Ciliary neurotrophic factor (CNTF) and its receptor (CNTFRα) signal through MAPK/ERK pathway in human prostate tissues: A morphological and biomolecular study

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ABSTRACT

Ciliary neurotrophic factor (CNTF) is a member of interleukin-6 type cytokine family. The CNTF receptor complex is a heterodimer including gp130 and CNTF receptor α (CNTFRα) proteins triggering the activation of multiple intracellular signaling pathways including AKT/PI3K, MAPK/ERK and Jak/STAT pathways. At present no data are available on the localization of CNTF and CNTFRα in prostate as well as on the role of CNTF in this organ. In this study we have analyzed the localization of CNTF and CNTFRα by immunohisto-chemistry and we have used PWR-1E cell line as a model for normal glandular cell to investigate the role of this cytokine. Our results show that CNTF and CNTFRα are expressed in the staminal compartment of the prostate and that CNTF selectively inhibits ERK pathway. In conclusion, we suggest that CNTF could be considered as key molecule to maintain the epithelium homeostasis *via* pERK downregulation by an autocrine mechanism. Further CNTF studies in prostate cancer could be useful to verify the potential role of this cytokine in carcinogenesis.

Key words: PWR-1E cell line; immunohistochemistry; Western blotting; cytokine.

Introduction

Ciliary neurotrophic factor (CNTF) was originally discovered in intraocular tissue of embryonic chick, having a role in promoting the survival of ciliary ganglion neurons during embryonic development.[1] It was subsequently identified also in mammals neuronal and non-neuronal cell types, showing trophic actions.[2] CNTF is a member of interleukin (IL)-6 type cytokine family including leukemia inhibitory factor (LIF), interleukin-6 (IL-6), IL-11, cardiotrophin 1 (CT-1), cardiotrophin-like cytokine (CLC), interleukin-27 (IL-27), and oncostatin M (OSM).[3] All IL-6 type family cytokines, need additional membrane-bound non-signaling receptors to activate signal transduction *via* gp130,[4-7] in particular CNTF acts by using the CNTF receptor α (CNTFRα).[6,8,9] The formation of the CNTF receptor complex triggers the activation of multiple intracellular signaling pathways such as MAPK/ERK[10], AKT/PI3K[11] and Jak/STAT[12] pathways, which mediate various biological effects, such as survival and/or differentiation in different cell types.[13-25] Most of the studies involving CNTF has been carried out on nervous system and adipose tissues concerning food intake and the association among obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia.[26-29] Recently, Chen *et al*.[30] provided new insights into the role of CNTF on the migration of corneal epithelial stem/progenitor cells and on AKT signaling pathway. At the best of our knowledge, less is known about the role of CNTF and its receptor CNTFRα in epithelial prostate tissues. The normal prostatic glandular epithelium consists of three cell types, *i.e.*, basal, luminal-secretory and neuroendocrine cells expressing specific markers and organized in a double layer surrounded by stromal tissue.[31-36]

The most widely accepted explanation for the balanced relationship between basal and secretory cells is due to the presence of non-altered stem cells in the basal cell compartment. The alteration of these normal stem cells leads to tumorigenesis.[37-40] At present, molecular and biological basis as well as the pathogenesis of prostate cancer are still a matter of debate although prostate cancer, *i.e.*, adenocarcinoma, is the second most common malignancy in males and the fifth leading cause of cancer mortality.[41-43] Generally, prostate adenocarcinoma is clinically localized and not aggressive. For patients with advanced prostate adenocarcinoma, androgen deprivation therapy is usually used, however, a part of these patients can have poor clinical outcomes for onset of castration resistant prostate cancer.[44] Thus, it is crucial to investigate the molecules and factors playing a pivotal role in processes such as cellular proliferation and migration. So, given the remarkable effects of CNTF on stem cell[30] and its potential on cell migration,

we performed a series of experiments with the aim: i) to identify the presence of CNTF and its receptor CNTFRα in human prostate tissues; ii) to characterize the signaling systems modulated by CNTF treatments using cultured prostate cells. We have investigated ERK, AKT and STAT3 pathways because they have an important role in triggering prostate cancer[45-48] and it has been proven that these three pathways can be involved in CNTF signaling through its receptor CNTFRα.[10-12,30] In addition, these pathways mediate processes such as cellular proliferation, survival, differentiation, migration that play a pivotal role in tissue homeostasis and in the cancer onset.[13-25]

Materials and Methods

Tissue collection

In this study we analyzed a total of 15 normal human prostate samples: 5 from benign prostatic hyperplasia (BPH), 5 normal-looking samples from radical prostatectomy (NL-RP) and 5 from cystoprostatectomy (CYP). A pathologist (RMa) reviewed the samples stained with hematoxylin and eosin for selecting the samples used in this study.

