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Binding of AR to SMRT/N-CoR complex and its co-operation with PSA promoter in prostate cancer cells treated with natural histone deacetylase inhibitor NaB

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Signaling through the androgen receptor (AR) plays a critical role in prostate cancer progression. The AR is a classical nuclear receptor (NR) providing a link between signaling molecule and transcription response. Histone deacetylase inhibitors (HDACI) have antiproliferative and proapoptotic effects on prostate cancer cells and their implication in silence AR signaling may have potential therapeutic use.

We aimed to study the inhibitory effects of the corepressor SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptors) which forms a complex together with nuclear receptor corepressor (N-CoR) and with histone deacetylase 3 (HDAC3) on AR activity.

The androgen-sensitive prostate cancer cell line LNCaP and androgen-insensitive prostate cancer cell line C4-2 both AR-positive, and androgen-insensitive DU145 and PC3 prostate cancer cell lines were treated with two HDACIs, sodium butyrate (NaB) and/or trichostatin A (TSA). We amplified immunoprecipitated DNA by conventional PCR and in the following step we used the chromatin immunoprecipitation (ChIP) analysis coupled with quantitative PCR for monitoring NaB induced formation of AR-SMRT/N-CoR complex binding on the PSA promoter. The co-immunoprecipitation assay revealed increase in AR-SMRT formation in NaB treated cells. Simultaneously, the Western blot analysis showed a significant decrease in AR protein expression.

In conclusion, the inhibitory effect of NaB on AR gene expression seems to be specific and unique for prostate cancer AR-positive cell lines and corresponds with its ability to stimulate AR-SMRT complex formation. We suggest that AR and SMRT/N-CoR corepressors may form a stable complex *in vitro* and NaB may facilitate the interaction between AR nuclear steroid receptor and SMRT corepressor prote

Key words: androgen receptor (AR), AR corepressor, HDAC inhibitor, histone deacetylases, chromatin immunoprecipitation (ChIP), sodium butyrate (NaB).

Abbreviations: (AR) androgen receptor, (CBP) CREB binding protein, (CPA) cyproterone acetate, (CT) Ct value, (FBS) fetal bovine serum, (D1) deiodinase 1 gene, (FC) fold-change, (HAT) histone acetyltransferase, (HDAC) histone deacetylase, (HDACi) HDAC inhibitors, (HDACs) histone deacetylases (HDACs), (HDAC3) histone deacetylase 3 (HDAC3), (N-CoR) nuclear receptor corepressor, (NaB) sodium butyrate, (NR) nuclear receptor, (PSA) prostate specific antigen, (qPCR) quantitative real-time PCR, (SAHA) suberoylanilide hydroxamic acid, (siRNA) small interference RNA, (SMRT) silencing mediator for retinoid and thyroid hormone receptors, (SD) standard deviation, (TR) thyroid hormone receptors, (TSA) trichostatin.

The androgen receptor (AR) plays a fundamental role in the development and progression of prostate cancer. In the initial stages, the growth of prostate cancers is dependent on androgens. However in advanced stages, the disease usually becomes progressive and unresponsive to androgen ablation therapies. This phase of the disease is known as hormone-refractory prostate cancer and it represents a major clinical problem.

The androgen receptor is a ligand-dependent transcription factor and a member of the nuclear receptor (NR) superfamily. The transcriptional activity of the AR is regulated not only by androgens, but also by coregulators, including both coactiva-

tors 40 and corepressors [1]. Several coactivators, such as the SRC (p160), CREB binding protein (CBP)/p300 and others enhance AR activity [2–4]. On the other hand, a group of corepressors inhibit AR activity through recruitment of histone deacetylase (HDAC) or inhibition of histone acetyltransferase (HAT) activity [5]. Histone deacetylases (HDACs) do not bind DNA directly but interact with DNA through multi-protein complexes that include coactivators and corepressors [6–7]. This study focused on the corepressor SMRT (Silencing Mediator for Retionid and Thyroid hormone receptors) which forms a complex with N-CoR (Nuclear receptor Co-Repressor) and histone deacetylase 3 (HDAC3) [8–10].

An increasing number of both histone- and non-histone proteins are being identified as substrates of the HDACs [8, 11–12]. The AR as the transcription factor is included as a non-histone proteins target of the HDACs. However, SMRT and N-CoR have been shown to interact with the AR, suppressing its transcriptional activity in the presence of the AR antagonists, flutamide and cyproterone acetate (CPA) [13]. Kang et al. [5] reported that antagonists of the AR such as bicalutamide, CPA and mifepristone (RU486) increased the binding capacity of N-CoR onto the AR promoter. However, bicalutamide antagonist activity seems to be independent of N-CoR and SMRT corepressors [14]. The mechanisms by which AR antagonists inhibit AR activity are apparently distinct from the functional mode of HDAC inhibitors (HDACi). Increase in AR-transactivation potential in the presence of inhibitors of HDACs is explained as increase in the acetylation of histone tails. Histone acetylation is assumed to result in a more open chromatin which is, in this way, activated for transcription. This suggests that like histones, epigenetic modifications of AR may be important for AR activity.

