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·Original Article·

Ectopic expression of neurotrophic peptide derived from saposin C increases proliferation and upregulates androgen receptor expression and transcriptional activity in human prostate cancer cells

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Abstract

Aim: To determine the effects of the functional domain of saposin C (neurotrophic peptide [NP]) on androgen receptor (AR) expression and transcriptional activity. **Methods:** We constructed DNA vectors expressing NP or a chimeric peptide of the viral TAT transduction domain and NP (TAT-NP) using gene cloning technology. The effects of ectopic expression of NP or TAT-NP on cell growth were examined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. Reverse transcription-polymerase chain reaction (RT-PCR), Western blot, transient transfection and reporter gene assays were used to determine the effects of NP on AR expression and activation. **Results:** NP stimulated proliferation of androgen responsive LNCaP cells in the absence of androgens. RT-PCR and Western blot analyses showed that ectopic expression of NP resulted in induction of AR gene expression, and that the NP-stimulated expression of AR could be synergistically enhanced in the presence of androgens. Furthermore, reporter gene assay results showed that NP could enhance AR transactivation by increasing androgen-inducible gene reporter activity. **Conclusion:** We provided evidence that ectopic expression of saposin C-originated NP could upregulate AR gene expression and activate the AR transcriptional function in an androgen-independent manner in prostate cancer cells. *(Asian J Androl 2007 Sep; 9: 601–609)*

Keywords: neurotrophic peptide; androgen receptor; saposin C; prostate carcinoma cell lines

1 Introduction

A tremendous amount of information supports the roles of androgens and androgen receptors (AR) in the development and progression of prostate cancer (PCa) [1, 2]. AR expression has been observed in primary, metastatic and hormone-refractory malignant stages of

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PCa. Molecular mechanisms that delineate the roles of AR in the development of recurrent hormone-refractory tumors have been proposed, including alterations in the AR gene mutations, amplification, or cross-talk with signal pathways that can be initiated by certain growth factors or cytokines [3–5]. As a consequence, growth factors are also implicated in the activation of AR in addition to androgens, and it is generally accepted that gain of function of AR activity appears to be essential for outgrowth and survival of hormone-refractory PCa.

Prosaposin is a highly conserved glycoprotein and the precursor of four small heat-stable sphingolipid activator proteins (saposin A, B, C and D), which are required for the enzymatic hydrolysis of sphingolipids in lysosomes [6]. As a neurotrophic factor *in vivo* and *in vitro*, the functional sequence of prosaposin is localized at the amino terminal end (14–22 residues) of saposin C. Several synthetic peptides, including prosaptide TX14A derived from this region, are equally bioactive as prosaposin [6–8]. Prosaposin, saposin C and prosaptides are proposed to exert their neurotrophic effects by binding to a putative high affinity G protein-coupled receptor (GPCR) [8] and thus they induce cell differentiation and prevent cell death in neuroglial-derived cells [9, 10]. In addition, prosaposin is also expressed as a secretory protein in various cell types and body fluids, including blood, seminal plasma, seminiferous tubular fluid and prostatic secretions [6]. The inactivation of the prosaposin gene affects the development of the prostate gland, suggesting that prosaposin plays an important role in formation of prostate at early stage [11]. Recently, prosaposin overexpression has been observed in PCa cells, tissues and xenografts [12]. Prosaposin, saposin C and prosaptide TX14A can stimulate PCa cell growth, migration and invasion through the activation of MAPK and PI3/AKT signal pathways [13–15]. Furthermore, Koochekpour *et al.* [16] reported that addition of prosaposin and saposin C resulted in activation of AR in LNCaP cells in the absence of androgens. Our main goal in the present study was to investigate whether ectopic expression of neurotrophic domain of saposin C could regulate AR expression and activity in PCa cells. To test this possibility, we constructed expression vectors encoding the neurotrophic sequence of saposin C (neurotrophic peptide [NP]).

