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ORIGINAL ARTICLE

Tadalafil modulates aromatase activity and androgen receptor expression in a human osteoblastic cell in vitro model

A. Aversa[1,2] · S. Fittipaldi[2,3] · V. M. Bimonte[2,3] · F. Wannenes[2,3] · V. Papa[2,3] · D. Francomano[1] · E. A. Greco[1,2] · A. Lenzi[1] · S. Migliaccio[3]

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Abstract
Purpose Phosphodiesterase type-5 inhibitor (PDE5i) tadalafil administration in men with erectile dysfunction is associated with increased testosterone/estradiol ratio, leading to hypothesize a potential increased effect of androgen action on target tissues. We aimed to characterize, in a cellular model system in vitro, the potential modulation of aromatase and sex steroid hormone receptors upon exposure to tadalafil (TAD).
Methods Human osteoblast-like cells SAOS-2 were chosen as an in vitro model system since osteoblasts are target of steroid hormones. Cells were tested for viability upon TAD exposure, which increased cell proliferation. Then, cells were treated with/without TAD for several times to evaluate potential modulation in PDE5, aromatase (ARO), androgen (AR) and estrogen (ER) receptor expression.
Results Osteoblasts express significant levels of both PDE5 mRNA and protein. Exposure of cells to increasing concentrations of TAD (10^{-8}–10^{-7} M) decreased PDE5 mRNA and protein expression. Also, TAD inhibited ARO mRNA and protein expression leading to an increase in testosterone levels in the supernatants. Interestingly, TAD increased total AR mRNA and protein expression and decreased ERα, with an increased ratio of AR/ER, suggesting preferential androgenic vs estrogenic pathway activation.
Conclusions Our results demonstrate for the first time that TAD decreases ARO expression and increases AR protein expression in human SAOS-2, strongly suggesting a new control of steroid hormones pathway by PDE5i. These findings might represent the first evidence of translational actions of PDE5i on AR, which leads to hypothesize a growing relevance of this molecule in men with prostate cancer long-term treated with TAD for sexual rehabilitation.

Keywords Tadalafil · Osteoblasts · Aromatase · Androgen receptor · Estrogen receptor

Introduction

It is well known that phosphodiesterases (PDEs) catalyze the hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), to the 5′ nucleotide monophosphate [1]. Eleven different PDE families (PDE1-11) have been described throughout the body, each family containing subfamilies and multiple splice variance, differing in selectivity for cyclic nucleotides, sensitivity to inhibitors and activators, physiological roles, and tissue distribution. In the last decade, several pharmaceutical compounds, which selectively inhibit the catalytic activities of PDE, have been developed for the treatment of various diseases, but only PDE5 inhibitors (PDE5i) reached clinical application mainly for treating male erectile dysfunction (ED) [2].

Interestingly, several studies demonstrated that small PDE5 amounts are present in different extra-genital tissues

✉ A. Aversa
antonio.aversa@uniroma1.it

S. Migliaccio
silvia.migliaccio@uniroma4.it

[1] Section of Medical Pathophysiology, Endocrinology and Nutrition, Department of Experimental Medicine, "Sapienza" University of Rome, Rome, Italy

[2] Lisa Laboratory, Policlinico of Catania, University of Catania, Catania, Italy

[3] Section of Health Sciences, Department of Movement, Human and Health Sciences, "Foro Italico" University of Rome, Largo Lauro De Bosis 15, 00195 Rome, Italy

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(i.e., skeletal muscle, heart, lung, adrenal gland) [3] and Ahlström and Lamberg-Allardt firstly demonstrated that PDE5 is present in UMR-106 osteoblast-like cells [4]. Successive studies have suggested that PDE5i might have some detrimental effects on osteoblastic activity both in vitro and in animal models [5]. Despite this, the chronic use of any PDE5 inhibitor has never been associated with reduced bone mineral density in humans.