Inhibitors of HDACs (HDACi) have a range of antitumor activities including induction of cell cycle arrest in G2-phase, differentiation and apoptosis. The structural diversity among HDACi suggests that the mechanisms of action of these compounds may involve the interaction of the HDAC with proteins independent of deacetylase activity [15]. Naturally occurring (*i.e.* sodium butyrate – NaB (Fig. 1), trichostatin – TSA) and synthetic HDACi such as hydroxamic acids (*i.e.* suberoylanilide hydroxamic acid – SAHA), cyclic tetrapeptides (*i.e.* depsipeptide) and/or benzamides (MS-275 and MGCD0103) have been identified [16]. Butyrates and phenylbutyrates, including sodium butyrate (NaB), are agents with demonstrated HDAC inhibitory effects. NaB potently inhibits colon cancer cells and has been suggested as a treatment for colon cancer [17–18]. NaB effects on the transcription and transactivation activities of the AR gene in androgen-dependent prostate cancer cell line have also been studied [19–22]. Overall, while it remains that the mechanisms of action of HDACi are complex and not completely elucidated [15, 23], an inhibitory effect of NaB on coregulatory proteins that play a role in regulating activity of the AR gene activities could make this agent attractive as a potential anti-cancer therapeutic.

Materials and methods

Cell culture and cell conditions. The four human prostate cancer cell lines LNCaP, C4-2, PC-3 and DU145 were used in the study. LNCaP is androgen-dependent cell line which expresses functional AR. However, its AR contains a single point mutation changing the sense of codon 868 (Thr to Ala) in the ligand binding domain [24]. On the other hand, C4-2 cell line is androgen-independent, although it is derivated from the LNCaP cells. C4-2 cells retain a functional AR and in comparison with the parental LNCaP cells, the C4-2 subline expressed lower steady-state levels of AR protein and mRNA transcript [25]. As showed Tilley et al. [26], the LNCaP cells express high levels of AR, while the PC-3 and DU145 cell lines products no detectable amount of AR [26–27].

The human prostate cancer cell lines LNCaP, PC3 and DU145 were purchased from the American Type Culture Collection (ATCC, Rockville, MD), and the C4-2 cell line was purchased from UroCor Labs (Oklahoma City, OK). LNCaP and C4-2 cells were maintained in RPMI 1640 Media (Sigma, St Louis, MO) supplemented with final 10 % concentration of fetal bovine serum (FBS), 0.01 % antibiotics, 2 mM L-glutamine and 1 mM sodium pyruvate. DU145 and PC3 cells were maintained in Dulbecco´s Modified Eagle´s Media – DMEM (Gibco) supplemented with 10 % fetal bovine serum (FBS), 0.01 % antibiotics and 2 mM L-glutamine. All cells were maintained at 37°C and 5 % CO_2 atmosphere.

Treatment with HDAC inhibitors. Cells were grown to approximately 60 % confluency on 100-mm dishes and treated with sodium butyrate – NaB (Sigma, St Louis, MO) and/or TSA (Sigma, St Louis, MO) for 24 hours and 48 hours. NaB and TSA were dissolved in 10 % DMSO and added to the media at final 1 mM and 5 mM concentrations of NaB, and/or final 0.1 μM and 0.5 μM concentrations of TSA. A corresponding volume of DMSO (0.1 % final concentration of DMSO) was added to the control untreated cells.

siRNA. Cells in six-well plates were seeded at a density such that cells reached about 60 % confluency were used for the small interference RNA (siRNA) transfection. The siRNAs for HDAC3, N-CoR and/or SMRT (Santa Cruz Biotechnology, Santa Cruz, CA) were annealed and used according to the manufacturer´s instructions. Cells were transfected with 0.25 – 1 μg of each siRNA per well. Transfection medium (Santa Cruz Biotechnology, Santa Cruz, CA) was removed and replaced by fresh RPMI 1640 medium. The cells were incubated at 37°C for additional 18 hours. The normal medium was aspirated and transfected cells were treated with 1 mM and/or 5 mM concentrations of NaB for 24 hours. The cells were collected and immediately re-suspended in SDS loading buffer. The proteins were separated on 10 % SDS-PAGE and immunoblotted with primary antibody against AR (clone AR441, Santa Cruz Biotechnology, Santa Cruz, CA).

