymes that are required to complete androgen *de novo* synthesis from cholesterol (9).

A number of previous studies have demonstrated that steroidogenic cells expressing endothelin receptor increase steroidogenesis when stimulated with endothelin-1 (ET-1) (10-12). Furthermore, patients with metastatic CRPC have been reported to exhibit increased ET-1 plasma levels compared with patients with localized PCa and healthy individuals (13-17), suggesting that ET-1 may contribute to the transition from androgen-dependent PCa to CRPC. ET-1, which is a potent vasoconstrictor peptide containing 21 amino acid residues, is associated with a number of aspects of PCa progression, including proliferation, escape from apoptosis, invasion, angiogenesis and new bone formation (16,18,19).

Leydig cells have been indicated to express high levels of endothelin receptors (12) and increase basal T secretion when stimulated with ET-1 (10,11). Additionally, endothelin receptor A (ET$_A$R) activation by ET-1 has been revealed to increase AR mRNA expression via c-myc in androgen-independent LNCaP cells, contributing to androgenic independence (20). However, to the best of our knowledge, the role of endothelin receptors in non-steroidogenic cells, including PCa cells, has not yet been described. In the present study, an ET-1-dependent increase in the expression levels of steroidogenic enzymes and an increase in AR and T production in PCa cells were observed.

Materials and methods

Chemicals and materials. ET-1 was purchased from Sigma-Aldrich; Merck KGaA. ET-1 receptor antagonists, 2-[(*3R,6R,9S,12R,15S*)-6-(*1H*-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazabicyclo[13.3.0]octadecan-3-yl] acetic acid or cyclo(D-tryptamine-D-aspartic acid-L-proline-D-valine-L-leucine) (BQ123) and 2,6-Dimethylpiperidinecarbonyl-γ-Methyl-Leu-N$_{in}$-(Methoxycarbonyl)-D-Trp-D-Nle,-N-[N-[N-[(2,6-Dimethyl-1-piperidinyl)carbonyl]-4-methyl-L-leucyl]-1-(methoxycarbonyl)-D-tryptophyl]-D-norleucine sodium salt (BQ788) were purchased from Cayman Chemical Company and bosentan was purchased from Sigma-Aldrich; Merck KGaA. The salts, chloroform and alcohols were obtained from MilliporeSigma and Hank's buffer was obtained from Thermo Fisher Scientific, Inc.

Endothelin receptor and AR blockade. Cells were incubated with BQ123 (ET$_A$R selective antagonist; 1 μM), BQ788 [endothelin B receptor (ET$_B$R) selective antagonist; 1 μM] or bosentan (ET$_A$R and ET$_B$R dual antagonist; 1 μM) for 30 min at 37°C with 5% CO$_2$. In all experiments, DMSO 0.1% was used.

Cell culture. PCa cells from androgen-dependent lymph nodal metastases (LNCaP) and PCa cells from bone metastases (PC3) were obtained from American Type Culture Collection. LNCaP cells, clone FDG (CRL1740), were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and PC3 cells (CRL1435) were maintained in DMEM F12 medium (Gibco; Thermo Fisher Scientific, Inc.). Both media were supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 1% penicillin, 1% streptomycin and 0.05% amphotericin B (Corning, Inc.). All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO$_2$. The media of PC3 cells were replaced with phenol red-free RPMI (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% activated charcoal (Sigma-Aldrich; Merck KGaA)-treated FBS, 1% streptomycin, 0.05% amphotericin B and 1% penicillin 24 h before the different treatments. Afterwards, cells were washed with PBS three times and harvested.

Tissue microarrays (TMAs). A TMA was constructed with 5-μm sections of formalin (10% v/v)-fixed for 24 h at room temperature and paraffin-embedded PCa samples from male patients (age range, 47-80 years; mean age, 63.7 years) with different Gleason Score (GS) (21) from the biopsy archive of our institutional Pathology Department (Clinical Hospital of the University of Chile, Santiago, Chile). All samples were diagnosis biopsies collected in different years (March 2016-January 2018) with a confirmed diagnosis and GS. The sample inclusion criterion was: PCa confirmed. All protocols for tissue archive use and processing have been approved by the institutional Ethical Committee of Faculty of Medicine, University of Chile (approval nos. 135-2015 and 083-2020). For diagnosis biopsies, patients were asked to sign a general consent when biopsies were obtained. For the use of the archive of biopsies from the Pathology Service, a signed authorization of the director of the Department of Pathology, University of Chile (authorization 03012016; Santiago, Chile) is required. TMAs with cores of 1 mm in diameter, including 7 samples with a low GS (GS<7), 14 samples with an intermediate GS (GS=7) and 11 samples with a high GS (GS>7) were obtained and evaluated by a pathologist.

Immunohistochemistry. TMA tissue sections were deparaffinized and rehydrated in decreasing concentrations of ethanol and distilled water [xylene (x2), ethanol 100% (x2), ethanol 95%, ethanol 70% and distilled water], and incubated for 40 min at 95°C in antigen recovery buffer (10 mM citrate buffer, pH 6.0). After cooling, endogenous peroxidase was inhibited by incubation with 0.3% H$_2$O$_2$ for 30 min and samples were blocked with 2.5% horse serum (Vector Laboratories, Inc.) for 30 min at room temperature. Then, sections were incubated with primary antibody against ET$_A$R (dilution, 1:300; cat. no. PA3-065; Thermo Fisher Scientific, Inc.) or ET$_B$R (dilution, 1:800; cat. no. PA3-066; Thermo Fisher Scientific, Inc.) overnight at 4°C. Subsequently, the samples were washed and incubated with secondary antibody conjugated to the HRP enzyme for 1 h at room temperature (ready to use; anti-rabbit-mouse-IgG; cat. no. PK-7200; Vector Laboratories,

Table I. Reverse transcription-quantitative PCR oligonucleotides.

Gene	Forward (5'-3')	Reverse (5'-3')
ET_AR	GAACATCTTAAGCAGCGTCGAG	ACCGATGTAATCCATGAGCAGT
ET_BR	GCTTGCTTCATCCCGTTCAGA	CTTCCCGTCTCTGCTTTAGGTG
ET-1	CAGGGCTGAAGACATTATGGAGA	CATGGTCTCCGACCTGGTTT
Pumilio	CGGTCGTCCTGAGGATAAAA	CGTACGTGAGGCGTGAGTAA

ET_AR, endothelin A receptor; ET_BR, endothelin B receptor; ET-1, endothelin-1.