Men suffering from erectile dysfunction (ED) have androgen levels significantly lower than normal one and it is demonstrated that the effects of two different PDE5 inhibitors treatments, Sildenafil and Tadalafil (TAD), are able to revert testosterone reduction, and thereby reducing LH levels, probably due to the higher frequency of full sexual intercourse [6]. The rationale of the present study started from a clinical observation suggesting that the continuous administration of the PDE5i tadalafil in men with ED is associated with an increase of serum testosterone/estradiol ratio [7]. In addition, our previous studies had shown that PDE5 is also expressed in human adipocytes in vitro and that TAD exposure modulates ARO expression and activity [8]. The actions of PDE5 inhibitors upon testosterone production have been studied extensively in rat models. Interestingly, it has been recently shown that inhibition of PDE5 activity upon prolonged sildenafil treatment, increased serum testosterone level and Leydig cells' steroidogenic capacity by stimulating cAMP and cGMP signaling pathway [9]. Also, acute administration of sildenafil stimulates testosterone production, suggesting implications for sexual activity regulation [10]. Due to these results, it might be postulated a modulation by TAD of steroid receptors through which hormone action is exerted. Thus, the present study was designed to evaluate the mechanism of action of PDE5i in human androgen/estrogen target tissues to further characterize in vitro the effects of exposure to the selective PDE5i TAD on both ARO expression and sex steroid receptors in osteoblastic cells, well-known steroid target tissue.

Materials and methods

Cell culture and treatment

Human SAOS-2 osteoblastic cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 10 % fetal bovine serum (FBS), at 37 °C and 5 % CO_2 in a humidified incubator. All buffers, media and reagents were purchased from Euroclone. Osteoblastic cells were switched to phenol red-free DMEM and treated with different doses of TAD (10^{-8}–10^{-7} M) for 24, 48 h. Tadalafil (kindly supplied by ELI Lilly ICOS Corporation, Indianapolis, IN, USA) was dissolved in dimethylsulfoxide and added to cells at the concentrations indicated for each experiment. At the end of incubations, media were removed and centrifuged at 250 g for 12 min, and supernatants were saved for the ELISA assays.

MTT assay

The effect of TAD on proliferation and cell vitality was quantified by 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide reduction assay (MTT assay, Sigma). Thus, 1.5×10^4 cells/well were seeded in 96-well culture plate and treated in quadruplicate with different doses of TAD (10^{-8}–10^{-7} M). After 1, 2, 3, 4 and 5 days of TAD treatment the assay was performed accordingly to the manufacturer's guidelines. Values from each treatment were calculated as percent of the viability versus the untreated control.

RNA isolation and quantitative real-time PCR

For RNA extraction, cells were washed twice with 1X phosphate buffer saline (PBS, Euroclone) and total RNA was immediately lysated with Trizol (Life Technologies) according the manufacturer's instructions. The purity and integrity of total RNA was monitored by electrophoretic analysis on denaturating agarose gel; ultraviolet spectrophotometry (Biorad) was used for RNA yield evaluation. Total RNA was treated as previously described [11]. Quantitative real-time PCR was performed in Abi Prism 7500 light cycler (Applied Biosystem) using Power SYBR Green PCR Master Mix (Applied Biosystem) as indicated by manufacturers. All primers were optimized as previously described [11]. Quantitative RT-PCR sample value was normalized for the expression of cyclophilin mRNA. The relative level for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method [12] and reported as arbitrary units (a. u.). Each sample was analyzed in duplicate in all experiments.

Protein extraction and Western blot analysis

Following experimental treatments, cells were washed with PBS and lysed in fresh ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 500 µM EDTA, 100 µM EGTA, 1.0 % Triton X-100, and 1 % sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Sigma).

Lysates were precleared by centrifugation and the protein amount was measured using Micro BCA Protein assay Reagent (Thermo Scientific). An equal amount of protein extracts (20 µg) was separated in SDS–polyacrylamide gels (10–14 %) and transferred to a nitrocellulose membrane (Amersham Biosciences).