Western blotting. Total cellular proteins (30 μg) were separated into 8 – 10 % SDS-PAGE and transferred to nitrocellulose membrane (Amershan Biosciences, Little Chalfont, UK). After

blocking in buffer containing 5 % (w/v) milk, the membranes were treated overnight at 4°C with primary antibodies against AR (clone AR441, Santa Cruz Biotechnology, Santa Cruz, CA), SMRTe (clone 1542/H7, Santa Cruz Biotechnology, Santa Cruz, CA), HDAC2 (clone #2545, Cell Signaling Technology®, Boston, MA), HDAC3 (clone #2632, Cell Signaling Technology®, Boston, MA) and α-tubulin (clone DM1A, Sigma, St Louis, MO) for loading control. Following a second incubation with peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), the proteins was visualized with Supersignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

Co-immunoprecipitation assay. The aliquots of chromatin protein lysates (400 μg) were incubated with 1 μg of antibody against AR (clone AR441, Santa Cruz Biotechnology, Santa Cruz, CA) with rotation at 4°C for one hour. Normal mouse IgG (sc-2025, Santa Cruz Biotechnology, Santa Cruz, CA) was used as negative control (1 μg). Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the samples and incubated with rotation at 4°C for overnight. The precipitates were collected by centrifugation and washed once with Low salt immune complex wash buffer, once with High salt immune complex wash buffer and twice with RIPA buffer. The final precipitate was dissolved in SDS loading buffer and analysed by 8 % SDS-PAGE and Western blotting. The anti-SMRT monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA).

Conventional PCR analysis of immunoprecipitated DNA. Fifty nanograms of immunoprecipitated DNA were used in 25-μl PCR reaction mixture with DyNAzyme™ EXT DNA polymerase (Finnzymes, Espoo, Finland). The reaction mixture was initially denaturated at 94°C for 15 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, elongation at 72°C for 90 s, and the final extension at 72°C for an additional 10 min after the last cycle. The 274-bp DNA fragment was amplified with the primer for PSA promoter 5'-GAGAGCTAGCACTTGCTGTT-3' and 5'-AGTTCTAGTTTCTGGTCTCA-3' [28]. PCR products were analyzed by gel electrophoresis with ethidium bromide in 1 % agarose gels.

ChIP immunoprecipitation. Cells in 100-mm dishes were cross-linked with 1 ml of 11 x Formaldehyde stock solution (1 % final concentration of formaledehyde). Cross-linking was stopped by addition of glycine (125 mM final concentration) and cells were washed with PBS. The cells were scraped in Chro-IP lysis buffer containing protease inhibitors (cOmplete Protease Inhibitor Coctail, Roche, Diagnostics, Basel, Switzerland). Chro-IP lysis buffer (50 mM Hepes-KOH, pH 8.0, 1 mM EDTA pH 8.0, 0.5 mM EGTA, 140 mM NaCl, 10 % glycerol, 0.5 % NP-40, 0.25 % Triton X-100) was used for a lysis of cells. The lysates were reversed on a rotator for 10 min and after centrifugation at 600 g for 5 min at 4°C, and followed washing. After centrifugation (600 g, 5 min, 4°C) pellets were resuspended in 1 x RIPA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0, 0.5 mM EGTA, 140 mM NaCl, 1 % Triton, 0.1 % Na-deoxycholate, 0.1 % SDS) and sonicated to break chromatin. Then the samples were centrifuged at 16,000 g for 10 min at 4°C. Either 2 μg of AR (N-20, Santa Cruz Biotechnology, Santa Cruz, CA) antibody or 2 μg of SMRTe antibody were added to aliquots of 700 μg of chromatin protein lysates and incubated with rotation at 4°C overnight. Salmon sperm DNA/Protein A agarose (Upstate Biotechnology, Temecula, CA) was added to the samples and incubated with rotation at 4°C for one hour. The samples were centrifuged at 600 g for 3 min at 4°C and the pellets were washed once with RIPA buffer containing 100 μg/ml salmon sperm DNA, once with Low salt immune complex wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once with High salt immune complex wash buffer (0.1 SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) and once with RIPA buffer containing 100 μg/ml salmon sperm DNA. After centrifugation, 100 μl of elution buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 % SDS) was added and samples were placed at 65°C for 20 min with occasional gentle agitation. The agarose beads were removed by centrifugation, when the supernatants were moved to new tubes. The cross-linking was reverted by heating at 65°C for 6 hours and then, proteinase K (100 μg/ml) was added to each tube and incubated at 55°C overnight. DNA was purified with QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions.

ChIP analysis coupled with quantitative PCR. Immunoprecipitated (40 ng DNA) and input samples (40 ng DNA) were amplified in a LightCycler® 480 Roche with detection system (Roche Diagnostics, Basel, Switzerland). There were using primers specific for the PSA promoter (forward: 5'-TTCAGGAGCATGAGGAATAAAAG-3'; reverse 5'-GACTCCCTGATCCCTGCAC-3) and probe (#54, Roche Diagnostics, Basel, Switzerland) corresponding to the primers. Cycling parameters for 20 μl reactions were 95°C 10 min, followed by 45 cycles of 95°C 10 s and 60°C 30 s, finished with 40°C 10 s.

