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Neurotensin downregulates the pro-inflammatory properties of skin dendritic cells and increases epidermal growth factor expression

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ABSTRACT

In the last decades some reports reveal the neuropeptide neurotensin (NT) as an immune mediator in the Central Nervous System and in the gastrointestinal tract, however its effects on skin immunity were not identified. The present study investigates the effect of NT on signal transduction and on pro/anti-inflammatory function of skin dendritic cells. Furthermore, we investigated how neurotensin can modulate the inflammatory responses triggered by LPS in skin dendritic cells. We observed that fetal-skin dendritic cells (FSDCs) constitutively express NTR1 and NTR3 (neurotensin receptors) and that LPS treatment induces neurotensin expression. In addition, NT downregulated the activation of the inflammatory signaling pathways NF-κB and JNK, as well as, the expression of the cytokines IL-6, TNF-α, IL-10 and the vascular endothelial growth factor (VEGF), while the survival pathway ERK and epidermal growth factor (EGF) were upregulated. Simultaneous dendritic cells exposure to LPS and NT induced a similar cytokine profile to that one induced by NT alone. However, cells pre-treated with NT and then incubated with LPS, completely changed their cytokine profile, upregulating the cytokines tested, without changes on growth factor expression. Overall, our results could open new perspectives in the design of new therapies for skin diseases, like diabetic wound healing, where neuropeptide exposure seems to be beneficial.

1. Introduction

Wound healing (WH) is a complex and well organized process involving different cells and mediators, which perfectly communicate to ensure that the inflammatory phase, important in the elimination of pathogens, does not culminate into infection. This process has received much attention by the scientific community since innumerous wound healing diseases have become critical or even impossible to cure. Atopic dermatitis, psoriasis and diabetic wound healing are examples of serious cutaneous diseases. It is therefore imperative to understand the molecular and cellular mechanisms of the disease in order to uncover better therapies. WH involves different cells, namely keratinocytes, fibroblasts, endothelial cells, Langerhans cells (LCs), macrophages, mastocytes and platelets, diverse inflammatory mediators, such as growth factors, chemokines and cytokines, and it also requires complex biological and molecular events that induce cell migration, cell proliferation and extracellular matrix deposition (ECM) [1]. Neuropeptides play an imperative role in wound healing, being key modulators of the inflammatory phase of WH. Neuropeptides can be produced by skin cells or released by sensory neurons when responding to stimuli, promoting different cellular responses [2]. Substance P, Calcitonin Gene Related Peptide (CGRP), Vasoactive intestinal peptide (VIP), Secretin, Secretoneurin and stressor neuropeptides induce migration and maturation of LCs, similar to lipopolysaccharide (LPS), a Toll-like receptor (TLR)4 agonist that has long been used as a potent inducer of dendritic cell (DC) maturation [3]. However, CGRP, VIP, Pituitary adenylate cyclase-activating peptide (PACAP) and α-Melanocyte-stimulating hormone (MSH) mediate an anti-inflammatory response and promote a Th2 cytokine polarizing profile in LCs. Previous studies have identified neurotensin (NT)-positive fibers and NT expression in the skin, suggesting important cutaneous functions [4,5]. Furthermore, the presence of NT in the skin has been implicated in the pathogenesis of skin disorders exacerbated by stress [6]. However, the role of NT in LCs and skin inflammation has not been explored so far. In the nervous system, NT has a pro-inflammatory role, inducing vasodilatation,

Abbreviations: (CGRP), Calcitonin Gene Related Peptide; (CNS), Central Nervous System; (DCs), Dendritic cells; (EGF), Epidermal growth factor; (ECM), Extracellular matrix; (FSDC), Fetal skin-dendritic cell; (IFN), Interferon; (IL), Interleukin; (LCs), Langerhans cells; (LPS), Lipopolysaccharide; (MSH), Melanocyte-stimulating hormone; (NT), Neurotensin; (NTR), Neurotensin receptor; (PACAP), Pituitary adenylate cyclase-activating peptide; (PDGF), Platelet derived growth factor; (TLR), Toll-like receptor; (TNF), Tumor necrosis factor; (VEGF), Vascular endothelial growth factor; (VIP), Vasoactive intestinal peptide; (WH), Wound Healing

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vascular permeability, mast cells' degranulation and enhancement of directional migration and phagocytosis of neutrophils [7,8]. In the gastrointestinal tract, NT is involved in the physiopathology of colonic inflammation, a tissue rich in neurotensin receptor 1 [9], stimulating NF-κB dependent Interleukin (IL)-8 secretion and IL-8 gene expression in human colonocyte cells [10]. In a recent report NT treatment induces an anti-inflammatory cytokine profile in colitis, characterized by the decrease of IL-6 and Tumor necrosis factor (TNF)-α expression [11].

Neurotensin is a tridecapeptide that binds to neurotensin receptors: neurotensin receptor 1, 2 and 3 (NTR1, NTR2 and NTR3). NTR1 and NTR2 are G protein-coupled receptors while NTR3 is an intracellular receptor with a single transmembrane domain, which is 100% homologous to gp95/sortilin [12]. NTR1, rather than NTR2, exhibits high affinity for NT [13] and NT effects on the Central Nervous System (CNS) are essentially mediated by this receptor, as NTR3 is predominantly localized in the trans Golgi network, although the mature protein can also be present in the plasma membrane [14].