Inc.). Then, samples were washed and incubated with ABC amplification system for 30 min at room temperature (ready to use; cat. no. PK-7 200; Vector Laboratories, Inc.). Finally, the chromogenic substrate 3,3'-diaminobenzidine (DAB) was added. Furthermore, the nuclei were stained with hematoxylin (ScyTek Laboratories, Inc.) for 1 min at room temperature. Images were obtained using a Leica DM2500 light microscope (Leica Microsystems GmbH), digitized and the DAB signal was quantified by ImageJ 1.51w software (National Institutes of Health), using the IHC toolbox plugin (https://imagej.nih.gov/ij/plugins/ihc-toolbox/index.html) and plotted using GraphPad Prism version 7.1 (GraphPad Software, Inc.). For semi-quantitative analysis of immunohistochemistry results, the gray level transformation method was implemented, using the logarithmic transformation technique. The digitized images were processed through gray level transformation techniques, since it operates directly on the pixels. The image involves 256 levels of gray, so in the histogram on the horizontal axis it ranges from 0 to 255 levels of gray. On the other hand, the vertical axis differs from the number of pixels in the image. The Log (255/average color/grays in the selected area) was determined as previously described (22).

RNA extraction and quantification. Total RNA was isolated from cells using TRIzol® (Thermo Fisher Scientific, Inc.), chloroform, isopropanol and 75% ethanol. Subsequently, nuclease free water (diethylpyrocarbonate-treated) was used to re-suspend extracted RNA. The purity and concentration of RNA were determined by spectrophotometry at 260/280 nm using a BioTeK Synergy HT plate reader (BioTek Instruments, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). A total of 1,000 ng total RNA from each sample was used for cDNA synthesis using the 5X All-In-One RT MasterMix kit (25°C for 10 min, 42°C for 15 min and 85°C for 5 min, 1 cycle; Applied Biological Materials Inc.). Subsequently, 50 ng/ml were amplified by qPCR using the Brilliant II SYBR Green qPCR Master Mix kit (95°C for 10 min, 1 cycle; 95°C for 15 min, 60°C for 15 min and 72°C for 15 min, 40 cycles; 95°C for 15 min, 65°C for 15 min and 95°C for 15 min, 1 cycle; Agilent Technologies, Inc.). RT-qPCR oligonucleotides are shown in Table I. The housekeeping gene Pumilio was used to normalize data and the results were analyzed using the $\Delta\Delta Cq$ method (23). To facilitate comparison, data were referred to those cells that express the highest levels of the receptor. PC3 cells expressed the highest levels of ET_AR mRNA and protein, so the expression levels of ET_AR of LNCaP cells were compared with PC3 cells. By contrast, LNCaP cells expressed the highest levels of ET_BR mRNA and protein, so ET_BR expression levels of PC3 cells were compared with LNCaP cells.

Intracellular calcium measurement. PC3 cells were grown on 25-mm diameter glass coverslips and kept in DMEM F12 medium for 24 h. Subsequently, the cells were washed with PBS and loaded with 2 μM Fluor-4 acetoxymethyl ester (Fluo4/AM; Thermo Fisher Scientific, Inc.) at room temperature for 15 min. Subsequently, the cells were maintained in Hank's Balanced Salt Solution at a final volume of 500 μl containing 142 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM HEPES and 5.6 mM glucose. On the one hand, 100 nM ET-1 was added at 60 sec after reaction was initiated with Fluo4/AM, at room temperature, to cells previously incubated with DMSO or with selective antagonists for endothelin receptors, at 37°C for 30 min. In all experiments DMSO (0.1%) was used. On the other hand, 17 mM carbachol (positive control) was added at 245 sec after reaction was initiated with Fluo4AM, at room temperature, to cells preincubated with BQ123 at 37°C for 30 min. The kinetics recording was carried out for 300 sec and the fluorescence intensity was measured in an AxioCam MRm (Carl Zeiss AG) coupled to a Carl Zeiss AG inverted fluorescence microscope AxioVert.A1 equipped for epifluorescence and the ImageJ 1.51w software (National Institutes of Health) was used.

Indirect immunofluorescence. PC3 and LNCaP cells were grown on 12-mm diameter glass coverslips seeded at a confluence of 60-70%. After 24-48 h, the cells were fixed with a fixing solution (4% paraformaldehyde) for 30 min at room temperature, washed and blocked with 3% BSA (Winkler, Ltd.) in PBS-glycine for 30 min at room temperature. Cells were incubated overnight at 4°C with the primary antibodies against ET_AR (dilution, 1:200; cat. no. PA3-065; Thermo Fisher Scientific, Inc.) and ET_BR (dilution, 1:200; cat. no. PA3-066; Thermo Fisher Scientific, Inc.), washed and incubated for 30 min at 37°C with the secondary antibody Alexa Fluor 594 (dilution, 1:500; cat. no. A21207; Thermo Fisher Scientific, Inc.). DAPI (dilution, 1:10,000; cat. no. sc3598; Santa Cruz Biotechnology, Inc.) was used for nuclear staining for 7 min at room temperature. The images were obtained using a Leica D2500 fluorescence microscope (Leica Microsystems GmbH).

Table II. Primary antibodies for western blotting.

Primary antibody	Supplier	Cat. no.	Species	Dilution
ET_AR	Thermo Fisher Scientific, Inc.	PA3-065	Rabbit anti-human	1:1,000
ET_BR	Thermo Fisher Scientific, Inc.	PA3-066	Rabbit anti-human	1:1,000
ET-1	Santa Cruz Biotechnology, Inc.	sc-517436	Mouse anti-human	1:500
CyP11A1	Abcam	ab-75497	Rabbit anti-human	1:1,000
CyP17A1	Merck KGaA.	ABC392	Rabbit anti-human	1:1,000
AKR1C2	Thermo Fisher Scientific, Inc.	PA5-36572	Rabbit anti-human	1:500
3β HSD2	Thermo Fisher Scientific, Inc.	PA5-27791	Rabbit anti-human	1:1,000
AR	Abcam	ab-9474	Mouse anti-human	1:1,000
Actin	MP Biomedicals, LLC	691002	Mouse anti-human	1:5,000

ET_AR, endothelin A receptor; ET_BR, endothelin B receptor; ET-1, endothelin-1; CyP11A1, cytochrome P450 family 11 subfamily A member 1; CyP17A1, cytochrome P450 family 17 subfamily A member 1; AKR1C2, aldo-keto reductase family member C2; 3β HSD2, 3β-hydroxysteroid dehydrogenase/isomerase 2; AR, androgen receptor.

Table III. Secondary antibodies.