Data analysis. In our study, the relative quantification of specific DNA fragments present in immunoprecipitated samples we measured by signal to noise normalized to percent input [29–31]. Samples of input (i), target region – PSA promoter region (tr) and negative control – sample without antibody (nc) were all from the same sonication. For each sample we ended up with an average Ct value (CT.i, CT.tr and CT.nc) and a standard deviation (SD.i, SD.tr and SD.nc). We calculated the delta Ct values (dCT.tr) and error associated for PSA promoter region (dSD.tr) and for our negative control (dCT.nc and dSD.nc) relative to input sample (i). Propagated error values of these dCTs were called dSD.tr and dSD.nc. These were calculated using the following formulas:

dCT.tg = CT.i – CT.tg
dCT.nc = CT.i – CT.nc
dSD.tr = sqrt((SD.i)^2 + (SD.tr)^2) / sqrt(n)
dSD.nc = sqrt((SD.i)^2 + (SD.nc)^2) / sqrt(n)
where n = number of replicate qPCR wells per sample (in this case is n = 3)

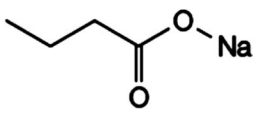

Figure 1. Sodium butyrate (NaB)

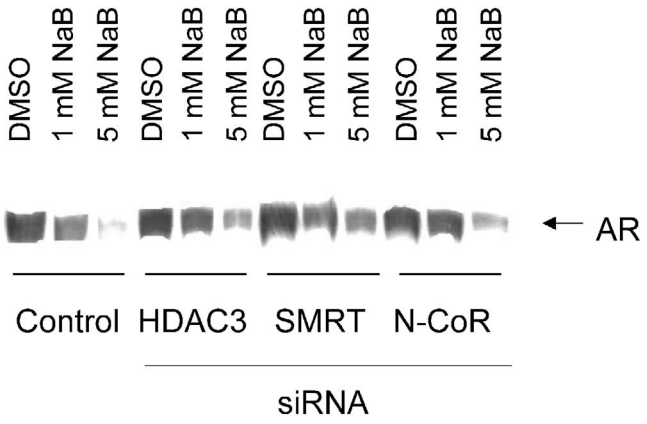

Figure 2. Knocking down HDAC3, SMRT and/or N-CoR in LNCaP cells after transfection with specific siRNAs. The cells were cultivated either in medium containing final 0.1 % concentration of DMSO (DMSO) or treated with 1 mM (1 mM NaB) and/or 5 mM concentrations (5 mM NaB) of NaB for 24 hours. Total cellular proteins were prepared 24 hours after treatment and analysed by Western blotting with antibody against AR (AR441, 110 kDa).

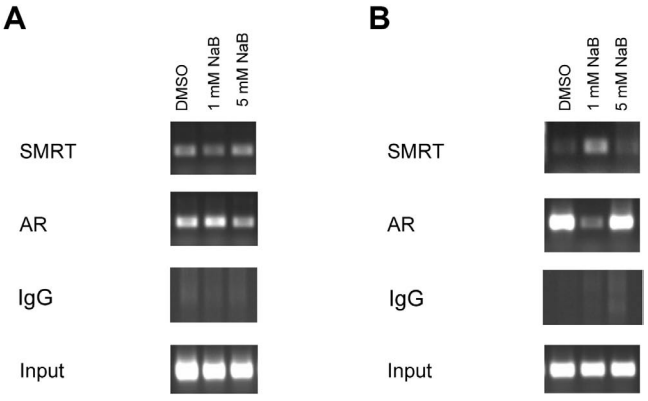

Figure 3. Conventional PCR analysis of immunoprecipitated DNA in LNCaP and C4-2 cells. Untreated cells (DMSO) and cells treated with 1 mM and/or 5 mM NaB for 24 hours were immunoprecipetated with AR (N-20) and/or SMRT antibodies. Immunoprecipitated DNA after ChIP assay were PCR-amplified with primers specific for *PSA* gene promoter (274 bp DNA fragment).

Once we got dCT values and the associated error values (dSD) in hand, we calculated the signal to noise (i.e. fold change over the negative control). This was done by finding the delta-delta CT (ddCT) of the target region (PSA promoter region); essentially the difference in CT values between the dCT.tr and dCT.nc. We used the following formulas:

ddCT = dCT.tr – ddCT.nc
ddSD = sqrt((dSD.tr)^2 + (dSD.nc)^2)
Once we had the ddCT and ddSD for the PSA promoter region, the transformation to linear "fold change" values was done using these formulas:

FC = 2^(ddCT)
FC.error = ln(2) * ddSD * FC

Table 1. Final calculated values of qPCR analysis.

Sample	FC	FC error
LNCaP DMSO AR (N-20)	0,95064751	0,494475967
LNCaP 1mM NaB AR (N-20)	2,242463045	0,576305155
LNCaP 5mM NaB AR (N-20)	1,263804405	0,293388388
LNCaP DMSO SMRT	0,385531325	0,118115768
LNCaP 1mM NaB SMRT	2,965298931	0,730937338
LNCaP 5mM NaB SMRT	1,849893249	0,215199088
LNCaP DMSO IgG	0,032516847	0,010208893
LNCaP 1mM NaB IgG	0,095637249	0,037921869
LNCaP 5mM NaB IgG	0,131386521	0,02268366
C4-2 DMSO AR (N-20)	1,083871804	0,398181709
C4-2 1mM NaB AR (N-20)	0,484662166	0,21241411
C4-2 5mM NaB AR (N-20)	1,023852081	0,095995906
C4-2 DMSO SMRT	1,051419335	0,188740104
C4-2 1mM NaB SMRT	0,84444694	0,35973198
C4-2 5mM NaB SMRT	2,813484509	0,736090046
C4-2 DMSO IgG	0,16583822	0,02901306
C4-2 1mM NaB IgG	0,238607391	0,101790318
C4-2 5mM NaB IgG	0,419486521	0,022688625