Neurotensin receptors mediate the activation of different signaling pathways. NTR1 induces intracellular signaling through Phospholipase C and the inositol phosphate signaling pathways. It also functions through the production of cGMP, cAMP and arachidonic acid, through the MAP kinase pathways and inducing the inhibition of Akt activity [15].

This work attempted to address the role of NT in skin dendritic cells in the absence and presence of an inflammatory stimulus, focusing on signal transduction, inflammatory mediators and pro/anti-inflammatory function of LCs. The knowledge of the molecular and cellular mechanisms of neuropeptides in the skin and its application on skin wounds could discern new therapeutic approaches for skin pathologies.

2. Materials and methods

2.1. Materials

Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6), an activator of inflammation, was obtained from Sigma Chemical Co. (St. Louis, MO, USA), NT was obtained from Bachem (Weil am Rhein, Germany) and NTR1 inhibitor SR48692 was obtained from Axon Medchem (Groningen, The Netherland). The protease inhibitor cocktail (Complete Mini) and the phosphatase inhibitor cocktail (PhosSTOP) were obtained from Roche (Carnaxide, Portugal). Bicinchoninic acid (BCA) kit assay was obtained from Novagen. 30% Acrylamide/BisSolution 29:1 (3.3% c), TEMED and SYBR green were obtained from BioRAD, and High Capacity cDNA Reverse Transcription kit was obtained from Applied Byosistems.

The polyvinylidene difluoride (PVDF) membranes and the antibody against b-actin were purchased from Millipore Corporation (Bedford, MA). The antibodies against phopho-(p-)AKT/PKB (Thr 308) and NTR were purchased from Santa Cruz (Frilabo), the antibodies against total JNK were obtained from UpState (Tape Group), the antibodies against p-JNK, p-ERK, p-p38 MAPK and total AKT/PKB were purchased from Cell Signaling and the antibody against total p38 MAPK was purchased from Biolegend (Tape Group). The alkaline phosphatase-linked secondary antibodies (anti-mouse and anti-rabbit) and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Carnaxide, Portugal). The Vectashield mounting medium was purchased from Vector, Inc. (Burlingame, CA, USA) and the Alexa Fluor 555 phalloidin antibody was purchased from Invitrogen (Barcelona, Spain).

TRIzol® was obtained from Invitrogen, diethyl pyrocarbonate (DEPC) was acquired from AppliChem. Methanol, ethanol, and isopropanol were obtained from Merck. All primers were obtained from MWG Biotech (Ebersberg, Germany). All other reagents were purchased from Sigma Chemical Co.

2.2. Culture of FSDC

The fetal skin dendritic cell line (FSDC), Langerhans cell analogs from mice, was kindly supplied by Dr. G. Girolomoni (Department of Biomedical and Surgical Science, Section of Dermatology and Venereology, University of Verona, Italy). This cell line is a skin DC precursor with antigen presenting capacity. This cell line was previously characterized [16] and the phenotype was confirmed in our lab [17]. FSDC was cultured in serum-containing endotoxin-free Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% (v/v) of inactivated fetal calf serum, 3.02 g/l sodium bicarbonate, 100 U/ml penicillin, 100 g/ml streptomycin and 30 mM of glucose, in a humidified incubator with 5% CO^2/95% air, at 37 °C. Along the experiments, cells were monitored by microscopic observation in order to detect any morphological changes.

2.3. MTT assay

Cells were stimulated with 1 μg/ml of LPS and treated with 10 and 100 nM of NT, for 24 h. Cell viability was determined by the reduction of MTT (Mosmann T., 1983), as previously described [17]. LPS or/and NT stock solutions were added to obtain the different final in-well concentrations studied.

2.4. Immunocytochemistry

Immunocytochemistry was performed as described previously by us [18]. Fluorescence labeling was visualized using a fluorescence microscope – Zeiss Axiovert 200 – and images captured with a coupled AxioCamHR camera. The filter set used included an excitation filter of 560 nm and an emission filter of 575 nm for Alexa Fluor 555 and an emission filter of 420 nm for DAPI.

2.5. Western blot

Cells (7.5×10^5) were plated in 12-well plates and treated with 10 nM of NT, or/and 1 μg of LPS, during 5, 15, 30 and 60 min. After

Table 1
Primer sequences for targeted cDNAs.