2 Materials and methods

2.1 Plasmid construction

Based on the functional domain sequence (14-mer peptide, TKLINDDKTEKEIL) of saposin C [17], we synthesized two complementary NP oligonucleotides containing KpnI and ApaI restriction enzyme sites at 5' and 3' ends, respectively and they are 5'-CATGACCAAGCTGATTGACAACAACAAGACTGAGAAAGAAATACTCTAATAGGGGCC-3' (sense) and 5'-CCTATTAGAGTATTTCTTTCTCAGTCTTGTTGTTGTCAATCAGCTTGGTCATGGTAC-3' (antisense). After annealing, the DNA fragment was inserted into pcDNA3.1 (+) and designated as pcDNA-NP. Similarly, double stranded DNA fragment was synthesized as above, containing a TAT protein transduction domain (TAT) sequence derived from HIV-1 and followed by the NP sequence. TAT was a transmembrane signal peptide and assumed to help NP run across cell membrane. An alanine was inserted between TAT and NP, serving as a hinge of two functional regions. Two complementary sequences containing HindIII and XbaI sites at 5' and 3' ends, respectively, were as follows: 5'-AGCTTATGTACGGCAGGAAGAAGCGTCGTCAGCGCAGGCGCGGTACCAAGCTGATTGACAACAACAAGACTGAGAAAGAAATACTCTAATAGT-3'(sense) and 5'-CTAGACTATTAGAGTATTTCTTTCTCAGTCTTGTTGTTGTCAATCAGCTTGGTACCGCGCCTGCGCTGACGACGCTTCTTCCTGCCGTACATA-3' (antisense). The annealed DNA fragment was cloned into pcDNA3.1 (+) to generate the pcDNA-TAT-NP plasmid. The authenticity of the above recombinant plasmids was confirmed by direct sequencing.

2.2 Cell culture and chemicals

Human PCa cell lines, LNCaP (The American Type Culture Collection, Rochville, MD, USA), PC3 and DU145 (The Cell Bank of Chinese Academy of Sciences, Shanghai, China) were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% newborn bovine serum, respectively, until reaching approximately 50–70% confluence. The cells were maintained in serum-free RPMI 1640 medium for 24 h to deplete endogenous steroid hormones before experiments. Unless specified otherwise, treatment with mibolerone (Mib, New England Nuclear), a synthetic androgen, was carried out for 24 h after transfection. Mib was dissolved in ethanol, and ethanol was also used as a control vehicle.

2.3 Cell proliferation assay

PC3 and DU145 cells were seeded in 96-well plates at a density of 0.5×10^4 cells/well, and LNCaP cells were seeded in 24-well plates at a density of 3×10^4 cells/well. The cells were cultured under the conditions described above. The expression vector pcDNA-NP (0.2 μg/well in 96-well plates, 0.8 μg/well in 24-well plates), or pcDNA-TAT-NP (0.2 μg/well in 96-well plates, 0.8 μg/well in 24-well plates) was transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent. The parental vector pcDNA3.1 (+) (0.2 μg/well in 96-well plates, 0.8 μg/well in 24-well plates) served as a negative control. After transfection, the cells were maintained in serum-free RPMI 1640 medium for an additional 24, 48 and 72 h. The number of living cells was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. Six replicates were used for each treatment and the assay was repeated at least three times.

2.4 RNA extraction and RT-PCR analysis

PC3 and LNCaP cells were seeded in 6-well plates as described above. After transfection of pcDNA-NP (4.0 μg/well), or pcDNA-TAT-NP (4.0 μg/well), the cells were maintained in the serum-free medium for an additional 24 h. Removal of the media was followed by a brief rinse with 1 mL cold phosphate buffered saline (PBS). The cells were used for total RNA extraction with TRIzol Reagent as specified by the manufacturer (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase I (Takara, Dalian, China) to degrade plasmid DNA. The first-strand synthesis of cDNA was made with 5 μg of total RNA and random hexamer primers using the RevertAid First-Strand cDNA Synthesis kit (Fermentas, Burlington, Ontario, Canada). Polymerase chain reaction (PCR) was then carried out under the following conditions: 28 cycles at 95ºC for 60 s, 58ºC for 60 s, 72ºC for 1.5 min, with a final 10-min extension cycle at 72ºC. Taq polymerase (Takara, Dalian, China) was used to detect desired genes in transcript abundance. Primers were synthesized by Invitrogen Corporation (Invitrogen). The oligonucleotides primers for NP and TAT-NP expression were as follows: 5'-TAATACGACTCACTATAGGG-3' (T7 promoter primer), and 5'-TAGAAGGCACA GTCGAGG-3' (pcDNA3.1/BGH reverse terminator primer). The expected lengths of PCR products are pcDNA-TAT-NP (193 bp), pcDNA-NP (157 bp) and pcDNA3.1 (+) (180 bp). The primers for AR gene transcript were: 5'-TTGGAGACTGCCAGGGAC-3' (forward), and 5'-TCAGGGGCGAAGTAGAGC-3' (reverse) (686 bp, according to the human AR sequence from the NCBI/genome data bank). The primers for the β-actin transcript were (359 bp, based on β-actin cDNA sequences from the NCBI/genome data bank): 5'-ACCAACTGGGACGACAT-3' (forward) and 5'-CG CTCGGTGAGGATCTTCAT-3' (reverse).