Transfer was verified by Ponceau S staining. The membrane was blocked 60′ at room temperature with 5 % non-fat dry milk (Cell Signalling Technology) in T-TBS (tris buffered saline plus tween 20, 0.01 %). Membranes were incubated with primary antibodies diluted as indicated by manufacturers, overnight at 4 °C. It was washed three times with T-TBS and incubated for 60′ at room temperature with HRP-labeled secondary antibody in 5 % non-fat dry milk. Membranes were then washed three times and the signals were visualized using ECL Prime Western blotting detection reagent (GE Healthcare) in the ImageQuant LAS4000 instrument (Amersham Biosciences). Quantitative analysis was performed using Imagequant TL Image analysis software (GE Healthcare) using beta-actin for normalization. The primary antibodies used were: anti-aromatase (Cell Signalling Technology) anti-androgen receptor (Santa Cruz Biotechnology), anti-estrogen receptor α (Santa Cruz Biotechnology), anti-phosphodiesterase type-5 (Cell Signalling Technology) anti-β-actin (Cell Signalling Technology).

ELISA assay

The culture medium of TAD-treated cells exposed to bovine serum was recovered for ELISA assays using Estradiol ELISA, Testosterone ELISA (Cusabio Biotech Co., LTD.). Each experiment was repeated three times. Hormones levels found in the supernatant were corrected per milligram of cellular protein of each dish. Data are expressed as pg/mg protein.

Statistical analysis

All results were expressed as means ± standard errors (SE) of at least three independent experiments. *p* values were calculated using the unpaired *t* test and statistically significant differences and were considered significant with values <0.05.

Results

Osteoblastic cells as tadalafil target

A first set of experiments was performed to evaluate a potential cytotoxic effect of TAD on SAOS-2 osteoblastic cells by checking cells viability by MTT assay. To address this point, cells were treated with different doses of TAD up to 5 days. TAD did not exert any toxic effect on SAOS-2 cells up to 5 days of exposure. By contrast, treated cells displayed higher viability in the MTT assay compared to the untreated cells ($p < 0.05$, Fig. 1), indicating that TAD stimulates osteoblastic cell proliferation.

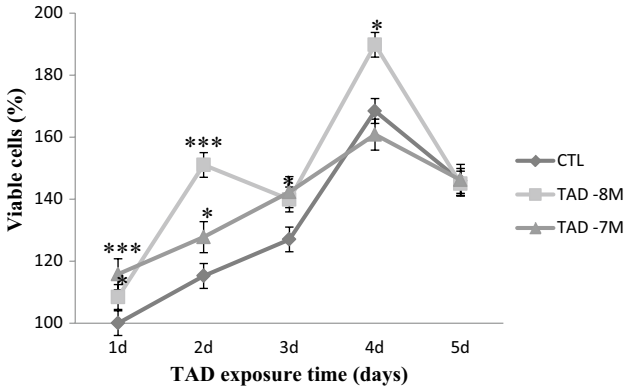

Fig. 1 Cytotoxic assay: Saos-2 cells were treated with different TAD concentrations (10^{-7}, 10^{-8} M) for several interval times (day 1 through 5). *$p < 0.05$, ***$p < 0.001$ versus CTL