Primer	5′-3′sequence (F:forward; R:reverse)	RefSeqID
HPRT1	F: GTTGAAGATATAATTGACACTG R: GGCATATCCAACAACAAAC	NM_013556
NT	F: AATGTTTGCAGCCTCATAAATAAC R:TGCCAACAAGGTCGTCATC	NM_024435
NTR1	F: GGCAATTCCTCAGAATCCATCC R: ATACAGCGGTCACCAGCAC	NM_018766
NTR2	F: GCCATTACTAACAGTCTAAGC R: GCAATTCGTCCTATTCTACAC	NM_008747
NTR3	F: ATGGCACAACTTCCTTCTG R: AGAGACTTGGAGTAGACAATG	NM_019972
IL-1β	F: ACCTGTCCTGTGTAATGAAAG R: GCTTGTGCTCTGCTTGTG	NM_008361
IL-6	F: TTCCATCCAGTTGCCTTC R: TTCTCATTTCCACGATTTCC	NM_031168
IL-10	F: CCCTTTGCTATGGTGTCCTTTC R: ATCTCCCTGGTTTCTCTTCCC	NM_010548
TNF-α	F: CAAGGGACTAGCCAGGAG R: TGCCTCTTCTGCCAGTTC	NM_013693
IFN-δ	F: CTTCTTGGATATCTGGAGGAACTG R: GGTGTGATTCAATGACGCTTATG	NM_008337
G-CSF	F: TCATTCTCTCCACTTCCG R: CTTGGTATTTACCCATCTCC	NM_009971
CCL5	F: CACTCCCTGCTGCTTTGC R: CACTTGGCGGTTCCTTCG	NM_013653
VEGF-A	F: CTT GTT CAG AGC GGA GAA AGC R: ACA TCT GCA AGT ACG TTC GTT	NM_001025250
EGF	F: GCA CAG TTT GTC TTC AAT GGC R: TGT TGG CTA TCC AAA TCG CCT TGC	NM_010113

incubation, cells were washed with ice-cold PBS and harvested in a sonication buffer containing 50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40 (Nonidet P-40), 0.5% Sodium Deoxycholate, 0.1% SDS, 2 mM EDTA, protease inhibitor cocktail, phosphatase inhibitor cocktail and DTT 1 mM. Cell lysates and protein quantification were performed as previously described [18].

The levels of IκB-α, p-ERK1/ERK2, p-p38 MAPK, p-SAPK/JNK and p-AKT/PKB protein levels were evaluated by western blots. Proteins were separated by electrophoresis on a 10% (v/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane, as previously described [18]. The immune complexes were detected by membrane exposure to the ECF reagent, followed by scanning for blue excited fluorescence on the VersaDoc (Bio-Rad Laboratories, Amadora, Portugal). Membranes were stripped and reprobed with antibodies against total ERK1/2, SAPK/JNK, p38 MAPK and AKT/PKB, or with an antibody for b-actin. The generated signals were analyzed using the Image-Quant TL software.

2.6. RNA extraction

Cells (2×10^6) were plated in 6-well plates in a final volume of 6 ml and treated with 1 μg/ml of LPS and/or 10 nM of NT during 6 h, or pre-treated with 10 nM of NT during 24 h and stimulated with 1 μg/ml of LPS during 6 h, or left untreated (control). Total RNA was isolated from these cells with the TRIzol® reagent according to the manufacturer's instructions. The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA). RNA was stored in RNA Storage Solution (Ambion, Foster City, CA, USA) at −80 °C.

2.7. Real time RT-PCR

One microgram of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription (RT), from Applied Biosystems, according to the manufacturer's instructions. After the cDNA synthesis, the samples were diluted with RNase-free water up to a volume of 100 μl and concentration of 0.01 μg/μl. Real-time RT-PCR was performed as previously described [18]. The results were normalized using a reference gene, hypoxanthine phosphoribosyltransferase 1 (HPRT-1) which was previously validated in our lab for FSDC [18]. Primers were designed using Beacon Designer® Software v7.2, from Premier Biosoft International and thoroughly tested (Table 1). Quantitative RT-PCR results were analyzed as in Neves et al. [18].