DMSO, 1mM NaB, 5mM NaB – treatment; AR (N-20), SMRT, IgG – antibodies; FC – fold change value

Results

Formation of AR-SMRT complex and reduced AR protein expressions after NaB treatment of AR-positive cells

SMRT silencing by specific siRNA seems to be the most effective in reducing AR protein expression after co-treatment of LNCaP cells with 5 mM NaB compared to siRNA specific for N-CoR and HDAC3 (Fig. 2). The results shown in Figure 3 indicated a reduced PCR amplification of PSA gene promoter by treatment with 5 mM NaB in LNCaP cells (Fig. 3A) and with 1 mM NaB in C4-2 cells (Fig. 3B). In contrast, C4-2 cells showed increased levels of PSA gene product under the same conditions.

For analysis of immunoprecipitated DNA by qPCR we calculated the fold-change (FC) and standard deviation of fold change (FC.error) with all error properly propagated and linearly transformed. The values from the qPCR analysis together with final values based the formulas above are shown in Tab. 1. Both

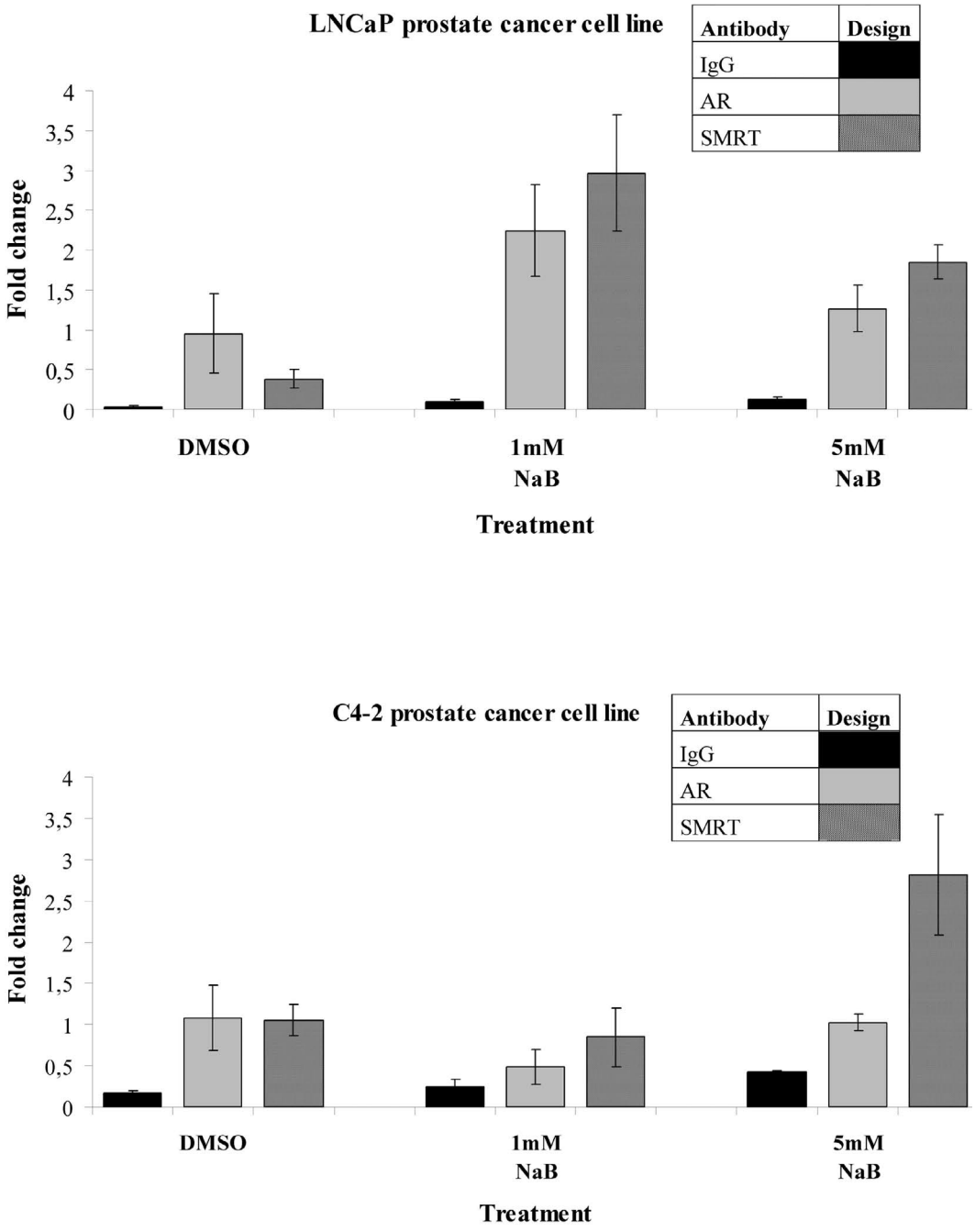

Figure 4. ChIP analysis coupled with quantitative PCR in LNCaP and C4-2 cells. The changes of formation AR-SMRT complex binding to *PSA* promoter and/or binding AR without SMRT to *PSA* promoter were analysed. Samples after chromatin immunoprecipitation and inputs (samples were prepared by isolation of DNA from sonicated chromatin sample without immunoprecipitation) were analyzed by qPCR using *PSA* promoter primers. Results fold changes were based upon above mentioned formulas (the final values presented in Tab. 1).