Secondary antibody	Supplier	Cat. no.	Dilution
Anti-rabbit	Jackson ImmunoResearch Laboratories, Inc.	111-035-003	1:10,000
Anti-mouse	Jackson ImmunoResearch Laboratories, Inc.	115-035-003	1:10,000

Western blotting. Proteins obtained from cell lines were extracted using RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% v/v NP-40, 1% w/v sodium deoxycholate, 2.5 mM Na_3PO_4, 1 mM b-glycerophosphate and 1 mM Na_3VO_4, pH 7.4) with a protease inhibitor cocktail (Roche Diagnostics). The homogenate was centrifuged at 16,708 x g for 15 min at 4˚C. Finally, the supernatant was quantified using a Bradford assay. A total of 25 μg of protein was loaded per lane, separated by SDS-PAGE (10 or 12% polyacrylamide for ET_AR and ET_BR), and transferred to a nitrocellulose membrane, except for detection of 3β HSD2, for which protein was transferred to a PVDF membrane. The membranes were blocked at room temperature with 5% BSA (Winkler, Ltd.) or milk of 0.2% TBS-Tween for 90 min and exposed to the primary antibody overnight at 4˚C. After washing, the binding of the primary antibodies was detected with secondary antibodies conjugated with the HRP enzyme for 90 min at room temperature, and detected using EZ-ECL chemiluminescence kit (Biological Industries) and a Vilber Lourmat equipment (Fusion FX5-XT 826.WL/superbright serial number 15200393; Vilber). Primary and secondary antibodies for western blotting are shown in Tables II and III, respectively. The densitometric analysis of western blot bands was performed using ImageJ v1.51 software (National Institutes of Health).

Determination of T levels in cell culture medium. PC3 cells were grown on 6-well plates in RPMI medium free of phenol red with 10% androgen-free FBS. After 24 h, DMSO (control) or endothelin receptor antagonist was added for 30 min at 37˚C, and then 100 nM ET-1 was added every 24 h at 37˚C.

In all experiments, DMSO (0.1%) was used. After 96 h, the culture medium was removed and centrifuged at 1,000 x g for 5 min at room temperature. The number of viable cells was quantified in each condition. Parameter Testosterone Assay (cat. no. KGE010; R&D Systems, Inc.) was used for T determination in culture supernatant according to the manufacturer's protocols. The assay was based on a competitive binding technique. A total of 50 μl of a monoclonal antibody specific for T (excluding non-specific binding wells) was added for binding the anti-mouse antibody coated microplate and incubated with shaking for 1 h at room temperature. After three washes with wash buffer (400 μl), 100 μl calibrator (non-specific binding wells and B0, zero standard), 100 μl standard, control or sample (remaining wells) were added. Subsequently, 50 μl conjugated T was incorporated into each well, and incubated for 3 h at room temperature on a horizontal orbital microplate shaker at 37 x g After three washes with wash buffer, 200 μl of the substrate were added for 30 min at room temperature. Afterwards, 50 μl of the stop solution was added to stop the reaction. Finally, the optical density was determined within 30 min by spectrophotometry at 450 nm using the BioTeK Synergy HT plate reader (BioTek Instruments, Inc.).

Statistical analysis. Plots were obtained using the GraphPad Prism 7.1 software (GraphPad Software, Inc.). Data are presented as the mean ± SD of at least three independent experiments for RT-qPCR and western blotting. Mann Whitney or Kruskal-Wallis (Dunn's multiple comparisons test) test was carried out. The box plots represent the signal intensity of ET_AR and ET_BR in each group. The points outside the box represent the outliers. The number of samples
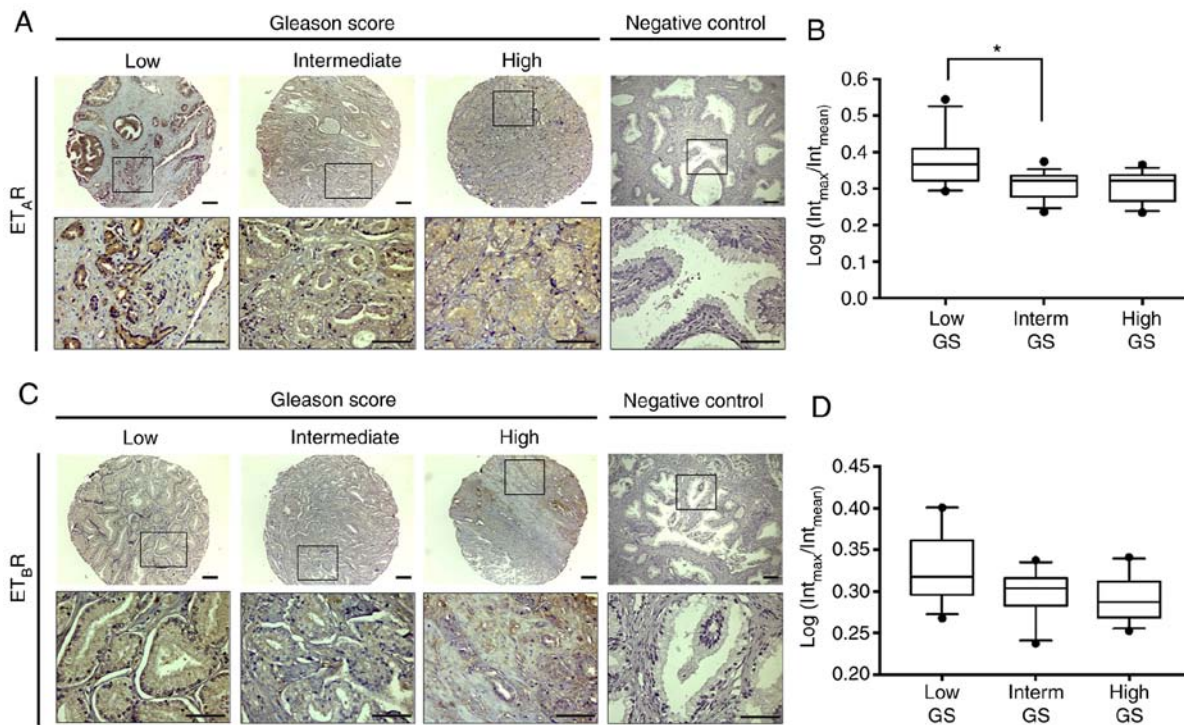
Figure 1. Expression levels of ET_AR/ET_BR in TMAs from PCa samples. The images indicate the expression levels of ET_AR/ET_BR in TMAs of PCa samples with low, intermediate, and high GS. (A) Immunohistochemistry analysis of ET_AR. (B) Semi-quantification of the DAB signal. (C) Immunohistochemistry analysis of ET_BR. (D) Semi-quantification of the DAB signal. The box plots represent the signal intensity of ET_AR and ET_BR in each group. The points outside the box represent the outliers. Total number of samples analyzed, n=32; low GS (<7), n=7; intermediate GS (=7), n=14; and high GS (>7), n=11. The box plots show the mean ± SD. *P<0.05 (Kruskal-Wallis test/Dunn's multiple comparisons test). Scale bar, 100 μm. PCa, prostate cancer; GS, Gleason Score; ET_AR, endothelin A receptor; ET_BR, endothelin B receptor; ET-1, endothelin-1; TMA, tissue microarray; DAB, 3,3'-diaminobenzidine; Interm, intermediate.