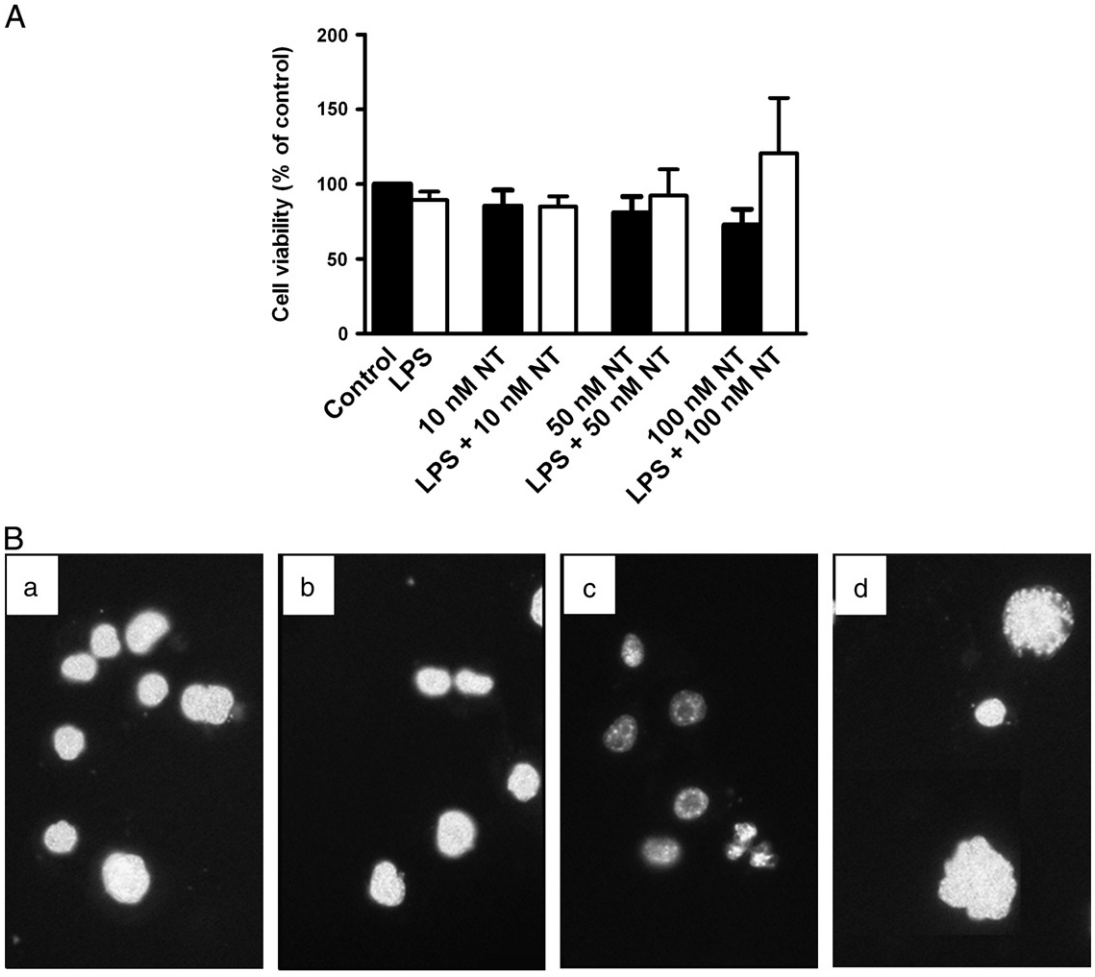

Fig. 1. FSDC viability. Cells were plated in 48/24-well plates, maintained in IMDM medium (control), incubated with 10, 50 and 100 nM of NT, 1 μg/ml of LPS alone, and with NT and LPS simultaneously, for 24 h, at 37 °C, with 5% CO^2. (A) MTT assay. After incubations, the MTT solution (5 mg/ml) was added to each well and cell viability was evaluated as described in experimental procedures. Student's *t*-test was used relative to control. Each value represents the mean ± SD from 3 experiments, performed in duplicate. (B) FSDC nuclei morphology. After incubation, cells were stained as described in experimental procedures. Images were acquired using a fluorescence microscope – Zeiss Axiovert 200 – and captured with a coupled AxioCamHR camera (400×). Image a is representative of the nuclear morphology of control cells and b of NT treated cells; image c represent the nuclei of some LPS treated cells and image d of LPS and NT treated cells, not observed in a and b.

2.8. Statistical analysis

The results are presented as mean ± S.D., and the means were statistically compared using the unpaired two-tailed Student's *t*-test, using Graphpad software. The significance level was $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

3. Results

3.1. Effect of neurotensin on FSDC viability

Cell viability was evaluated by the MTT assay which measures the metabolic activity of the cells, as well as through the examination of nuclei morphology after cell treatment for 24 h with 10, 50 or 100 nM of neurotensin alone or in the presence of LPS. As indicated in Fig. 1A, NT treatment did not significantly affect cell viability when compared with non-treated cells. The higher cell viability, however not significant, observed in cells treated with LPS and 100 nM of NT may be a response to the inflammatory stimulus of LPS, as cells modify their metabolic activity to express different inflammatory molecules like cytokines, chemokines and growth factors, to act in inflammation. Nuclei morphology was assessed by DNA staining with DAPI and NT treated cells did not change nuclei morphology in comparison to the control (Fig. 1B — a and b), although cells treated with LPS or LPS and NT displayed some apoptotic nuclei (Fig. 1B — c and d).

3.2. Cell morphology

FSDC morphology was analyzed by fluorescence microscopy in order to evaluate actin and nuclei staining (Fig. 2). In control conditions and NT treated cells, cells showed actin arrangements dispersed through the entire cell, but with sutured actin filaments in the periphery of the cytoplasm, evidencing numerous vesicles related to the high endocytic activity of these immature cells. Some cells also showed dendritic-like projections (Fig. 2A, B and C). On the other hand, when cells were stimulated with LPS or LPS and NT for 24 h, the actin in the periphery of the cytoplasm was more sutured in some regions than in others and the actin cortical network appeared to be disassembled, probably demonstrating FSDC adjustments for migration. Some dendritic-like projections were also seen in LPS or LPS and NT stimulated cells (Fig. 2D, E and F). Indeed, NT alone did not promote FSDC adjustment for migration.