The PCR products were confirmed as a single band through 1.2% agarose gel electrophoresis and normalized with β-actin. The experiments were repeated for at least three times independently and each PCR experiment included non-template control.

2.5 Western blot analysis

PC3 and LNCaP cells were seeded in 75 mL culture flasks using the same treatment described above. Removal of the media was followed by a brief rinse with 3 mL cold PBS, and cell pellets were obtained by centrifugation. Whole cell lysates were prepared according to the method described previously [18]. Freshly prepared protease inhibitors (0.5 mmol/L phenylmethanesulfonyl fluoride (PMSF), 50 μg/mL aprotinin, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride and 10 mmol/L β-glycerolphosphate) were also added. The Bradford protein assay (Bio-Rad, Hercules, CA, USA) was used for quantifying the protein content. Fifty micrograms of protein for each sample was separated on an SDS polyacrylamide gel (8%), and the gel was electrotransferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) for Western analysis. The blots were blocked with 5% non-fat milk in TBST buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.1% Tween 20, pH 8.0) prior to incubation with specific antibody of AR (BD Biosciences, 554225) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After washing with TBST buffer for three times, the membranes were incubated with an anti-rabbit or anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h and visualized by enhanced chemiluminescence substrate (ECL, Amersham, Arlington Heights, IL, USA).

2.6 Transient transfection and reporter gene activity assays

PC3 and LNCaP cells were incubated in 24-well plates under the conditions described above for 48 h before transfection. For transfection into LNCaP cells, pGL3

basic vector with 6-kb prostate-specific antigen (PSA) promoter (PSA promoter-Luc, 0.8 μg/well), or pGL3-SV40 with three copies of androgen response elements (ARE) of hk2 gene (hk2-3ARE-Luc, 0.8 μg/well) [18], and pcDNA-NP (0.3 μg/well) or pcDNA-TAT-NP (0.3 μg/well) were cotransfected with Lipofectamine 2000. For transfection into PC3 cells, the human AR expression vector pSG5-hAR (0.2 μg/well) was included for cotransfecting with the plasmids described above. The parental vectors pcDNA3.1 (0.3 μg/well), pGL3 basic (0.8 μg/well) and pGL3-SV40 (0.8 μg/well) were used as controls. The phRL-TK vector (0.1 μg/well, Renilla luciferase, Promega, Madison, WI, USA) served as an internal control to normalize transfection efficiency. Twenty-four hours after transfection, cells were either treated with 1 nmol/L of Mib or remained untreated for an additional 24 h in serum-free medium. Cell extracts were prepared and used for dual-luciferase assay (Dual-Luciferase Reporter assay system, Promega, Madison, WI, USA). At least three independent transfection experiments were performed.

Statistical analysis was performed using the 2-tailed Student's *t*-test. $P < 0.05$ was accepted as the level of significance.

3 Results

3.1 Identification of NP mRNA expression in PCa cells

We first constructed expression vectors containing NP or TAT-NP. Expression levels of NP and TAT-NP after transfection were investigated at mRNA level using RT-PCR. The time course study revealed that the plasmids of pcDNA-TAT-NP (lane 2 in Figure 1A) and pcDNA-NP (lane 3 in Figure 1A) can be continuously expressed at the three measured time points (12, 24 and 48 h) in LNCaP cells, which were maintained at least up to 48 h, as shown in Figure 1A. No bands were seen in the negative control (lane 1). The bands observed in lane 4 were from a transcript containing the multiple clone site sequence in pcDNA3.1 vector. It seemed that the mRNA expression levels of NP and TAT-NP had no significant difference among various time-periods. The mRNA expressions of NP and TAT-NP were further analyzed in PC3 cells. As shown in Figure 1B, the bands could be observed in cells transfected with pcDNA-TAT-NP (lane 2) and pcDNA-NP (lane 3) with a similar expression pattern to that in LNCaP cells. These results suggested that the plasmids of NP and TAT-NP can be expressed in PCa cells.