analyzed was 32 for low GS (<7), 14 for intermediate GS (=7) and 11 for high GS (>7). The bar shows the mean ± SD. P≤0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of ET_AR and ET_BR in TMAs of samples. In the present study, quantitative immunohistochemistry for ET_AR and ET_BR was performed on constructed TMAs. Intracellular staining of ET_AR and ET_BR was observed in PCa samples (Fig. 1A and C). Furthermore, semi-quantification analysis indicated that the intensity of ET_AR immunostaining was significantly higher in samples with low GS (Kruskal-Wallis test; P<0.05) compared with in samples with intermediate GS (Fig. 1A and B). No changes in ET_BR were observed to be associated with GS in primary tumor samples (Fig. 1C and D). However, in benign prostatic hyperplasia samples, used as a non-neoplastic control, ET_AR was revealed to be located in epithelial and stromal cells (Fig. S1A), and ET_BR was revealed to be primarily located in epithelial cells (Fig. S1B).

Expression levels of ET-1, ET_AR and ET_BR in PCa cell lines. Basal expression levels of ET-1, ET_AR and ET_BR in LNCaP and PC3 cells were determined using RT-qPCR and western blotting. In addition, ET_AR and ET_BR levels were also determined by immunofluorescence. PC3 cells were demonstrated to exhibit the highest levels of ET_AR and ET-1 expression at the mRNA (ET_AR; Mann-Whitney test; P=0.05; Fig. 2A;

ET-1; Mann-Whitney test; P=0.05; Fig. 3A) and protein levels (ET_AR; Mann-Whitney test; P<0.05; Fig. 2B and E; ET-1 Mann-Whitney; P=0.05; Fig. 3B). By contrast, LNCaP cells were revealed to express increased levels of ET_BR mRNA (ET_BR; Mann-Whitney test; P=0.05; Fig. 2C) and protein (ET_BR; Mann-Whitney test; P=0.05; Fig. 2D and F) compared with PC3 cells. Negative controls are shown in Fig. 2G.

Intracellular Ca^{2+} measurement revealed that activity of ET_AR is induced by ET-1 in PC3 cells. Since ET_AR is a Gq protein-coupled receptor (15,17), the activity of this receptor was evaluated via intracellular calcium measurement. The results in Fig. 3C indicated that activation of ET_AR by ET-1 induced an intracellular Ca^{2+} transient signal, which was reflected as an increase in relative fluorescence intensity (F/F0) when adding ET-1 at 60 sec compared with carbachol (positive control; Fig. 3D), suggesting the exit of calcium from the endoplasmic reticulum and/or the subsequent entry of calcium from the extracellular medium. This effect was inhibited by a selective antagonist for ET_AR, BQ123 (Fig. 3D), and the non-selective antagonist Bosentan (Fig. 3E).

Effect of ET-1 on the expression levels of steroidogenic pathway enzymes and AR in PC3 cells. In the present study, the effect of ET-1 on the enzymes of the steroidogenic pathway in PC3 cells was investigated. The effect was not determined in LNCaP cells since PC3 cells expressed higher levels of ET-1 and ET_AR. Therefore, by using PC3 cells, the effects could be observed more clearly. It was demonstrated
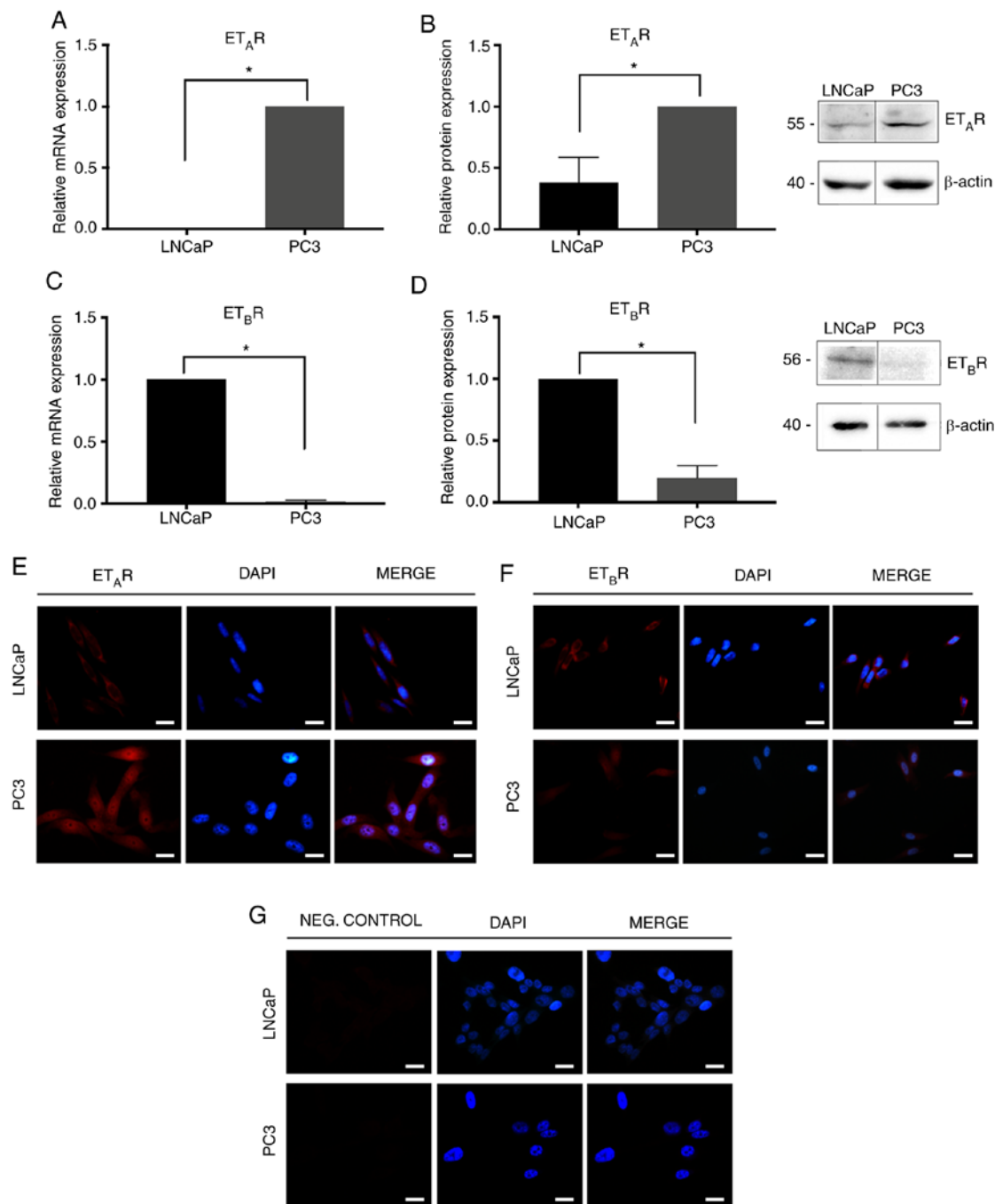
Figure 2. ET_AR and ET_BR expression in prostate cancer cells. (A) ET_AR mRNA expression normalized to Pumilio compared with PC3 cells. (B) ET_AR protein expression in PC3 and LNCaP cells. Quantification was normalized to β-actin and PC3 cells. (C) ET_BR mRNA expression normalized to Pumilio compared with LNCaP cells. (D) ET_BR protein expression in PC3 and LNCaP cells normalized to Pumilio compared with LNCaP cells. Immunofluorescence analysis of (E) ET_AR and (F) ET_BR in PC3 and LNCaP cells. (G) Negative controls. Data are presented as the mean ± SD (n=3 independent experiments) of arbitrary units. *P<0.05 (Mann-Whitney test). Scale bar, 20 μm. The values 55, 56 and 40 correspond to mass units (kDa). ET_AR, endothelin A receptor; ET_BR, endothelin B receptor; Neg., negative.