3.3. NTR expression in FSDC

Protein expression of neurotensin receptors was determined in untreated cells (control), by Western Blot analysis. Only NTR1 and NTR3 were constitutively expressed in FSDC, where NTR3 seems to be the most expressed receptor among the NTRs (Fig. 3A). Furthermore, NTR gene expression was also assessed by real-time RT-PCR in untreated cells (control) and cells exposed to LPS for 6 h. Comparing the relative amounts of the transcripts, NTR3 was the most abundant among the analyzed NTR receptors, as shown in Fig. 3B, in accordance to the WB results. The treatment of cells with LPS for 6 h caused a decrease in transcription of all NTRs, by -1.14 ± 0.55 ($n=3$), -1.88 ± 0.77 ($^{*}p<0.05$, $n=3$) and -0.85 ± 0.78 ($n=3$) fold, for NTR1, NTR2 and NTR3 respectively, while expression of NT was increased by 3.75 ± 0.50 ($^{***}p<0.0001$, $n=3$) relative to control (Fig. 3C).

3.4. Intracellular signaling pathways modulated by NT in FSDC

To evaluate the intracellular pathways stimulated by NT in skin dendritic cells, Western blot analysis was performed to detect the activated/phosphorylated forms of MAP kinases (ERK1/ERK2, SAPK/

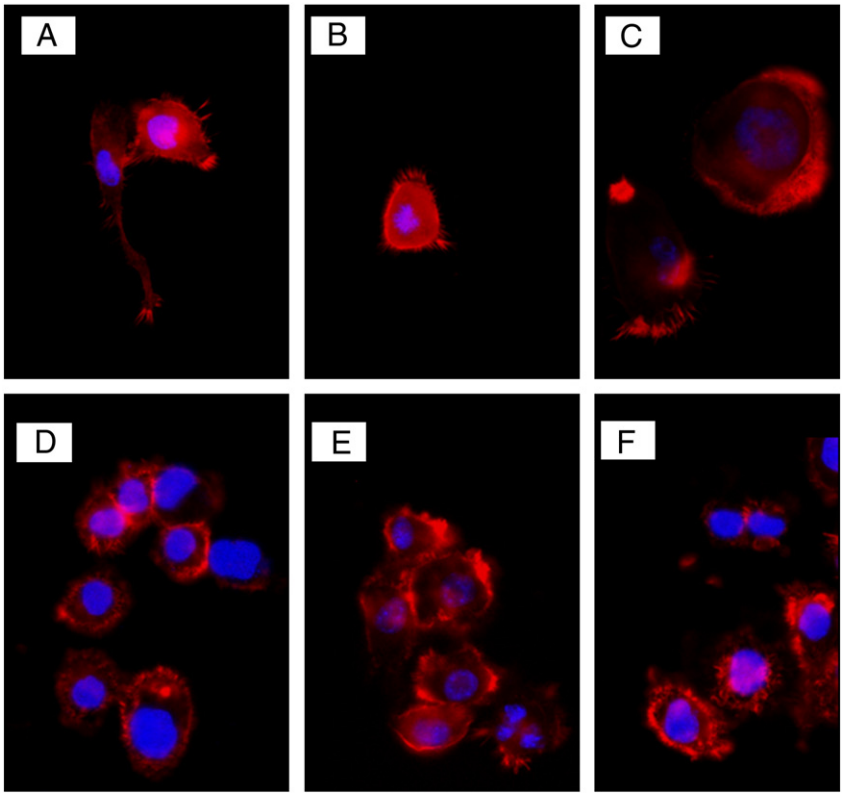

Fig. 2. Cytoskeleton and nuclei morphology of FSDC. Cells were immunostained with Alexa Fluor 555 phalloidin (red) antibody for actin and stained with DAPI (blue) for the nuclei. Images A and B represent cells in control conditions and C represents neurotensin treated cells; D and E are representative of LPS treated cells and F represents LPS and NT treated cells. Immunostaining was performed as described in "Materials and methods". The images were acquired by fluorescence microscopy and photographs were taken at 400× magnification.