All the samples were obtained from pathological files from the Pathology Services of the Polytechnic University of the Marche Region-United Hospitals. The procedures followed for the collection of samples were in accordance with the Helsinki Declaration of 1975, as revised in 2013.

Immunohistochemistry

All prostate samples were fixed in 10% neutral buffered formalin and routinely processed for paraffin embedding. Immunohistochemical staining was performed as previously described.[49] Briefly, after dewaxing, paraffin sections were rinsed in phosphate buffered saline (PBS), incubated with 3% hydrogen peroxide for 40 min to block endogenous peroxidase. Pre-treatment by heat in 10 mM citrate buffer, pH 6.0 for 5 min was used for CNTF and p63 while pre-treatment by 100 ng/mL Proteinase K (Sigma-Aldrich, St. Louis, MO, USA) 5 min at 37°C was used for CNTFRα. After pre-treatment, sections were rinsed with PBS and incubated with normal horse serum (Vector Laboratories, Burlingame, CA, USA) diluted 1:75 in PBS for 1 h at room temperature (RT). Sections were then incubated with anti-CNTFRα, anti-CNTF and anti-p63 (Table 1) primary antibodies diluted in PBS, overnight at 4°C. After a thorough rinse in PBS, sections were incubated with the appropriate biotinylated secondary anti-

Table 1. Primary antibodies used in this study.

Antibody	IHC	WB	IF	Company
pAb Rabbit anti-human CNTF (#ab190985)	1:500	//	1:100	Abcam, Cambridge, UK
pAb Rabbit anti-human CNTFRα (#PA5-45053)	//	1:400	//	Thermo Fisher Scientific, Waltham, USA
mAb Mouse anti-human CNTFRα (#ab89333)	1:150	//	1:100	Abcam, Cambridge, UK
mAb Rabbit anti-human pAKT (#4060)	//	1:1000	//	Cell Signaling Technology, Danvers, USA
pAb Rabbit anti-human AKT (#9272)	//	1:1000	//	Cell Signaling Technology, Danvers, USA
mAb Rabbit anti-human pERK1/2 (#4377)	//	1:800	//	Cell Signaling Technology, Danvers, USA
mAb Rabbit anti-human ERK1/2 (#4695)	//	1:1000	//	Cell Signaling Technology, Danvers, USA
mAb Mouse anti-human pSTAT3 (#4113)	//	1:800	//	Cell Signaling Technology, Danvers, USA
mAb Rabbit anti-human STAT3 (#4904)	//	1:1000	//	Cell Signaling Technology, Danvers, USA
mAb Mouse anti-human p63 (#M7317)	1:50	//	//	DAKO, Glostrup, Denmark

mAb, monoclonal antibody; pAb, polyclonal antibody; IHC, immunohistochemistry; WB, Western blotting; IF, immunofluorescence.

body (Vector Laboratories) diluted 1:200 v/v solution for 30 min at RT. Vectastain ABC Kit (Vector Laboratories) for 1h at RT and 3′,3′- diaminobenzidine hydrochloride (Sigma-Aldrich) were used to develop the immunohistochemistry reaction. Sections were counterstained with Mayer's hematoxylin, dehydrated and mounted using Eukitt solution (Kindler GmbH and Co., Freiburg, Germany). Negative controls were performed by omitting the first or secondary antibody for all the immunohistochemical reactions performed in this study. p63 was used as nuclear marker of basal prostate cells. Nervous tissues (fibers and ganglions) are used as positive internal control for CNTF and CNTFRα.[50-52]

Cell culture

Normal human prostate epithelial cells PWR-1E (ATCC/LGC Standards, Manassas, VA, USA) were cultured in serum-free keratinocyte cell culture media (K-SFM) supplemented with human EGF (5 ng/mL), bovine pituitary extract (25 mg/mL), 100 U/mL penicillin and streptomycin (Gibco, Thermo Fisher Scientific, MA, USA) at 37°C, 95% humidity and 5% CO_2. The medium was changed 3 times a week and cells were split 1:4 every 3/4 days.