that ET-1 treatment increased the expression levels of CyP11A1 (Kruskal-Wallis test; P<0.05; Fig. 4A and B-D), 3β HSD2 (Kruskal-Wallis test; P<0.05; Fig. 4A and H-J) and AR (Kruskal-Wallis test; P<0.05; Fig. 5A-C) in PC3 cells compared with the control without ET-1. Blocking ET-1 receptors with BQ123 and/or Bosentan prevented the stimulatory effect of ET-1 on the expression of CyP11A1 (ET-1/BQ123 + ET-1; Kruskal-Wallis test; P<0.001; Fig. 4B), 3β HSD2 (ET-1/Bosentan + ET-1; Kruskal-Wallis test; P<0.05;

Fig. 4J) and AR (ET-1/BQ123 + ET-1; Kruskal-Wallis test; P<0.01; Fig. 5B; ET-1/Bosentan + ET-1; Kruskal-Wallis test; P<0.05; Fig. 5D). Additionally, blocking ET_BR with BQ788 decreased, with respect to ET-1, the expression levels of 3β HSD2 (ET-1/BQ788 + ET-1; Kruskal-Wallis test; P<0.05; Fig. 4I). There were no changes observed in CyP17A1 and aldo-keto reductase family member C2 (AKR1C2) protein expression in the three antagonist treatment groups, with respect to ET-1 (Fig. 4E-G and K-M). The aforementioned
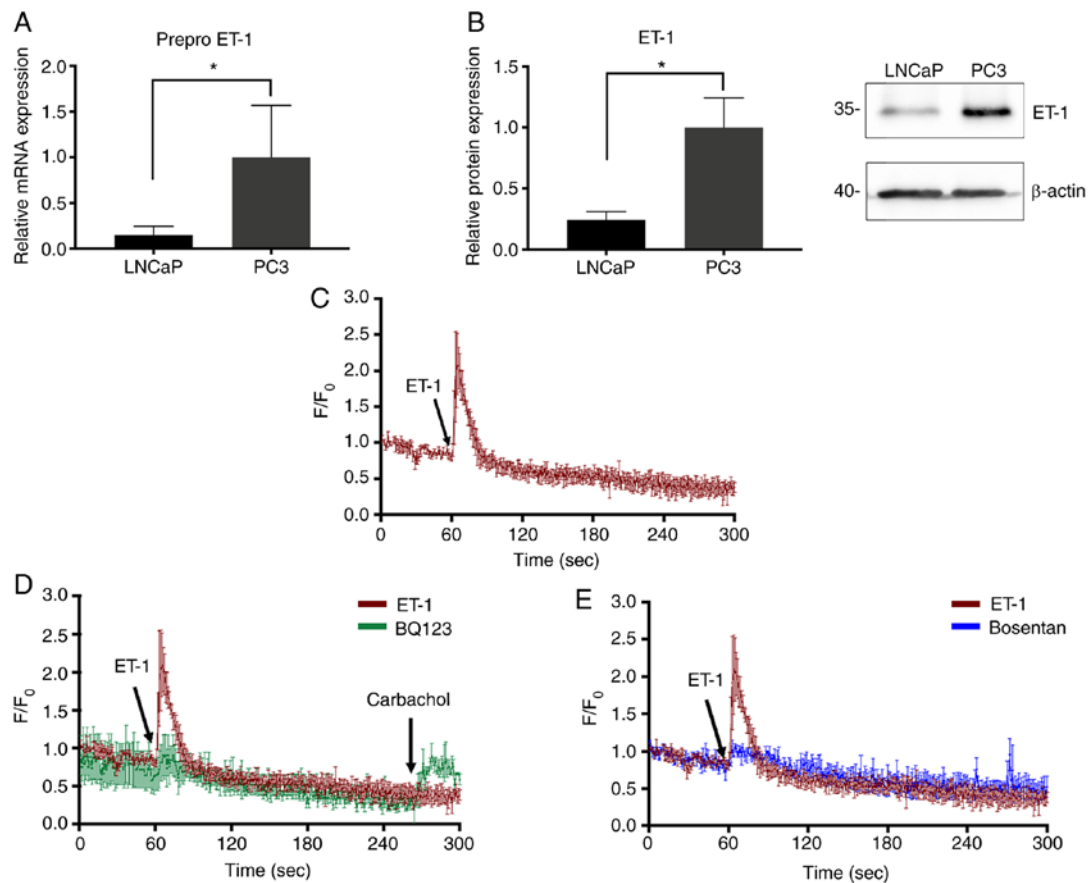
Figure 3. ET-1 expression and effect of ET-1 on calcium signaling in PCa cells. (A) Prepro-ET-1 mRNA expression normalized to Pumilio compared with PC3 cells. (B) ET-1 protein expression in PC3 and LNCaP cells. Quantification was normalized to β-actin and PC3 cells. (C) Addition of 100 nM ET-1. (D) Cells were incubated with 1 μM BQ123 and stimulated with ET-1. After 240 sec, cells were stimulated with carbachol. (E) Cells were incubated with 1 μM Bosentan and stimulated with ET-1. Data are presented as the mean ± SD (n=3 independent experiments). *$P<0.05$ (Mann-Whitney test). The values 35 and 40 correspond to mass units (kDa). F/F_0, relative fluorescence intensity; PCa, prostate cancer; ET-1, endothelin-1; BQ123, 2-[(3R,6R,9S,12R,15S)-6-(1H-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazabicyclo[13.3.0]octadecan-3-yl] acetic acid.