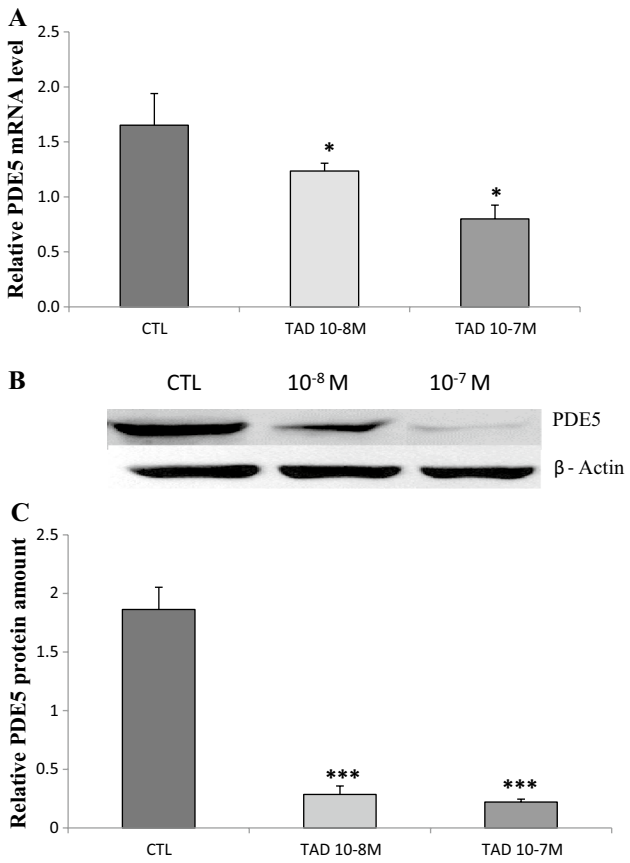

Fig. 2 Expression of mRNA (**a**) and protein (**b**, **c**) PDE5 in osteoblasts. Cells were grown in presence of different TAD concentrations (10^{-7}, 10^{-8} M) for 24 and 48 h, respectively. *$p < 0.05$, ***$p < 0.0001$ versus CTL

To verify whether bone tissue could represent a specific target tissue of PDE5i, we firstly evaluated the PDE5 expression in human SAOS-2. PDE5 mRNA and protein were detected in osteoblasts by RT-PCR and Western Blot (Fig. 2a, b), in

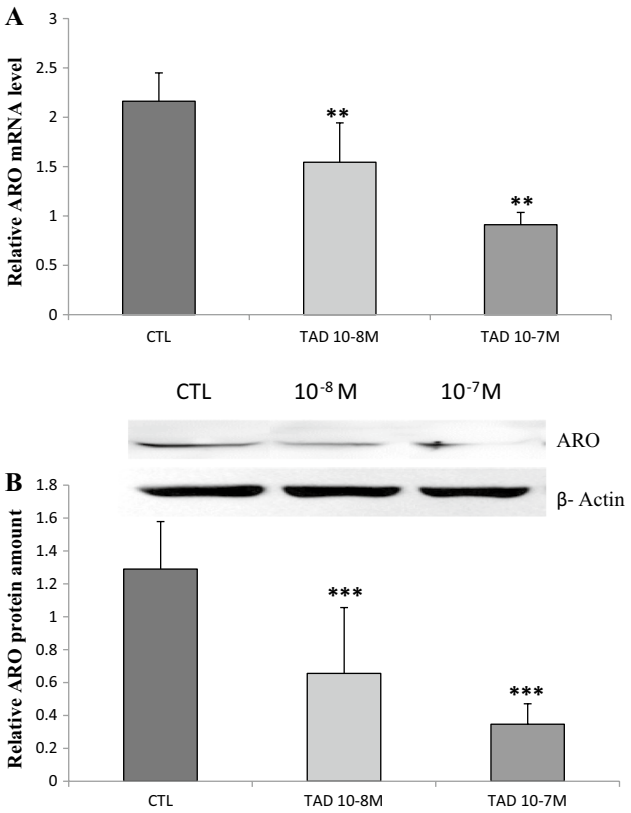

Fig. 3 Expression of mRNA (**a**) and protein (**b**) ARO in osteoblasts, grown as described in Fig. 2. $^{**}p < 0.01$; $^{***}p < 0.001$ versus CTL