1 mM and 5 mM concentrations of NaB induced formation of AR-SMRT complex binding on PSA promoter and decreased binding formation of AR on PSA promoter without SMRT in LNCaP and/or in C4-2 cells (Fig. 4).

We found that total protein extracts obtained from affected LNCaP and C4-2 cells exhibited significantly lower expression of AR protein after 5 mM NaB and/or 0.5 μM TSA for 24 hours (Fig. 5) and 48 hours (Fig. 6) treatment. In contrast, in affected

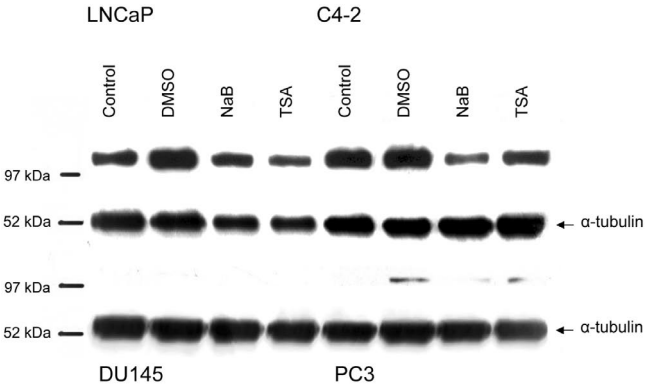

Figure 5. AR protein expression in prostate cancer cell lines treated with NaB and TSA after 24 hours. Control – untreated cells cultivated in medium; DMSO – untreated cells cultivated in medium with final 0.1 % concentration of DMSO; NaB – cells treated with 5 mM NaB (NaB was dissolved in 0.1 % final concentration of DMSO); TSA – cells treated with 0.5 μM TSA (TSA was dissolved in 0.1 % final concentration of DMSO).

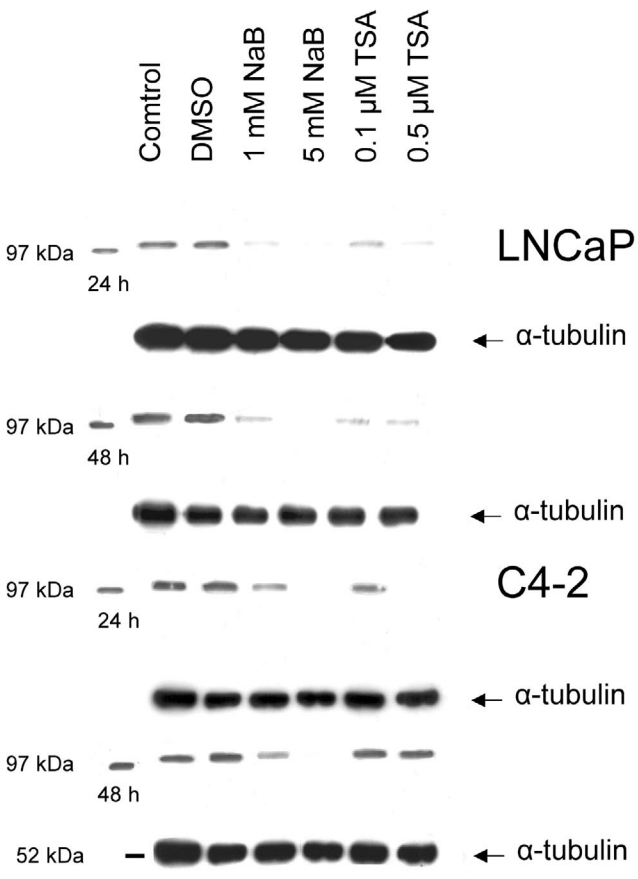

Figure 6. AR protein expression in LNCaP and C4-2 cell lines treated with NaB and TSA after 24 and 48 hours. Control – untreated cells cultivated in medium; DMSO – untreated cells cultivated in medium with final 0.1 % concentration of DMSO; 1 mM NaB and 5 mM NaB – cells treated with 1 mM and/or 5 mM NaB (NaB was dissolved in 0.1 % final concentration of DMSO); 0.1 μM TSA and 0.5 μM TSA – cells treated with 0.1 μM and/or 0.5 μM TSA (TSA was dissolved in 0.1 % final concentration of DMSO).