results demonstrated that ET-1 may regulate the protein expression levels of CyP11A1, 3β HSD2 and AR via ET_AR or ET_BR.

Effect of ET-1 on T secretion in PC3 cells. To determine if the increase in the expression levels of steroidogenic enzymes was associated with the steroidogenic process, T production was evaluated in PC3 cells. Blocking of endothelin receptors was indicated to induce a decrease in T concentration (ET-1/BQ123 + ET-1; Kruskal-Wallis test; $P<0.05$; Fig. 5E; ET-1/BQ788 + ET-1; Kruskal-Wallis test; $P<0.05$; Fig. 5F).

Discussion

ADT is the first-line treatment for patients with localized and advanced PCa (4). However, patients with PCa may develop resistance and this can lead to CRPC (8,9). Upregulation of the expression levels of androgen biosynthesis enzymes has been identified in tissues from patients with CRPC (9). Additionally, high plasma concentrations of ET-1 have been reported in patients with CRPC (13-17). It has been previously proposed that ET-1 may contribute to the transition from androgen-sensitive PCa to CRPC (24). However, to the best of our knowledge, the factors that may promote androgen

resistance are yet to be determined. In 2009, Lee *et al* (20) demonstrated that ET-1 increases c-myc expression via ET_AR, leading to increased AR expression in PCa cells within androgen-free medium. Furthermore, steroidogenic cell lines expressing endothelin receptor have been previously indicated to increase basal T secretion following ET-1 stimulation (10,11).