Immunofluorescence

PWR-1E cells were washed in Dulbecco's PBS (Lifetechnology, Monza, Italy), fixed in 4% paraformaldehyde in PBS for 10 min at RT, and permeabilized in PBS 0.1 M added with 0.1% Triton X-100 (Sigma, Milan, Italy) for 5 min. After washing in PBS at RT, cells were blocked with 10% Normal Donkey Serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS 0.1 M and incubated overnight at 4° C with the anti-human CNTF (1:100) and CNTFRα (1:150) antibodies (Table 1). Cells were then washed three times in PBS and incubated with the FITC-conjugated donkey anti-rabbit (for CNTF) and TRITC-conjugated anti-mouse (for CNTFRα) IgG secondary antibodies (both from Jackson ImmunoResearch) for 30 min at RT. TOTO3 probe was used for nuclear staining. Finally, the slides were cover-slipped with propyl gallate and evaluated with a Leica TCS-SL spectral confocal microscope.

CNTFRα detection in cell lines by Western blotting

Once PWR-1E cells reached 80% confluence, cells were lysed by using the following lysis buffer: 0.1M PBS, 0.1% (w/v) SDS, 1% (w/w) NONIDET-P40, 1mM (w/v) Na orthovanadate, 1mM (w/w) PMSF (phenyl methane sulfonyl fluoride), 12 mM (w/v) Na deoxycholate, 1.7 μg/mL Aprotinin, pH 7.5. Cell lysates were centrifuged at 20,000 g for 20 min at 4°C and the supernatants were aliquoted and stored at −80°C. Viable counts using the Trypan blue dye exclusion test were routinely performed. All experiments were performed in duplicate and were repeated at least three times. The proteins concentrations were determined by a Bradford protein assay (Bio-Rad Laboratories, Milan, Italy). All protein samples were analyzed by Western blotting technique. They were fractionated on 10% SDS-polyacrylamide gels (SDS-PAGE) and elec-
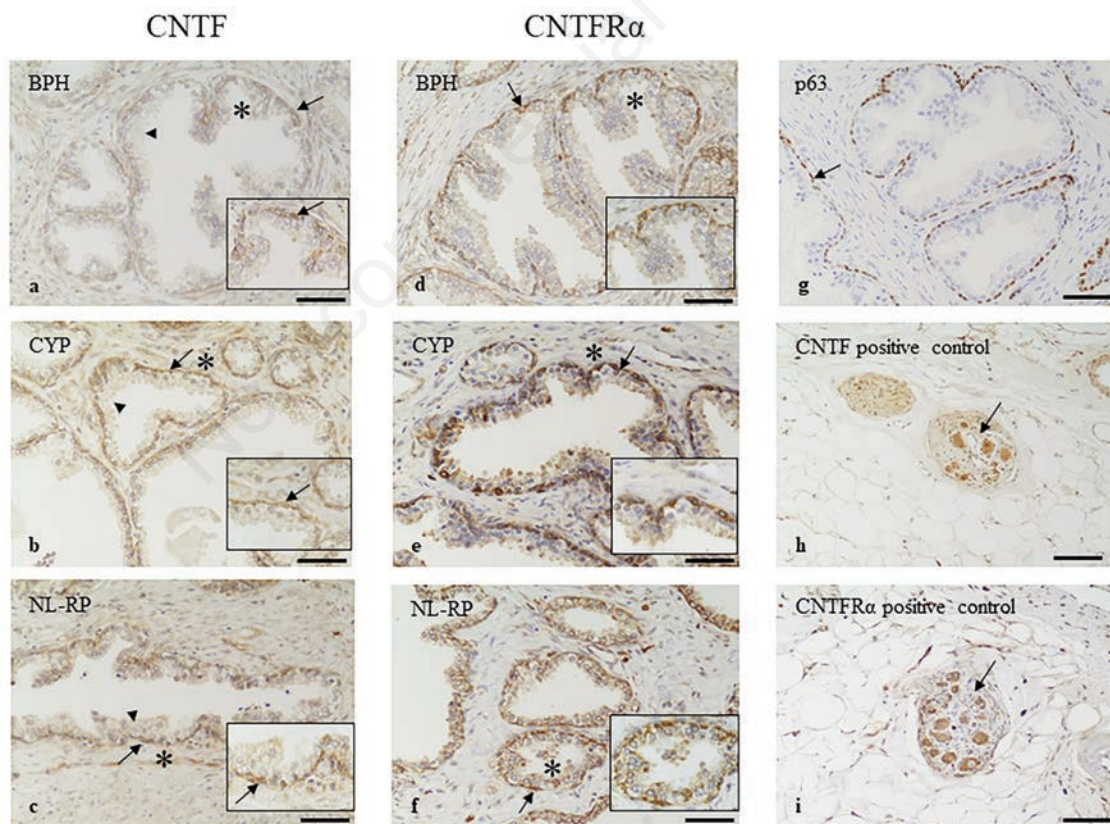