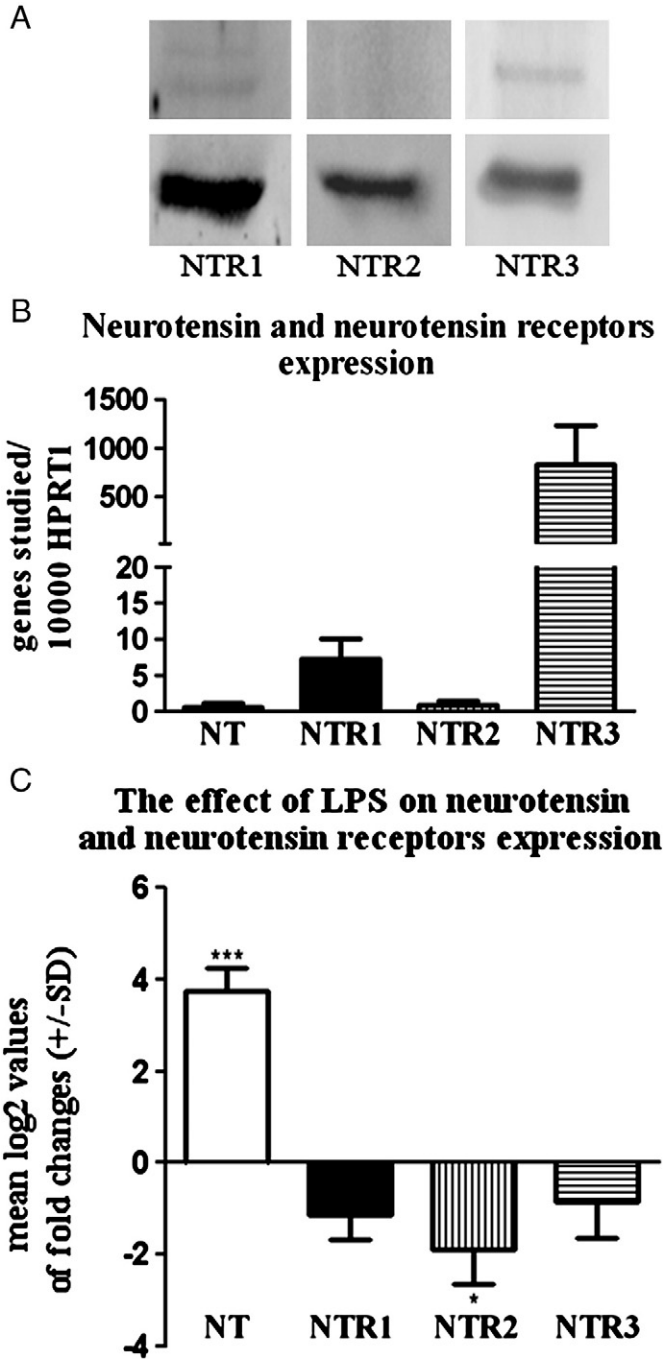

Fig. 3. Neurotensin receptors (NTRs) expression in FSDC. Cells were maintained in IMDM medium (control) or treated with 1 μg/ml of LPS, at 37 °C, with 5% CO_2. (A) After 30 min of incubation, cell extracts were subjected to WB analysis using NTR1, NTR2 and NTR3 antibodies, with normalization to b-actin. The blot shown is representative of 3 independent experiments yielding similar results (B/C). After 6 h of incubation, total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as genes studied/10,000 molecules of the reference gene HPRT1 (3B) or mean log2 values of fold changes relative to the control (C). Each value represents the mean ± S.D. from three independent experiments (*$p<0.05$; ***$p<0.001$ unpaired two-tailed Student's *t*-test).

JNK, p38 MAPK) and AKT/PKB. Modulation of the transcription factor NF-κB was also evaluated. For that, FSDC was incubated, during short time periods, with 10 nM of NT. In these conditions we observed that NT upregulated IκB-α levels (Fig. 4A), although no alterations were observed for the MAP kinases (data not shown). The NF-κB inhibitory protein – IκBα – was not degraded and its expression increased in a time-dependent manner until 30 min, indicating an inhibition of the NF-κB signaling pathways (Fig. 4A).

FSDC was also stimulated with LPS being a maximal phosphorylation for ERK1/ERK2, SAPK/JNK, p38 MAPK and AKT/PKB observed between 15 and 30 min, with a more notorious effect on the ERK1/ERK2 signaling pathway (Fig. 4B). The expression of IκBα decreased in a time-dependent manner until the 30 min time point, indicating activation of the NF-κB (Fig. 4B). We next tested the ability of NT to modulate LPS-triggered intracellular signals. When NT was added simultaneously with LPS, p38 MAPK and ERK1/ERK2 activation was upregulated essentially at 30 min, while the SAPK/JNK signaling pathway was downregulated at 30 min, when compared with the results obtained with LPS alone (Fig. 4B). Between 5 and 15 min, NT also caused an increase in the expression of IκBα, diminishing NF-κB activation (Fig. 4B). No alterations were observed in the AKT/PKB signaling pathway (data not shown). Furthermore, in the presence of