3.2 NP stimulates PCa cell growth

To test proliferative ability of ectopic expression of NP or TAT-NP, MTT assays were used to analyze the cell growth. The result in Figure 2A shows that ectopically expressed NP and TAT-NP can stimulate proliferation of LNCaP cells by 15% and 10% at 24 h, 14% and 20% at 48 h, and 10% and 16% at 72 h, respectively, as compared with the control group. It appeared that there was no obvious difference between NP and TAT-NP in terms of their proliferative stimulation activity. In addition, stimulatory effects of NP on cell proliferation were examined by using PC3 and DU145 cell lines, and similar results were obtained as shown in Figure 2B and 2C. The NP and TAT-NP-stimulated effects were observed increased by 20% and 19% at 24 h, 15% and 21% at 48 h, 39% and 35% at 72 h on PC3 cells (Figure 2B), whereas by 13% and 17% at 24 h, 7% and 20% at 48 h, and 24% and 34% at 72 h on DU145 cells (Figure 2C). Taken together, the data showed that expressions of NP could increase proliferation of prostate cancer cells in the absence of androgens.

3.3 NP upregulates androgen receptor expression

AR plays an important role in the proliferation and survival of PCa cells. Once the expression level of AR was reduced or eliminated specifically, the cell proliferation was consequently decreased [19]. In an attempt to investigate whether AR expression could be affected by NP, the AR mRNA and protein expression levels were evaluated by RT-PCR and Western blot in LNCaP cells following transfection of NP or TAT-NP. The time course induction of AR mRNA expression was detected as early as 12 h after transfection, and the bands in cells transfected with TAT-NP (lane 1 in Figure 3A) and NP (lane 2 in Figure 3A) were all stronger than that of the control (lane 3 in Figure 3A) in the absence of androgens. The stronger induction by NP and TAT-NP were maintained at least up to 48 h.

Protein expression levels are shown in Figure 3B. The NP-mediated and TAT-NP-mediated induction of AR protein expressions were observed in the absence of androgens in LNCaP cells (lane 1 and lane 2 in Figure 3B, respectively). Both NP and TAT-NP displayed some stimulatory effects on AR expression. In addition, the NP-induced and TAT-NP-induced expressions of AR were further enhanced when cells were treated with Mib