Figure 1. Immunohistochemistry localization of CNTF and CNTFRα in prostate samples. CNTF is highly expressed in basal layer (arrows) of BPH (a), CYP (b) and NL-RP (c) while the secretory layer (arrowheads) is mainly negative. The stromal tissues are weakly stained for CNTF in all samples analysed. CNTFRα is highly expressed in basal layer (arrows) of BPH (d), CYP (e) and NL-RP (f) while the other tissues are mainly negative. The basal layer of glandular epithelium is identified by p63 marker (arrow, g). Pictures in h) and i) show a ganglion (arrow) positive for CNTF (h) and for CNTFR (i) used as positive internal controls. The insets show higher magnification of the area indicated by asterisk, scale bars: 30 μm. Scale bars: a,b,c,d,f,g) 100 μm; e,h,i) 200 μm.

trophoretically transferred (Trans-Blot® Turbo™ Transfer System; Bio-Rad Laboratories Inc., Richmond, CA, USA) to nitrocellulose membranes, and subjected to Western blot analysis. Non-specific protein binding was blocked with 5% (w/v) non-fat-dried milk (Bio-Rad Laboratories) in Tris-buffered saline (TBS/0.05% Tween 20 (TBS-T) for 1 h. Blots were incubated with 1:400 anti-CNTFRα (Thermo Fisher Scientific, Waltham, MA, USA) primary antibody overnight at 4°C. After washing, blots were incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Italia Srl, Milan, Italy) diluted 1:5000 in TBS-T. Detection of bound antibodies was performed with the Clarity Western ECL Substrate (Bio-Rad Laboratories) and images were acquired with Chemidoc (Bio-Rad Laboratories). Bands were analyzed using the ImageJ software (https://imagej.nih.gov/ij/download.html) for quantification, and normalization was completed using β-actin band intensities.

CNTF signaling pathway in PWR-1E cell line

After verified the presence of the CNTFRα in PWR-1E cells, a dose/responsive curve was performed to test the best CNTF concentration showing a significant response for cellular treatments. These cells were treated with 0, 2, 10 and 20 ng/mL by recombinant human CNTF (rhCNTF) for 15 min, to detect which of the following signaling pathway was trigged: pERK/ERK, pAKT/AKT and pSTAT3/STAT3. These signaling pathways were analyzed by Western blotting as above described using the primary antibodies shown in Table 1.

Results were calculated in arbitrary units (AU) and reported as

bars of a histogram. pERK1/2, pAKT and pSTAT3 quantities were normalized using total ERK1/2, AKT and STAT3 respectively.

Statistical analysis

Data represent the mean ± SD, and were analyzed for statistical significance ($p<0.05$) using Student's *t*-test by Graphpad Prism ver. 8 program.

Results

CNTF and CNTFRα localization in human prostate tissue by immunohistochemistry

CNTF and CNTFRα were localized mainly in the basal layer of the prostate epithelium in BPH (Figure 1 a,d), CYP (Figure 1 b,e) and NL-RP (Figure 1 c,f), while the secretory luminal layer was mainly negative or very weakly positive for the two molecules in all samples analyzed (Figure 1 a-f). Stromal components of prostate tissue were weakly positive for CNTF (Figure 1 a-c) and mainly negative for CNTFRα (Figure 1 d-f).