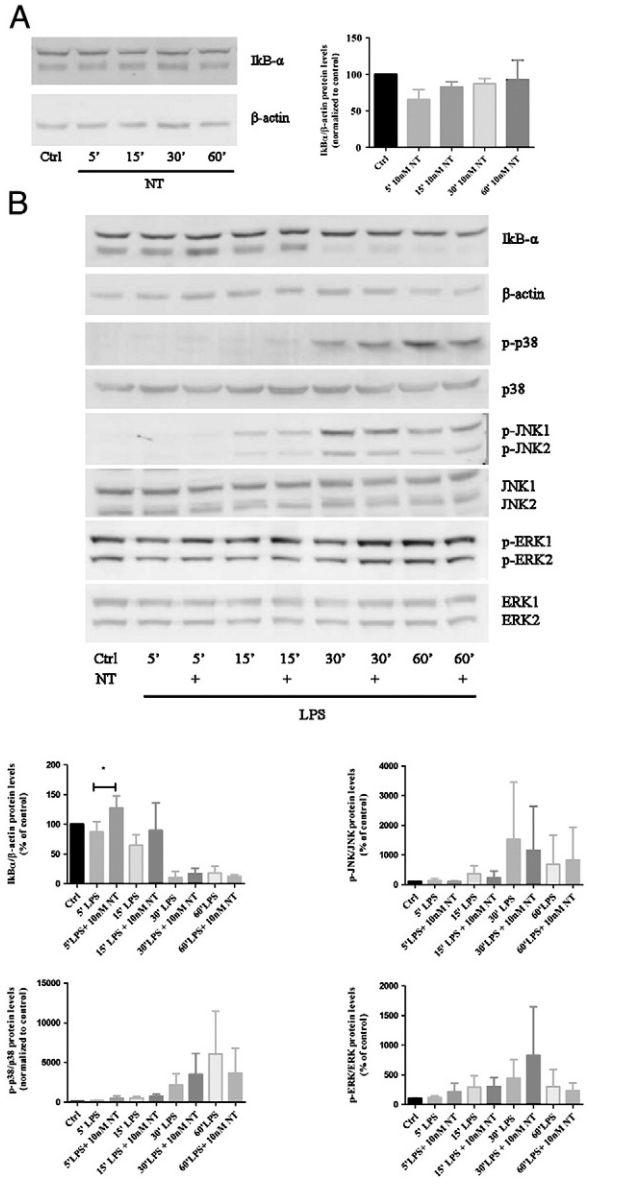

Fig. 4. Activation of intracellular signaling pathways by NT and LPS in FSDC. Cells were maintained in culture medium (control), or incubated with 10 nM of NT alone (A) or simultaneously with LPS (B) for 5, 15, 30 or 60 min. Total cell extracts were subjected to WB analysis, as described in "Materials and methods", using antibodies to p-ERK1/2, p-JNK1/2 and p-p38MAPK. The activation of NF-κB was evaluated by the determination of the levels of its inhibitory protein, IkB-α (band on the bottom). Equal loading was evaluated with antibodies to total ERK1/2, SAPK/JNK and p38 MAPK, or with an anti-β-actin antibody. The blots shown are representative of at least three independent experiments yielding similar results. The quantification of WB bands was evaluated with normalization to ERK1/2, JNK1/2, p38MAPK and IkB-α antibodies. Each value represents mean ± SD (t-student test, comparing treatments and stimulus of the same time set).

the NTR1 inhibitor SR48692, activation or inactivation of the described signaling pathways returned to normal (data not shown), suggesting that the observed effects occurred through NTR1 receptor.

3.5. Modulation of gene expression by neurotensin

To evaluate the effects of LPS on FSDC gene expression after 6 h of incubation, IL-1β, TNF-α, Interferon (IFN)-γ, Granulocyte colony-stimulating factor (G-CSF), the chemokine CCL5, IL-10, Epidermal growth factor (EGF), Vascular endothelial growth factor (VEGF) and Platelet-derived growth factor (PDGF) were measured, as shown in Table 2. LPS highly induced the expression of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and G-CSF, as well as, the chemokine CCL5. We observed that the anti-inflammatory cytokine IL-10 was slightly induced, while growth factors were almost unaffected (Table 2). To further evaluate the effect of NT on key inflammatory cytokines and chemokines, cells were subjected to different NT and LPS incubation protocols to mimic different physiopathological conditions that occur *in vivo*. Cells were treated with 10 nM of NT alone for 6 h or 30 h; incubated with LPS alone or simultaneously with 10 nM of NT and LPS for 6 h, and finally incubated with 10 nM of NT for 24 h before an additional stimulus of LPS for 6 h. Pre-treatment of FSDC with NT simulated *in vivo* treatments with NT immediately after skin injury (before inflammation starts). Simultaneous cell treatment with LPS and NT for 6 h simulated a treatment with NT after injury where inflammation is present. Total RNA was isolated from cells, quantified and reverse transcribed to cDNA, to perform real-time RT-PCR.

Table 2
Expression of cytokines, chemokines and growth factors triggered by LPS in FSDC.

Gene	LPS (mean log2 values of fold changes relative to the control)
IL-1β	4.94 ± 3.18 (***p<0.001, n = 14)
IL-6	8.00 ± 3.22 (***p<0.001, n = 17)
TNF-α	3.58 ± 1.17 (***p<0.001, n = 16)
IFN-γ	0.72 ± 1.34 (n = 4)
G-CSF	6.65 ± 3.13 (**p<0.01, n = 5)
CCL5	7.52 ± 2.44 (***p<0.001, n = 16)
IL-10	2.28 ± 2.29 (***p<0.001, n = 17)
EGF	−0.35 ± 1.26 (n = 16)
VEGF	−0.15 ± 1.38 (n = 16)
PDGF	0.73 ± 0.74 (**p<0.01, n = 10)

Cells were plated in 6-well microplates in culture medium and treated with 1 μg of LPS during 6 h, at 37 °C, with 5% CO_2. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relative to the control. Values represent the mean ± S.D. from at least three independent experiments (**p<0.01; ***p<0.001 unpaired two-tailed t-student test relative to the corresponding control).

After 6 h of FSDC exposure to neurotensin, the expression of the majority of the cytokines diminished, namely IL-6, TNF-α and IL-10, by −0.34 ± 0.26 (n = 3), −1.10 ± 0.62 (*p<0.05, n = 3) and −1.77 ± 0.91 (*p<0.05, n = 3) fold, relative to control (Fig. 5A). In addition, when cells were exposed to NT for long-time periods (30 h), the observed effects were similar but with smaller intensity. The expression of CCL5 was significantly increased by 0.74 ± 0.50 (*p<0.05, n = 4) fold, relative to control (Fig. 5A).