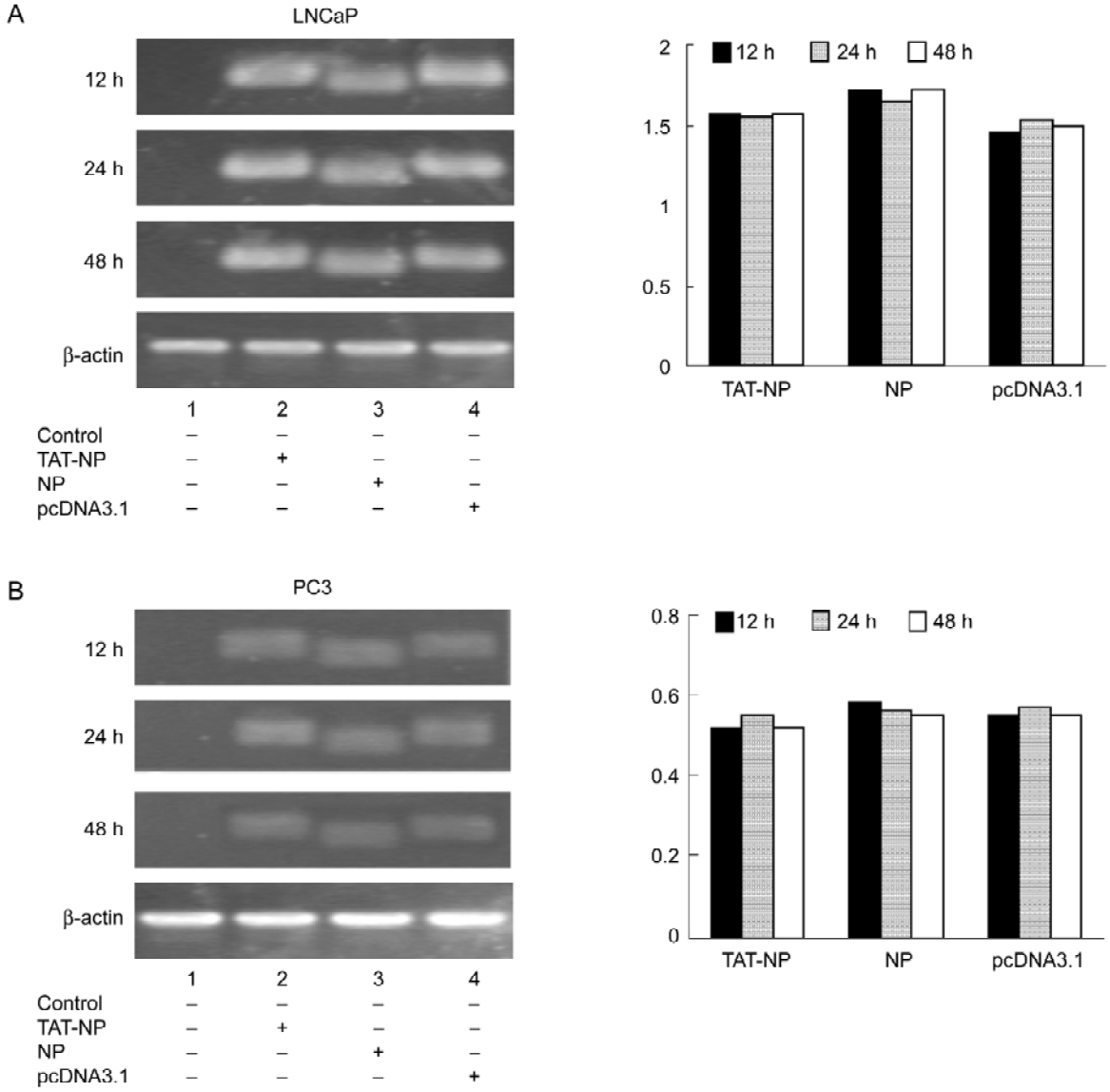

Figure 1. Analysis of mRNA expression levels of neurotrophic peptide (NP) by reverse transcription-polymerase chain reaction (RT-PCR). Transfection of pcDNA-NP or pcDNA-TAT-NP vector into LNCaP cells (A) and PC3 cells (B). The total RNA were extracted at varying periods of time and, as indicated, were used for RT-PCR analysis. The parental vector pcDNA3.1 was included as a control. The bands observed in lane 4 were from a transcript containing the multiple clone site sequence in pcDNA3.1 vector. The expression of β-actin was served as an internal control to monitor sample loading. Densitometric measurements for NP mRNA levels were normalized to β-actin and expressed as a relative number where 1 equals 100% of control.

following transfection. As seen in Figure 3C, the synergistic effect of androgen and NP on the expression of AR is evident (lanes 4 and 6, lanes 5 and 6), as compared with Mib treatment alone. Furthermore, a human AR expression vector pSG5-hAR was cotransfected with TAT-NP or NP plasmid into PC3 cells to evaluate the NP enhancing effect on AR expression. As expected, the result (Figure 3D) shows that the AR protein expressions were induced in cells cotransfected with TAT-NP (lane 1) or NP (lane 2) in the absence of androgen, and less AR protein was detected in the control group. These findings suggest that NP and TAT-NP might upregulate AR

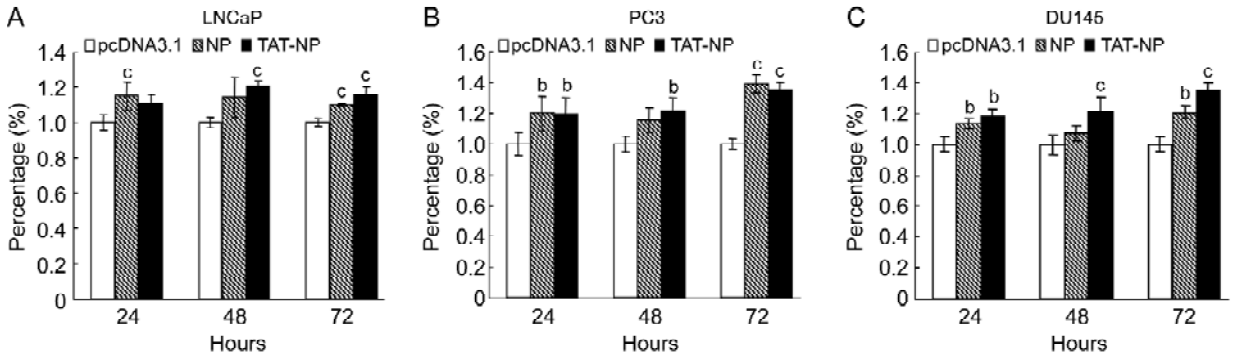

Figure 2. Effect of ectopic expression of neurotrophic peptide (NP) on prostate cancer cells (PCa) proliferation. LNCaP cells (A), PC3 cells (B) and DU145 cells (C) were transfected with pcDNA-NP or pcDNA-TAT-NP vector. The parental vector pcDNA3.1 was also included as a control. After transfection, the cells were maintained in serum-free medium for 24, 48 and 72 h. The percentage of viable cells were measured by MTT assay. Values are expressed as the mean ± SD (n = 3). $^{b}P < 0.05$, $^{c}P < 0.01$, compared with the control.