CNTF and CNTFRα localization in PWR-1E cell line by immunofluorescence

In Figure 2 the immunopositivity for CNTF is mainly nuclear (on chromatin, the nucleoli are negative; inset in panel c), while the cytoplasm is weakly stained (panels b and c). The green signal for
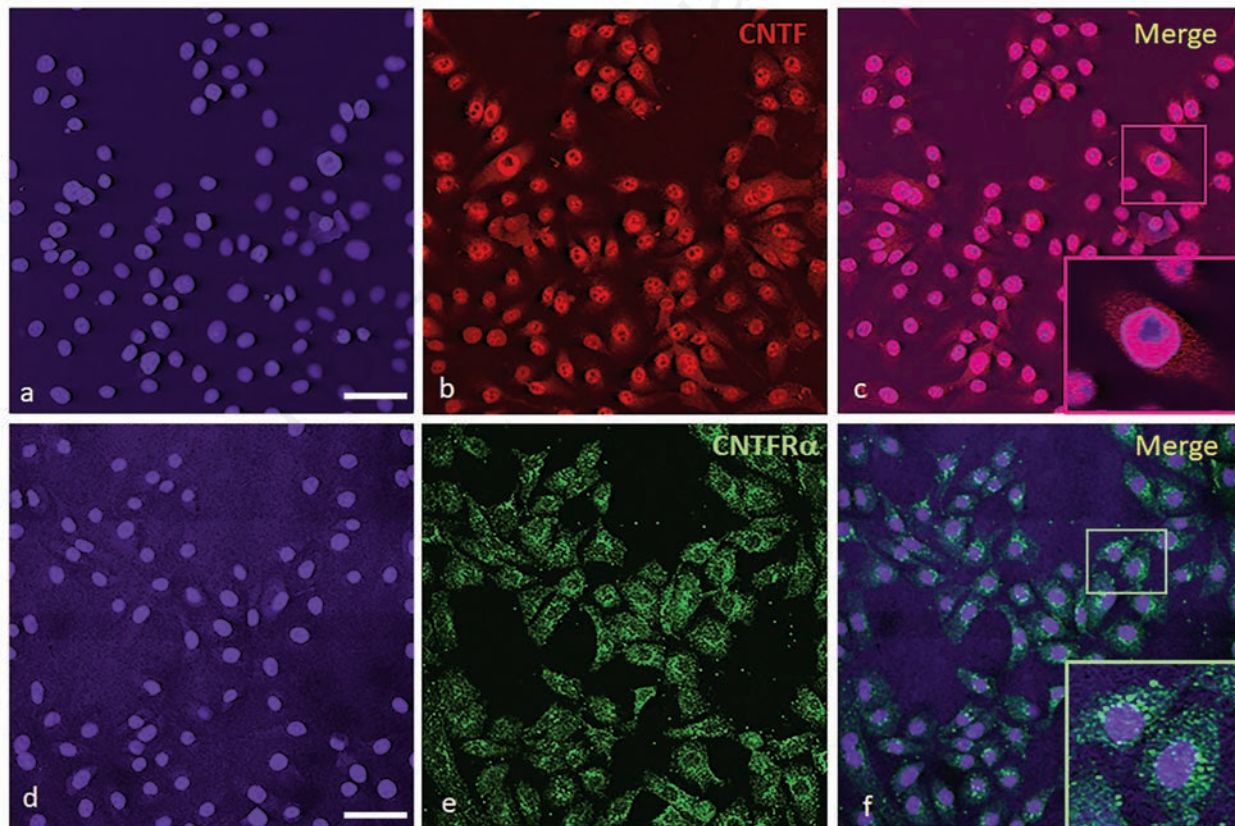

Figure 2. Immunofluorescence of CNTF and CNTFRα in PWR-1E cell line. In (a) and (d) are stained the nuclei in blue. CNTF (b, red staining) is localized mainly in nuclei while the cytoplasm is weakly stained for CNTF (b,c). In (c), the nucleoli are negative as depicted in the inset (c, Merge). CNTFRα (e, green staining) is localized in the cytoplasm of the cells as shown in (f) and it is especially intense in the perinuclear region (see inset in f, Merge). Scale bars: a,b,c,d,e,f) 50 μm; Insets in c,f) 17 μm.

CNTFRα is cytoplasmic (panels e and f) and especially intense in the perinuclear region (inset in panel f).