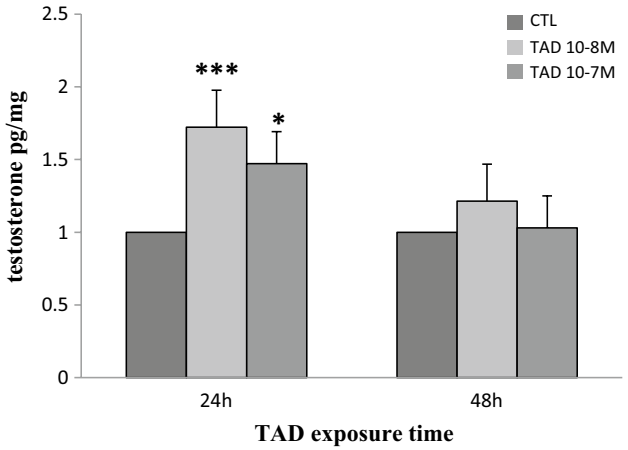

Fig. 4 ELISA quantification of testosterone in supernatant of osteoblastic cells grown as described in Fig. 1. $^{*}p < 0.05$; $^{***}p < 0.001$ vs. CTL

accordance with previous published results in other osteoblastic cells lines [5]. Moreover, 24 h treatment with increasing concentration of TAD induced a decrease of PDE5 mRNA ($p < 0.05$, Fig. 2a), also confirmed, after 48 h of treatment, by a decreased protein level ($p < 0.001$, Fig. 2b).

Dose-dependent effect of TAD on ARO mRNA and protein expression

Since our previous data indicated a modulation of ARO by TAD in human adipocytes [7], experiments were performed to evaluate potential modulation of this enzyme in human osteoblastic cells. After 24 h, the exposure of osteoblasts to different concentrations of TAD down-regulated ARO mRNA expression in a concentration-dependent manner ($p < 0.01$, Fig. 3a). Moreover, TAD also inhibited ARO protein expression in a concentration-dependent manner, strongly suggesting an inhibition of this enzyme activity ($p < 0.001$, Fig. 3b), as previously suggested in vivo [6].

Androgen and estrogen receptors expression

To evaluate potential modulation of ARO by TAD, testosterone level in the cellular medium of osteoblastic cells exposed to TAD was measured by ELISA. Interestingly at 24 h testosterone levels were significantly increased in a concentration-dependent manner (Fig. 4, $p < 0.05$). Conversely, TAD treatment did not significantly modify estradiol levels at any experimental point (data not shown).

Then, a potential modulation of androgen and estrogen receptor by TAD was evaluated. Interestingly, TAD treatment increased total AR expression in osteoblastic cells after 48 h exposure with a maximal effect obtained at a concentration of 10^{-7} M, as depicted in Fig. 5. Moreover, an increased nuclear translocation of AR was also observed, as evaluated by Western blot analysis, (data not shown), strongly suggesting an activation of androgen pathway. Estrogen receptor α (ERα) expression was also evaluated while ERβ mRNA and protein were barely detectable in these cells (data not shown), confirming results previously shown by others [13].

Interestingly, the ratio of total AR/ERα increased in a concentration-dependent manner strongly indicting a preferential activity of androgen rather than estrogen pathway upon TAD exposure of osteoblastic cells (Fig. 6, $p < 0.001$). Also, Western blot analysis showed an increased expression of cytoplasmic and nuclear fractions of AR after different concentrations of TAD exposure at 48 and 72 h (Fig. 7).

Discussion

Our results confirm that significant amounts of PDE5 are expressed in human osteoblast SAOS-2 cells. Further, treatment with the selective PDE5i TAD in vitro modulates aromatase enzyme activity. More notably, exposure of osteoblastic cells to TAD induced an increase in total AR expression and AR/ERα ratio. In particular, this effect was