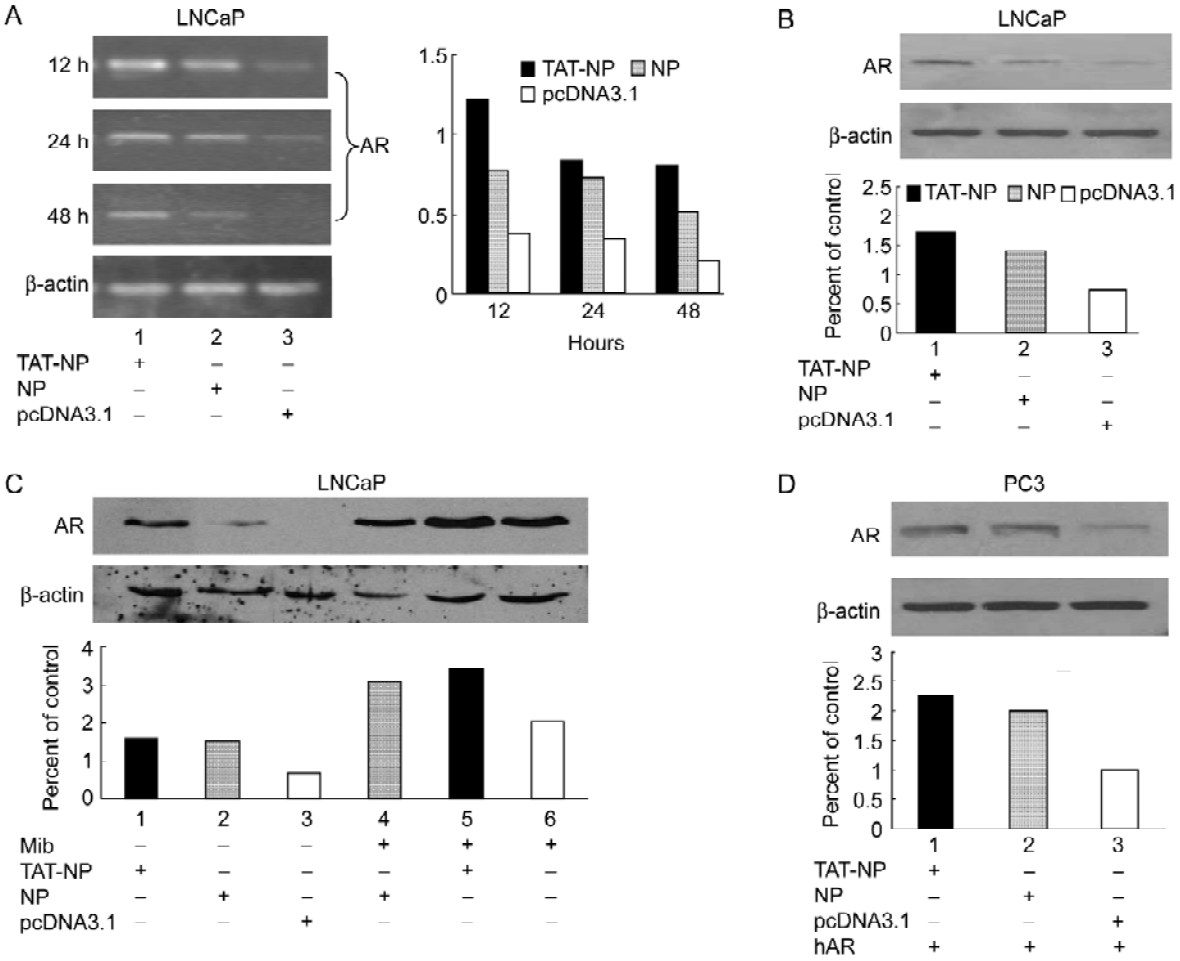

Figure 3. Induction of androgen receptor (AR) mRNA and protein expression by neurotrophic peptide (NP) in prostate cancer (PCa) cells. (A): Transfection of expression vector pcDNA-NP, pcDNA-TAT-NP, or pcDNA3.1 (a control) into LNCaP cells for 24 h, total RNA were prepared and then AR mRNA levels were determined by RT-PCR. LNCaP cells (B, C) and PC3 cells (D) were transfected with vectors as indicated for 24 h. The AR protein expression levels in whole cell lysates were analyzed by Western blot. The expression of β-actin was served to monitor mRNA and protein loading and transferring efficiency. Densitometric measurements for AR mRNA and protein levels were normalized to β-actin and expressed as a relative number where 1 equals 100% of control.

gene expression at the mRNA and protein levels.