The present study determined the expression levels of ET_AR and ET_BR in PCa TMAs, including in samples with different GSs (<7, 7 and >7). The results demonstrated that low GS samples exhibited higher ET_AR expression compared with intermediate GS samples, indicating that in primary tumors from patients without treatment, the expression levels of ET_AR were decreased and may be associated with the progression of PCa. Upregulation of ET_AR and ET-1 expression has been demonstrated in the early stages of PCa (16) and higher expression levels of ET_AR and ET-1 are associated with advanced tumor stage (25). Furthermore, androgen-sensitive cells (LNCaP) cultured in androgen-deprived medium for 5 months have been indicated to exhibit increased levels of ET_AR and ET-1 mRNA, suggesting that this axis may serve an important role in the progression of PCa to CRPC (26). However, in the present study, no variation of ET_BR was observed in PCa samples with different GSs. Previous immunohistochemical
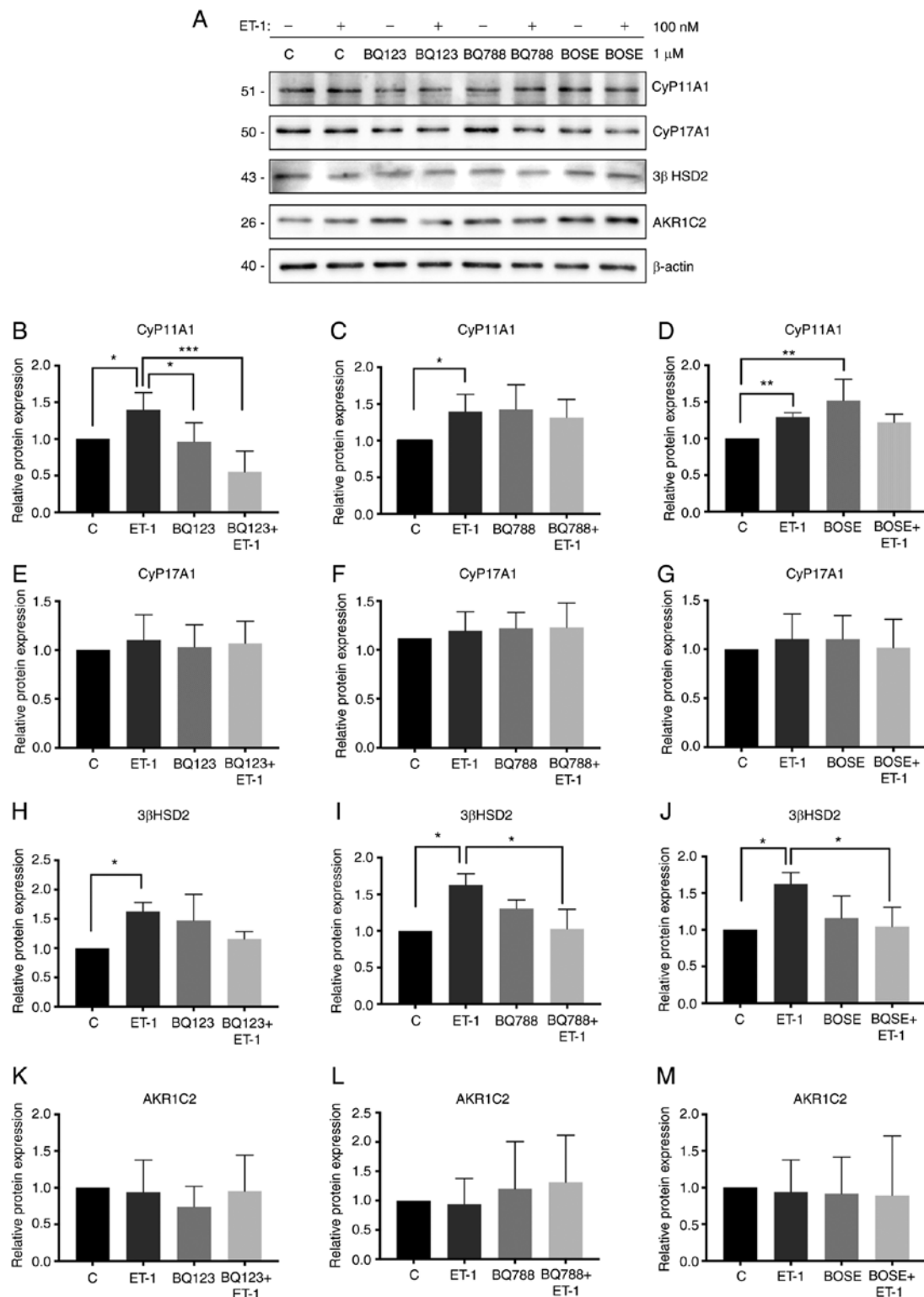
Figure 4. Effect of ET-1 on the expression levels of steroidogenic enzymes. (A) Lysed PC3 cells were analyzed by western blotting and membranes were incubated with antibodies against CyP11A1, CyP17A1, 3β HSD2, AKR1C2 and β-actin. (B) Protein expression levels of CyP11A1 in the presence or absence of BQ123. (C) Protein expression levels of CyP11A1 in the presence or absence of BQ788. (D) Protein expression levels of CyP11A1 in the presence or absence of BOSE. (E) Protein expression levels of CyP17A1 in the presence or absence of BQ123. (F) Protein expression levels of CyP17A1 in the presence or absence of BQ788. (G) Protein expression levels of CyP17A1 in the presence or absence of BOSE. (H) Protein expression levels of 3β HSD2 in the presence or absence of BQ123. (I) Protein expression levels of 3β HSD2 in the presence or absence of BQ788. (J) Protein expression levels of 3β HSD2 in the presence or absence of BOSE. (K) Protein expression levels of AKR1C2 in the presence or absence of BQ123. (L) Protein expression levels of AKR1C2 in the presence or absence of BQ788. (M) Protein expression levels of AKR1C2 in the presence or absence of BOSE. Quantification was normalized to β-actin tand control PC3 cells. Data are presented as the mean ± SD (n=3 independent experiments). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (Kruskal Wallis test/ Dunn's multiple comparisons test). The values 51, 50, 43, 26 and 40 correspond to mass units (kDa). ET-1, endothelin-1; BQ123, 2-[(3R,6R,9S,12R,15S)-6-(1H-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazabicyclo[13.3.0]octadecan-3-yl] acetic acid; BQ788, 2,6-Dimethylpiperidinecarbonyl-γ-Methyl-Leu-N$_{in}$-(Methoxycarbonyl)-D-Trp-D-Nle,-N-[N-[N-[(2,6-Dimethyl-1-piperidinyl)carbonyl]-4-methyl-L-leucyl]-1-(methoxycarbonyl)-D-tryptophyl]-D-norleucine sodium salt; BOSE, bosentan; CyP11A1, cytochrome P450 family 11 subfamily A member 1; CyP17A1, cytochrome P450 family 17 subfamily A member 1; AKR1C2, aldo-keto reductase family member C2; 3β HSD2, 3β-hydroxysteroid dehydrogenase/isomerase 2.
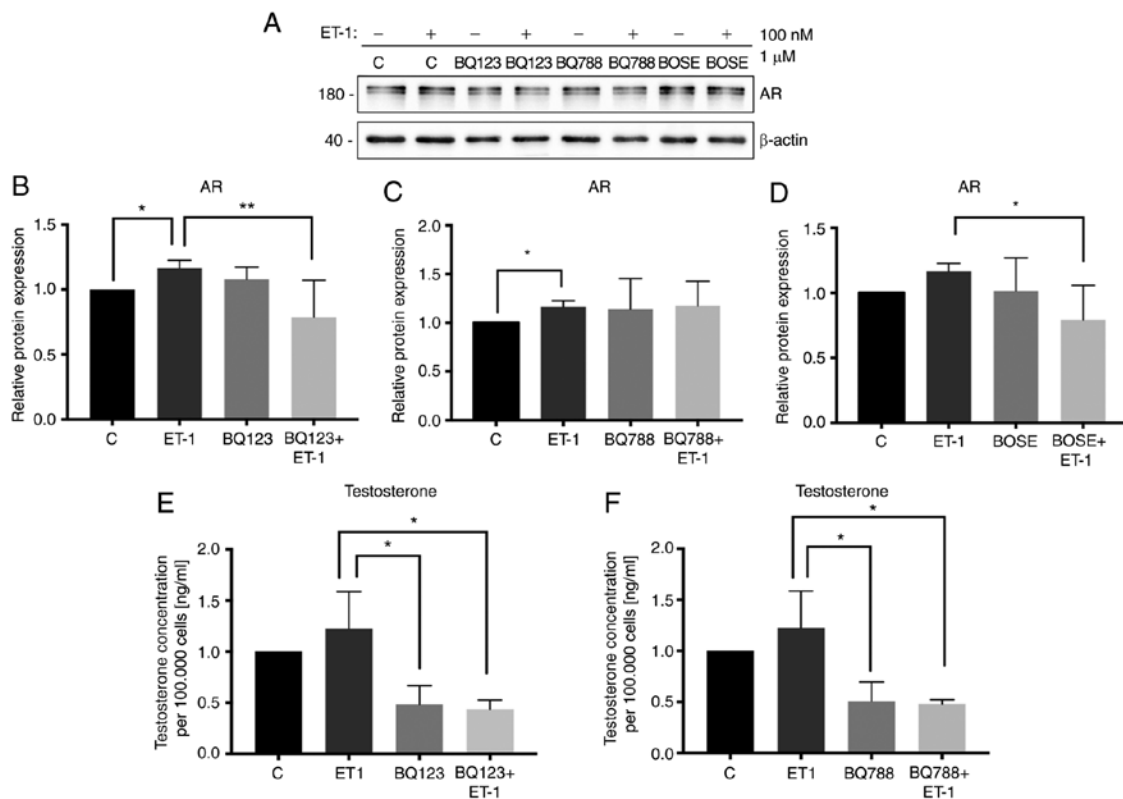
Figure 5. Effect of ET-1 on expression levels of AR and testosterone secretion. (A) Lysed PC3 cells were analyzed by western blotting and membranes were incubated with antibodies against AR and β-actin. (B) Protein expression levels of AR in the presence or absence of BQ123. (C) Protein expression levels of AR in the presence or absence of BQ788. (D) Protein expression levels of AR in the presence or absence of BOSE. Quantification was normalized to β-actin and control PC3 cells. The cells were stimulated with 100 nM ET-1 and previously incubated with BQ123 or BQ788 for 96 h. (E) Testosterone secretion in presence or absence of BQ123. (F) Testosterone secretion in presence or absence of BQ788. Data are presented as the mean ± SD (n=3 independent experiments). *P<0.05, **P<0.01 (Kruskal Wallis test/Dunn's multiple comparisons test). Quantification was normalized to control PC3 cells. The values 180 and 40 correspond to mass units (kDa). ET-1, endothelin-1; BQ123, 2-[(3R,6R,9S,12R,15S)-6-(1H-indol-3-ylmethyl)-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazabicyclo[13.3.0]octadecan-3-yl] acetic acid; BQ788, 2,6-Dimethylpiperidinecarbonyl-γ-Methyl-Leu-N$_{in}$-(Methoxycarbonyl)-D-Trp-D-Nle,-N-[N-[N-[(2,6-Dimethyl-1-piperidinyl)carbonyl]-4-methyl-L-leucyl]-1-(methoxycarbonyl)-D-tryptophyl]-D-norleucine sodium salt; BOSE, bosentan; AR, androgen receptor.

studies of prostate adenocarcinoma tissue have revealed the decreased expression of ET_BR compared with ET_AR levels (27,28).