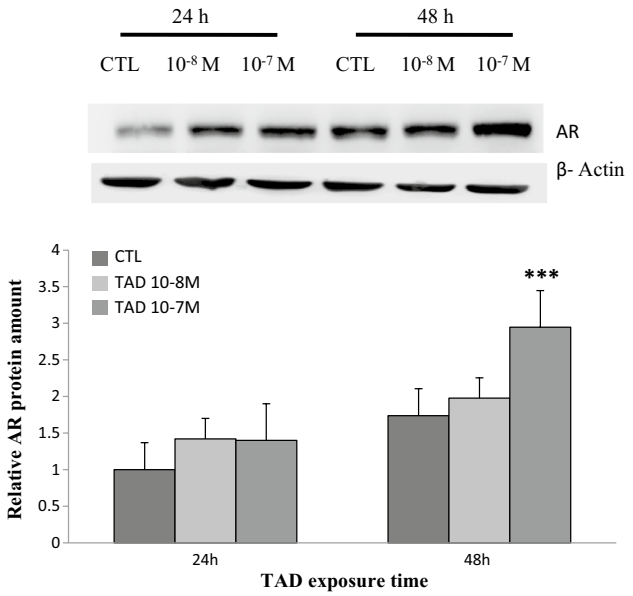

Fig. 5 Western blot analysis of total expression of AR. Protein levels were evaluated in osteoblasts grown as described in "Materials and methods" section by Western blot analysis (*top panel*). Quantification of the data obtained from Western blot analysis is depicted in the graph (*bottom panel*; ***$p < 0.001$ vs. CTL)

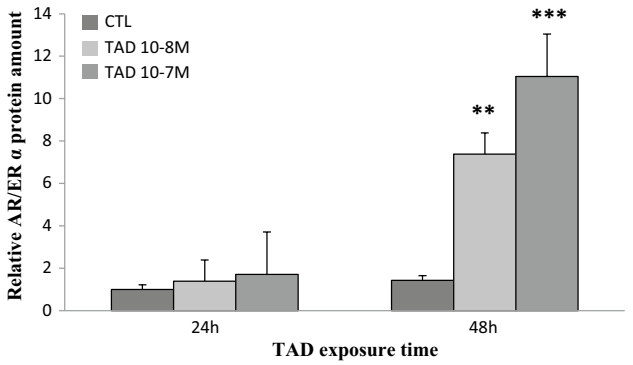

Fig. 6 Expression of ratio AR/ERα in Saos-2 osteoblastic cells grown as described in "Materials and methods" section (**$p < 0.01$ and ***$p < 0.001$ vs. CTL)

maximal after 24–48 h of treatment with different concentrations of TAD (10^{-8} and 10^{-7} M) and, accordingly, protein expression was increased after 24–48 h of exposure. Interestingly, a significant increase of testosterone level in the supernatant was observed after 48 h of TAD exposure, suggesting an overall decrease in ARO enzymatic activity after long-term in vitro human osteoblastic cells exposure to TAD. Interestingly, our findings show for the first time that exposure of osteoblastic cells to the PDE5i TAD can significantly decrease ARO expression and function.

PDE5 inhibitors promote their pharmacological effects by inhibiting PDE5, an enzyme responsible for the degradation of cGMP. The raised levels of this cyclic nucleotide are thereby involved in the activation of the steroidogenic pathway and testosterone secretion, as recently PDE5 was identified in Leydig cells [14]. In the testis, cGMP signal transduction pathways are involved in a variety of local functions, based on autocrine or paracrine effects. In particular, cGMP has been suggested to influence motility in spermatozoa, development of testicular germ cells, relaxation of peritubular lamina propria cells, testosterone synthesis in Leydig cells and dilatation of testicular blood vessels [15]. Thus, chronic treatment with sildenafil is able to exert stimulatory actions on testosterone production via cGMP accumulation [16]. In the present study, we did not investigate cGMP accumulation, but it is speculated that testosterone accumulation in the supernatant induced by TAD exposure might be either a direct effect via cGMP or indirect via aromatase inhibition.

The effects of aromatase inhibitors (AIs) on the human skeletal system due to systemic estrogen depletion are becoming clinically important due to their increasing use as an adjuvant therapy in postmenopausal women with breast cancer [17]. Cytochrome P450-ARO converts androgens into estrogens, and the role of estrogens in the pathophysiology of male osteoporosis is still under evaluation [18]. Results published by Lormeau et al. confirmed the hypothesis according to which estrogens do not play a major role in the regulation of bone turnover in men [19]. Interestingly, they found that plasma levels of estradiol