3.4 NP enhances AR transcriptional activity

PSA and hk2 are androgen-inducible genes that contain ARE to which AR binds. The expressions of PSA and hk2 genes are highly dependent on androgen-mediated activation of AR [18]. Next we examined whether transactivation of AR could be modulated by NP independent of androgens. The LNCaP cells were transiently cotransfected with a PSA promoter luciferase reporter and NP, or TAT-NP plasmid. The luciferase activities of the reporter were monitored to show the AR transactivation. As seen in Figure 4A, production of PSA promoter reporter was observed in the cotransfection of NP or

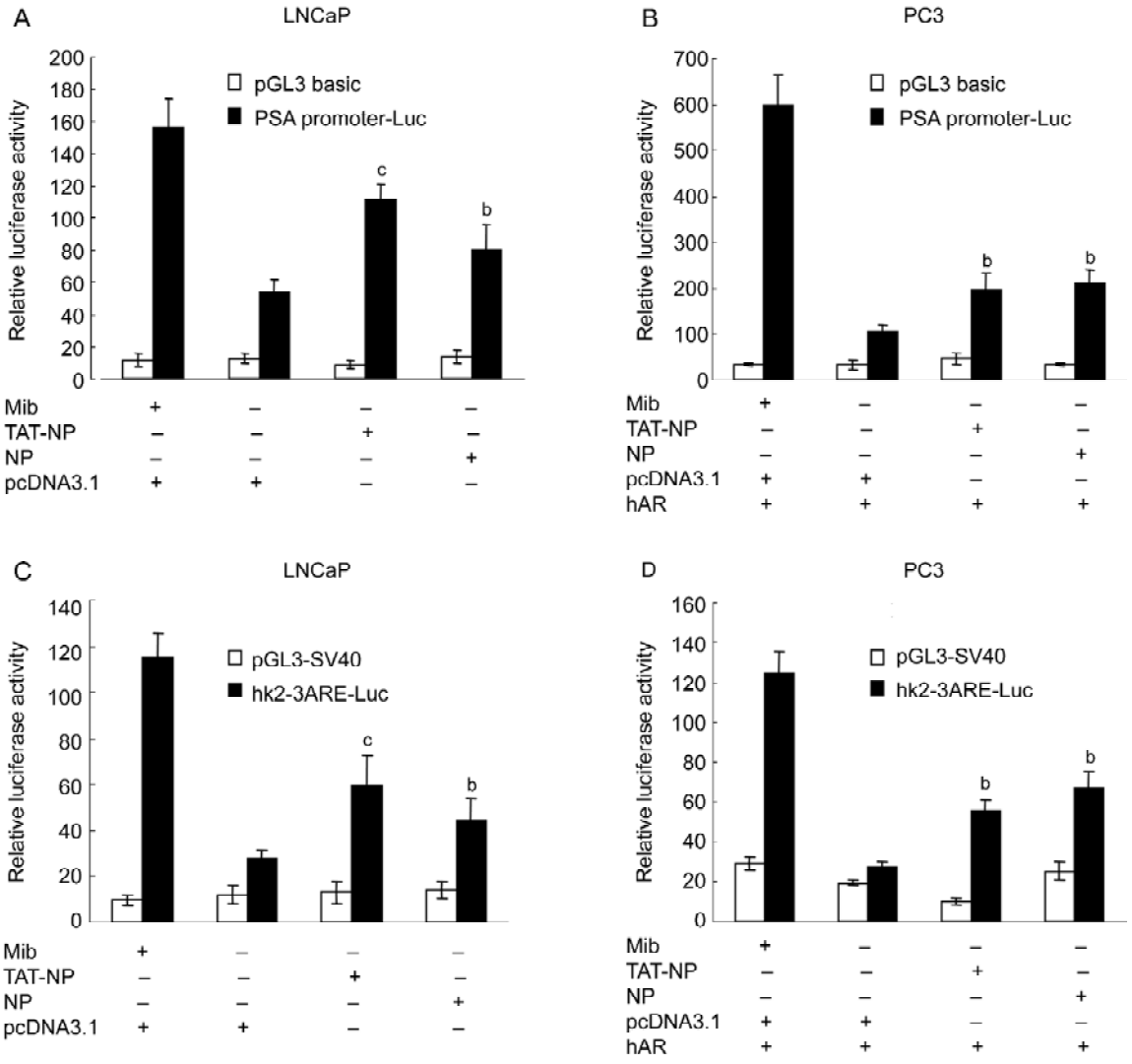

Figure 4. Upregulation of androgen receptor (AR) transcriptional activity by neurotrophic peptide (NP) in prostate cancer (PCa) cells. (A): LNCaP cells were cotransfected with prostate-specific antigen (PSA) promoter-Luc plasmid and pcDNA-NP, or pcDNA-TAT-NP vector. (B): PC3 cells were cotransfected with pSG5-hAR plasmid together with PSA promoter-Luc and pcDNA-NP, or pcDNA-TAT-NP vector. (C): LNCaP cells were cotransfected with hk2-3ARE-Luc reporter plasmid and pcDNA-NP, or pcDNA-TAT-NP vector. (D): PC3 cells were cotransfected pSG5-hAR plasmid together with hk2-3ARE-Luc and pcDNA-NP, or pcDNA-TAT-NP vector. The phRL-TK vector served as an internal control to monitor transfection efficiency. The pGL3 basic vector, pGL3-SV40 vector and parental vector pcDNA3.1 were included as controls. The normalized relative luciferase activities (mean ± SD) of at least three independent experiments were shown. $^{b}P < 0.05$, $^{c}P < 0.01$, compared with the control.

TAT-NP plasmid without androgens, whereas basal luciferase activity of PSA promoter reporter was shown in cotransfection of pcDNA3.1 plasmid. The strongest induction of the PSA promoter reporter activity was observed in response to androgen treatment, followed by cotransfection of NP or TAT-NP. We then cotransfected the human AR expression vector into AR-negative PC3 cell line with the PSA promoter reporter to verify the NP effect on the AR transactivition. As seen in Figure 4B, higher PSA promoter reporter activity was shown in the cells cotransfected with NP or TAT-NP plasmid. To further demonstrate the role of NP in regulation of AR transactivity, hk2-3ARE-Luc vector containing three tandem ARE in the hk2 promoter was used in the cotransfection experiment. The increased reporter activities stimulated by NP or TAT-NP were observed in both LNCaP (Figure 4C) and PC3 cells (Figure 4D). The results are consistent with those in Figure 4A and 4B. Because the reporter construct hk2-3ARE-Luc only contains three copies of ARE, production of luciferase activity induced by NP might result from stimulation of NP on AR transactivation. The data suggest that the stimulatory effect of NP on AR expression and transactivation could be achieved by the transfection of NP expression vector into prostate cancer cells in the absence of androgen.