CNTF signaling pathways analyzed by Western blotting

rhCNTF induced pERK de-phosphorylation at 10 ng/mL and 20 ng/mL, whereas 2 ng/mL of rhCNTF was insufficient to induce de-phosphorylation of pERK (Figure 3a, see the representative Western blotting image). The expression level of pERK was very weakly or negative detected performing Western blotting analyses. The quantitative analysis of pERK expression levels normalized on ERK expression levels was represented by the histogram on the right side of the Western blotting (Figure 3a). The statistical analysis showed a significant pERK decrease after rhCNTF treatments at 10 ng/mL (p=0.008) and 20 ng/mL (p=0.006) (Figure 3a). RhcNTF did not have any effect on phosphorylation/de-phosphorylation of pAKT/AKT (Figure 3b) and pSTAT3/STAT3 (Figure 3c). The histograms representing the quantitative analysis of pAKT expression levels normalized by AKT (Figure 3b) and pSTAT3 expression levels normalized by STAT3 (Figure 3c) did not show any statistical differences (p>0.05) among treatments at the different rhCNTF concentrations (see pAKT/AKT and pSTAT3/STAT3 histograms in Figure 3b c, respectively).

Discussion

This is the first study that describes the localization of CNTF and its receptor CNTFRα in human prostate tissues, and investigates the activation pathways of CNTF in human normal prostate cell line.

In order to characterize the location of CNTF and its receptor CNTFRα we have used prostate tissues obtained from different zones of the prostate, *i.e.* transitional and peripherical zones. In particular, we analyzed benign prostatic hyperplasia (BPH) from transitional zone and normal-looking samples, from both radical prostatectomy (NL-RP) and cystoprostatectomy (CYP), from peripheral zone where at least 75% of cancers originate.[53] The normal glandular epithelium of prostate is highly organized containing, basal cells, which account for ~40% of total epithelial cell numbers, and a layer of luminal-secretory cells that make up the rest of the epithelium.[54] We have demonstrated by immunohistochemistry the presence of CNTF and CNTFRα in the basal cell layer in the prostatic normal glandular epithelium, in which reside stem cells.[55] Under normal conditions the basal cells through differentiation replace the terminally differentiated luminal cells that regularly shed in the lumen of the gland, while an impaired differentiation of the normal epithelial lineage is present in prostate cancer.[55,56] It has been supposed that the genesis of prostate cancers takes into account both genetic and epigenetic changes, such as DNA methylation, chromatin remodeling and transcriptional regulation, involving a single cell or a cancerous progenitor cell which sources a dysregulated differentiation program to form the tumor.[54,57,58] Although the prostate cancer presents a dominant luminal phenotype, recently, a progenitor cell on the basal layer seems to be an increasingly supported alternative for the origin of prostate cancer and basal to luminal cellular differentiation appears a critical event.[54,57]