DU145 cells, and in control untreated cells, no AR protein was obtained. In PC3 cells, a weak AR protein expression was observed (Fig. 5).

To determine whether SMRT was responsible for inhibition of AR protein expression in cells treated with NaB, we performed co-immunoprecipitation assays in LNCaP cells. We found that treatment of LNCaP cells with 1 mM and/or 5 mM NaB caused increased the of AR-SMRT formation after 24 hours (Fig. 7).

Reduction of HDAC2 and HDAC3 protein expressions in AR-positive cell lines after treatment by HDAC inhibitors NaB and TSA

No differences in HDAC2 or HDAC3 protein expression were found between control samples and samples acquired from cells treated with 5 mM NaB and/or 0.5 μM TSA in LNCaP, C4-2 and PC3 cell lines. We found reduced HDAC2 and HDAC3 expression in samples treated with 5 mM NaB and/or 0.5 μM TSA only in the DU145 cell line (Fig. 8).

Discussion

We investigated the role of SMRT/N-CoR corepressor complex in the regulation of AR protein expression in prostate cancer cells treated with the HDAC inhibitors, NaB and/or TSA. We focused attention on the effect of corepressor SMRT on AR activation in AR-positive prostate cancer cells after treatment with NaB. Initially, we amplified immunoprecipitated DNA from cells treated with 1 mM and/or 5 mM NaB by conventional PCR. The limitation of the conventional amplification of immunoprecipitated DNA by PCR is quantification of the PCR product only at the end of the PCR reaction. For this reason it is not possible to interpret the results shown in Fig. 3 as quantitative. Analysis of ChIP immunoprecipitates by quantitative real-time PCR (qPCR) (Fig. 4) is more accurate then conventional PCR. In the co-immunoprecipitation assay, increase in AR-SMRT/N-CoR complex formation in NaB treated cells was found (Fig. 7). We suggest that AR and SMRT/N-CoR corepressors may form a stable complex and that NaB may facilitate this interaction between AR nuclear steroid receptor and SMRT corepressor protein.

Additionally, we found that the HDAC inhibitors had no affect on HDAC2 or HDAC3 protein expressions in AR-positive prostate cancer cells (LNCaP, C4-2 and PC3 cell lines). Treatment with HDAC inhibitors may trigger chromatin modifications with potential epigenetic implications involving SMRT/N-CoR complex formation with HDAC3. We believe that HDAC3 associated with SMRT/N-CoR corepressors may

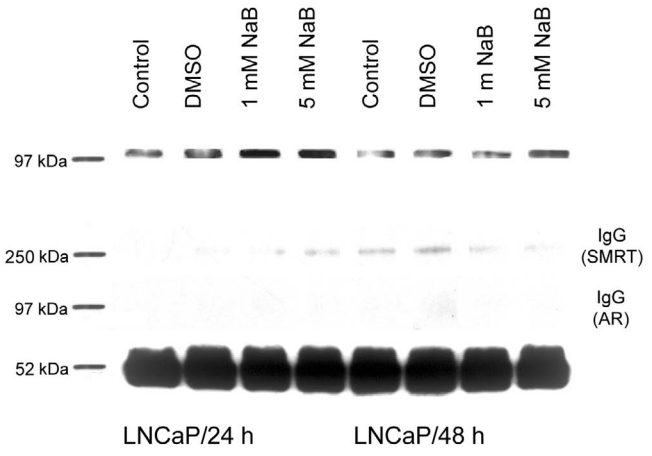

Figure 7. NaB effect on forming AR-SMRT complex *in vitro* in LNCaP cell line. Untreated cells cultivated in medium (Control), untreated cells cultivated in medium containing 0.1 % concentration of DMSO (DMSO) and the cells treated with 1 mM and/or 5 mM NaB for 24 and 48 hours were used. Proteins from the lysates were immunoprecipitated by anti-SMRT antibody. The precipitates were resolved into 8 % SDS-PAGE followed by Western blotting with antibody against AR (AR441, 110 kDa) and the AR-SMRT (275 kDa) complex was detected. Immunoprecipitation was performed using negative control normal mouse IgG and immunodetection with AR (IgG-AR, 110, 112 kDa) and/or SMRT (IgG-SMRT, 275 kDa) antibodies followed. The α-tubulin (55 kDa) for loading control was used.