4 Discussion

Numerous reports revealed how diversity of AR signaling could be modulated. Regulatory mechanisms, including overexpression/amplification, mutations and androgen-independent activation of AR, are all believed to play important roles in the progression of androgen-independence of PCa. Several growth factors have been implicated in the induction of AR activity independent of androgens. Recent data have indicated prosaposin and its active derivatives (i.e. saposin C, prosaptide TX14A), which have been known as neurotrophic factors [7], to be functional as cell survival and anti-apoptotic factors for PCa cells [13–15]. Subsequently, purified human milk-prosaposin and recombinant saposin C were shown to promote AR expression and activity [16]. Our present study found that ectopically expressed NP, which encoded the functional sequence of saposin C, stimulated PC3, DU145 and LNCaP cell proliferation. Furthermore, expression of NP was also involved in regulation of AR expression and transcriptional function. The RT-PCR and Western blot analyses results indicated that NP increased AR gene expression (Figures 2 and 3) and subsequently enhanced AR transcriptional activity in an androgen-independent manner (Figure 4). These results are consistent with the data obtained from synthesized prosaptides or purified recombinant human saposin C [13–16], which were added to cell culture media. In addition, our results provide evidence that stimulatory effect of NP on AR expression is synergistically enhanced by androgen (Figure 3C); however, the NP-mediated induction was less than that mediated by androgen treatment. It suggested that NP might play a role in regulation of AR transactivity independent of androgens, which might be achieved by its regulation of a complex network and signaling pathways in PCa.

In sum, our observations indicate that NP, as a growth stimulating peptide to PCa cells, exerted activity by increasing the expression and enhancing the transactivation of AR in a ligand-independent manner. Investigations focusing on how NP regulates AR will provide further insight into its role in PCa progression and a new cancer therapeutic target.

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