Multiple signaling pathways normally involved in prostate differentiation can be also linked to prostate cancer development,
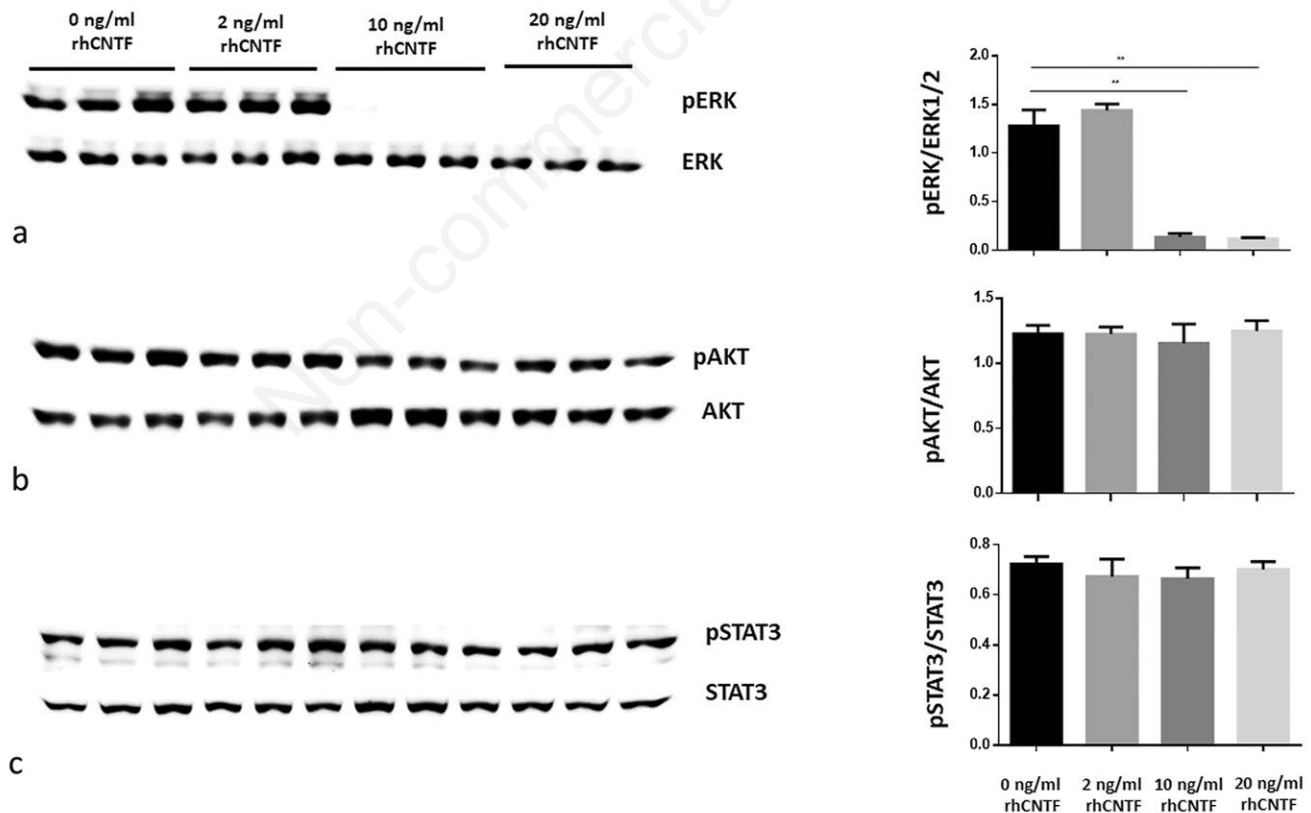

Figure 3. Dose-dependent response of PWR-1E normal prostate cell line to rhCNTF. Densitometrical analysis of the bands show a significant downregulation of pERK1/2 (a) while pAKT (b) and pSTAT3 (c) do not show any significant modulation. Results were calculated in arbitrary units (AU) and reported in the histograms. Note the significant decrease (**) of pERK1/2 in PWR-1E cell lines treated with 10 and 20 ng/mL rhCNTF compared to the untreated control (mean ± SD; **p<0.01).

including the MAPK/ERK, AKT/PI3K, and Jak/STAT pathways.[45-48] In addition, it has been reported that alteration of the axis PTEN/AKT/PI3K together with activation of RAS/MAPK/ERK signaling are correlated with prostate cancer progression and metastasis and that RAS/MAPK/ERK pathway alone is significantly elevated in both primary and metastatic lesion.[59,60] Moreover, it is known that the activation of Jak/ STAT3 pathway by IL-6 is crucial for maintenance of tumor progenitor cell phenotype and that CNTF binding to its receptor CNTFRα can active the three pathways described above.[10-12,29,30]

Our *in vitro* results showed that AKT and STAT3 phosphorylation were not modified under rhCNTF treatments while rhCNTF selectively inhibits ERK pathway, in fact we have detected a decrease of ERK activation, *i.e.* the decrease of pERK under rhCNTF stimulation, in normal prostate cellular model. Our data are in line with that of Nickols and colleagues[61] showing a correlation of prostate cancer recurrence with the increase of pERK expression levels. Moreover, the same authors demonstrated that the activation of ERK is characteristic of castration resistant prostate cancer while others found a correlation between increased ERK phosphorylation with both stage T and Gleason grade of prostate cancer.[61,62] So, we can hypothesize that inhibition of ERK phosphorylation can decrease migration and invasion processes downstream of this pathway. As a result, we can speculate that CNTF promotes epithelium homeostasis *via* pERK downregulation by an autocrine mechanism in normal prostate glandular basal compartment and that dysregulation of this mechanism could contribute to the onset of prostate cancer. All these findings suggest that further CNTF studies in prostate cancer are needed to verify the potential role of this cytokine in carcinogenesis.

Acknowledgments

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