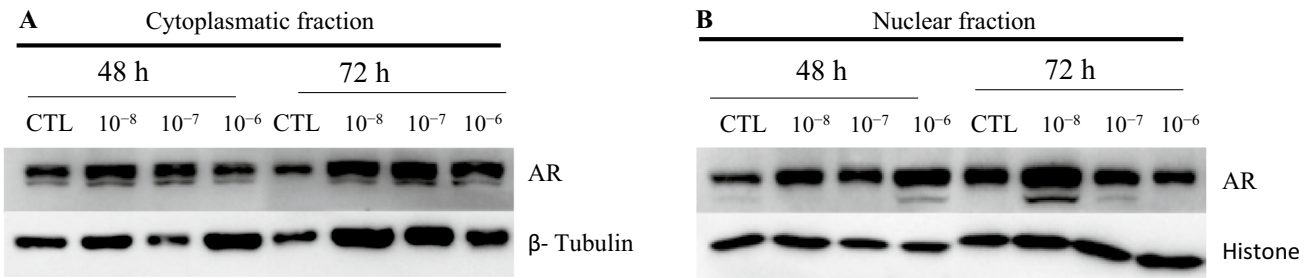

Fig. 7 Western blot analysis of cytoplasmatic (**a**) and nuclear (**b**) fractions of AR after different concentrations of TAD exposure at 48 and 72 h

were similar in both patients and controls. In our study, we provide for the first time evidence that chronic exposure to TAD in vitro is able to increase testosterone levels and, to interfere with androgen/estrogen receptor expression, suggesting a significant modulation and increase of AR expression versus ER intracellular pathway.

Osteocytes and osteoblasts express high amount of ERα, but also AR as well. The increased androgen levels occurring upon TAD exposure, might lead to the speculation that the increased AR expression could be responsible for a bone protective effect of TAD, since in our experience testosterone appears to play a predominant role in preserving male trabecular bone more than female bone [20]. Moreover, our recent unpublished observation shows that TAD increases AR expression in muscle cells in vitro (manuscript in preparation) and other steroid responsive cells, further indicating that TAD can modulate, accordingly, sex steroid pathways in different cellular systems, known target of steroid hormones actions.

Using osteoblastic cells which express aromatase, AR, and ERα, Miki et al. demonstrated that the steroidal AI exemestane stimulated cell proliferation via both AR-dependent and AR-independent pathways [21]. Interestingly, recent in vitro data had demonstrated that the AI anastrozole was able to exert more remarkable antiproliferative effects by prior activation of the AR by testosterone in breast cancer cells in vitro [22]. These findings further corroborate our results on the inhibitory effects of TAD on ARO and further highlight a potential new therapeutic use for the PDE5i molecules as potential agents for the treatment of prostate cancer. It is known that men with prostate carcinoma initially respond to treatments designed to inhibit androgen secretion or to block their action in target cells. Later the tumors may become refractory to androgen-related therapies. Indeed, anastrozole [23] and selective AI [24] had been proposed for the treatment of men with advanced prostate cancer with inconsistent results. We herein speculate that chronic exposure to TAD, by enhancing AR after common rehabilitative treatments following prostate cancer surgery, might be one of the mechanisms involved in prostate cancer cell maintenance of responsiveness to anti-androgen therapy.

The study has some limitations. We did not investigate PDE5 or cGMP activities, as well as any transduction mechanism involved in these effects. In addition, we did not evaluate PDE11 transcript levels and potential cross-talk inhibition of this isoenzyme by tadalafil in our experimental model.

In conclusion, this is the first demonstration that TAD modulates ARO activity and AR expression in human steroid target cells in an in vitro model system. Further in vivo studies are then required to fully characterize the role of TAD in both ARO and AR expression and activity modulation to fully understand the biological implication in other androgen/estrogen target tissues as well as its potential clinical implication in patients with androgen-resista1nt prostate cancer.

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Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest.

Ethical approval The study in object has been approved by the local ethical committee of Sapienza University of Rome; Compliant with the Ethical standards.

Informed consent Each subject signed a complete and detailed informed consent before entering the study.

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