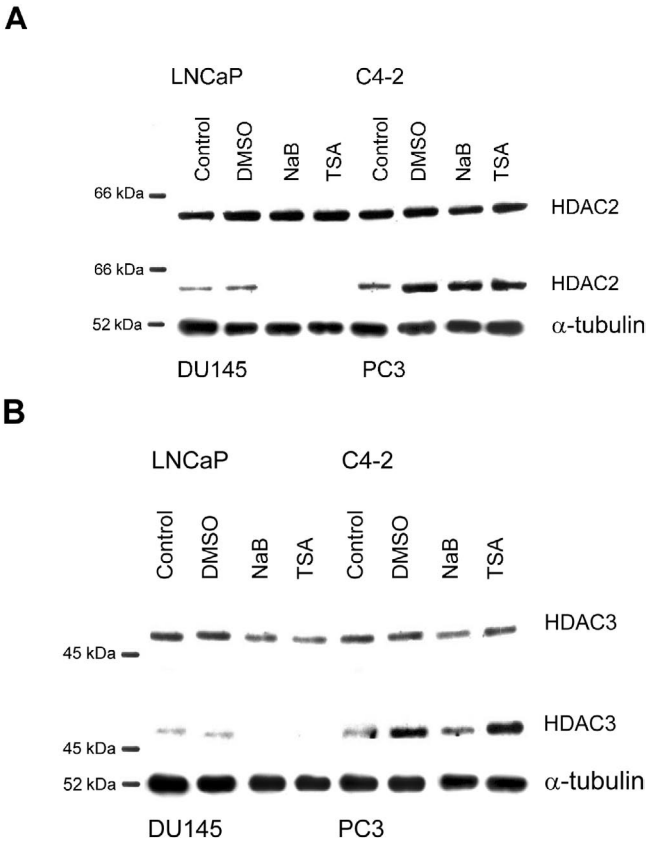

Figure 8. Effect of inhibitors HDAC on HDAC protein expressions. The HDAC2 (A) and HDAC3 (B) protein expressions were detected in control samples (Control – untreated cells cultivated in medium; DMSO – untreated cells cultivated in medium with final 0.1 % concentration of DMSO) and cell samples treated with 5 mM concentration of NaB (NaB) and/or 0.5 μM concentration of TSA (TSA) acquired from LNCaP, C4-2, DU145 and PC3 cells. Total cellular proteins were resolved by 10 % SDS-PAGE followed by Western blotting using HDAC2 (60 kDa) and HDAC3 (49 kDa) antibodies.

be protected against the inhibitory effect of NaB or TSA. We found that treatment of DU145 cells with 5 mM NaB and/or 0.5 μM TSA after 24 hours, inhibited HDAC2 and HDAC3 protein expressions (Fig. 8). Since it is known that the AR gene expression in this cell line can be suppressed by hypermethylation [32], it is possible that this mechanism, mediated by interaction between AR and SMRT/N-CoR/HDAC3 complex, is defective in this cell line.

Gene transcription roughly correlates with degree of histone acetylation [33-34]. This suggests that the recruitment of histone acetylases could be critical in the assembly of the AR transcription complex. NaB effects on AR-mediated PSA gene transcription have been examined and as expected, histone acetylase activity was found to be inhibited by NaB [35]. Given the evidence that SMRT and N-CoR form complexes with HDAC3 [8-10, 36], these corepressors could have an additive effect on the inhibition of transcription and histone acetylation. However, despite the roles of SMRT and N-CoR in regulating the transcriptional activity of several NRs, the significance of SMRT and/or N-CoR on AR transcriptional activity is less clear. Both SMRT and N-CoR proteins interact with AR and bind to the PSA promoter or to various AREs of AR target genes [37-38]. We analyzed binding of AR-SMRT complex to PSA promoter and/or binding AR alone to PSA promoter using various NaB concentrations. The ChIP analysis coupled with qPCR of LNCaP and C4-2 cells demonstrated that NaB promoted the formation of the AR-SMRT complex (Fig. 4). Simultaneously, increased formation of AR-SMRT complex after NaB treatment indicates that SMRT may be responsible for the suppression of AR transcription activity. It has been suggested that changes in histone acetylation status play an important role in nuclear receptor activity. In addition, Kang et al. [5] found that transcriptional activation of AR is accompanied by a cascade of distinct covalent histone modifications. Consistent with this, HDAC inhibitors can repress transcription [39]. Korkmaz et al. [19] studied the role of histone acetylation on AR function. Using three independent HDAC inhibitors: depsipeptide (FR901228), NaB and TSA, they found that inhibition of HDAC activity caused significant increase in the transcription ability of AR in the LNCaP cell. They found dose-dependent effects of NaB and depsipeptide on AR activity: low doses caused increase in levels of PSA mRNA, whereas high doses of NaB completely inhibited PSA expression. This implies that HDAC inhibitors

repress both AR expression and AR-dependent expression of PSA in a dose-dependent manner [19, 38]. Another mechanism of AR suppression by HDAC inhibitors was shown by Welsbie *et al.*, [40]. Synthetic HDAC inhibitors, Vorinostat (SAHA) and LBH589 block AR activity through suppression of the coactivator/RNA polymerase II complex assembly after binding of AR to the promoters of target genes. Rokhlin *et al.*, [20] found that TSA sharply reduced AR gene expression after 24 hours treatment, with partial recovery after 48 hours and return to normal levels after 72 hours later. Similarly, we observed the same changes in cell lines with functional AR. However, we found more intense NaB effects after 24 hours treatment (Fig. 6).

In conclusion, the inhibitory effect of NaB on AR gene expression (Fig. 5 and Fig. 6) appears to be specific to prostate cancer AR-positive cell lines. This corresponds with its ability to stimulate AR-SMRT complex formation (Fig. 3, Fig. 4) and SMRT protein expression (Fig. 7).

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