In the present study, the expression levels of ET_AR, ET_BR and ET-1 were also measured in PCa cell lines, indicating that the androgen-insensitive cell line PC3 expressed the highest mRNA and protein levels of ET_AR and ET-1. LNCaP cells expressed higher levels of ET_BR compared with PC3 cells. ET_BR is a receptor, which is mainly expressed in prostatic epithelial cells and is associated with the regulation of extracellular ET-1 via lysosomal degradation (29). The present study indicated that androgen-sensitive LNCaP cells expressed lower levels of ET-1 but higher levels of ET_BR compared with PC3 cells, which was in agreement with previous studies (17,30). These results suggested that the ET-1 axis may serve a role in PCa progression and may contribute to the development of androgen resistance.

The activity of ETs is mediated by the activation of ET_AR and ET_BR, which are Gq protein-coupled receptors (15,17). Therefore, the present study revealed an expected increase in intracellular calcium in ET-1-stimulated PC3 cells. Accordingly, this effect was suppressed by preincubating the cells with endothelin receptor antagonists (BQ123 and Bosentan).

The ET-1 signaling pathway may be associated with the transition to an androgen-insensitive stage in patients with PCa (24). This can be hypothesized as an increase in AR expression in LNCaP cells after 3 months of androgen deprivation has been previously reported (20). However, the effect of ET-1 on steroidogenesis in non-steroidogenic cells, such as PCa cells, is yet to be determined. Therefore, the basal expression of steroidogenic pathway enzymes in PCa cell lines was examined in the present study.

Steroidogenesis is a physiological process, which mainly occurs in the adrenal glands and gonads (31,32), and different enzymes, including CyP11A1, CyP17A1, 3β HSD2, 17β-hydroxysteroid dehydrogenase type 3 and steroid 5 α-reductase 1, are involved in T production. Steroidogenesis can be acute or chronically regulated depending on the tissue type, and both types of regulation are controlled by different factors or hormones (31,33). An increase in the expression levels of enzymes that are involved in the synthesis of androgens, including CyP11A1, CyP17A1 and 3β HSD1, has been demonstrated in samples from patients with advanced PCa (9,34,35). Therefore, the effect of ET-1 on CyP11A1, CyP17A1, AKR1C2, 3β HSD2 and AR was evaluated in the present study. The results indicated that CyP11A1, 3β HSD2 and AR protein expression increased with ET-1 treatment and this effect was attenuated by a selective (BQ123) and non-selective antagonist (Bosentan) of the endothelin receptor. Other enzymes of the steroidogenic pathway could be tested, and this point could

be considered a limitation of the present study. However, the enzymes evaluated in the present study are those showing significant expression changes in tissues of patients with CRPC (9). In addition, endothelin receptor blockade induces a decrease of T concentration in the culture medium of PC3 cells. In 2006, Alimirah *et al* (36), reported that both PC3 and DU145 cells effectively express AR. Their study revealed that this receptor does not have the same characteristics of normal AR and, depending on the antibodies used, which bind to different regions of AR, it is possible to find double bands in these cell lines (36). Some of the results regarding the levels of the AR and T may be reproduced in DU145 cells (PCa cells from brain metastasis), since they express ET_AR (data not shown) and AR and produce T (37). To the best of our knowledge, the aforementioned results are the first to indicate that ET-1 regulates steroidogenesis via ET_AR or ET_BR in PC3 cells. On the other hand, there are cell lines derived from vertebral (VCaP) and lymphonodular (LAPC4) metastases of patients with PCa refractory to hormone therapy; however, these cell lines exhibit high levels of AR and androgen sensitivity (38). The objective of the present study was to analyze the effect of ET-1 in an androgen-insensitive cell line (PC3), in which high expression levels of ET_AR were observed.

The results of the present study indicated that ET-1 may serve an important role in the production of T in PCa cells via the canonical pathway as the main pathway for T synthesis in PCa. Changes in the protein expression levels of steroidogenic enzymes may be induced by ET-1 or ET_AR via two potential mechanisms: A cyclic AMP (cAMP)/protein kinase A (PKA)-dependent signaling pathway or a cAMP-independent signaling pathway with protein kinase C involvement. These signaling pathways may allow phosphorylation of transcriptional factors (steroidogenic factor 1, GATA binding protein 4 or cAMP responsive element binding protein), which are associated with the transcription of encoding genes for steroidogenic enzymes (31,39,40). However, cAMP/PKA signaling has been indicated to be the main regulatory mechanism for steroidogenesis, since the cAMP-independent pathways are usually associated with the modulation or enhancement of the biosynthetic process, acting synergistically with PKA (31,33).

These findings suggested that ET-1, via the activation of its receptors, may be actively associated with T production in PCa. This mechanism may contribute to the progression of PCa to CRPC, which indicated that ET-1 and its receptors may be potential therapeutic targets that can be used in the treatment of advanced PCa.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MJT performed the experimental design, experiments and statistical analysis, and wrote the manuscript. FLM participated in the design of testosterone measurement and drafted the manuscript. DH helped with the experimental design and writing of the manuscript. SI designed and helped in the immunohistochemical assays. AL assisted in the performance of some reverse transcription-quantitative PCR experiments of enzymes of the steroidogenic pathway. PL participated in intracellular calcium measurement design. EAC participated in the experimental design and helped to draft the manuscript. JT helped with the experimental design. HRC designed the general study and assisted in the writing of the manuscript. HRC and EAC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All protocols for tissue collection, use and processing have been approved by the institutional Ethical Committee of Faculty of Medicine, University of Chile (approvals nos. 135-2015 and 083-2020; Authorization for biopsy archive use of the Department of Pathology, University of Chile, 03012016, Santiago, Chile).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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