In cells incubated simultaneously with LPS and 10 nM of NT for 6 h, the cytokine profile was similar to the profile induced by NT alone, as shown in Fig. 5B. In addition, we observed a tendency for a decrease in IL-6 and TNF-α expression by −0.57 ± 1.21 (n = 4) and −0.97 ± 0.89 (n = 3) fold and a tendency for an increase in CCL5 by 0.54 ± 1.37 (n = 3) fold, relative to control, while the expression of IL-10 was unaltered (Fig. 5B). In contrast, when cells were pre-treated with 10 nM of NT during 24 h and then incubated with LPS for 6 h, the cytokine profile changed. We observed an upregulation of the expression of all cytokines, with a clear tendency in the upregulation of IL-6 expression by 0.70 ± 1.66 (n = 6), relative to control (Fig. 5B).

Concerning growth factors expression, after 30 h of NT incubation, EGF was increased by 1.45 ± 1.18 (*p<0.05, n = 4) fold, while the expression of VEGF decreased by −0.29 ± 0.18 (*p<0.05, n = 4) fold, relative to control (Fig. 5A). When cells were simultaneously treated with NT and LPS for 6 h, the expression of EGF increased by 2.37 ± 2.20 (*p<0.05, n = 3) fold, relative to control, while VEGF expression was unaltered (Fig. 5B). However, growth factors expression did not change when cells were pre-treated with 10 nM of NT during 24 h and then incubated with LPS for 6 h, as shown in Fig. 5B. Moreover, to determine if the increased expression of EGF observed had an autocrine or paracrine effect on these cells, the expression of the EGF receptor by FSDC, as well as its phosphorylation by NT and/or LPS was determined by real time

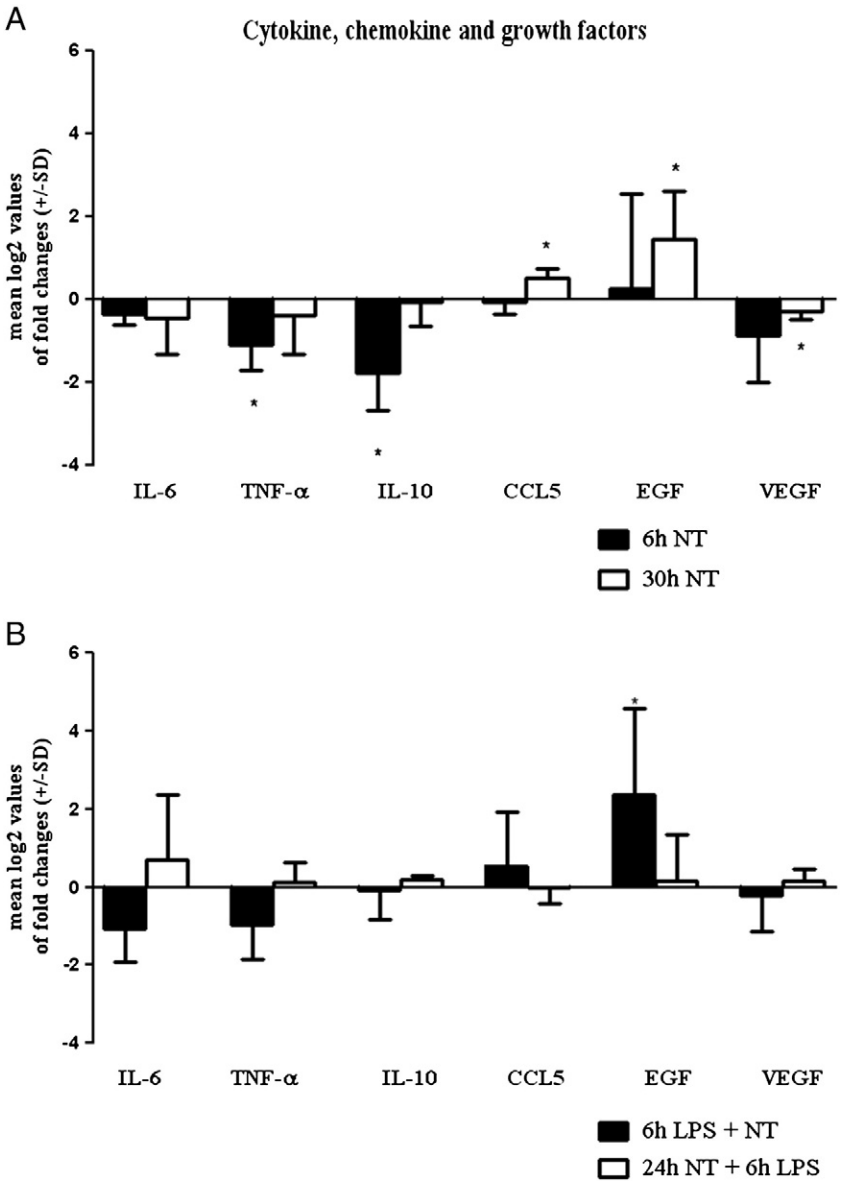

Fig. 5. Modulation of gene expression by neurotensin and LPS in FSDC. Cells were maintained in IMDM medium (control) and treated with 10 nM of NT during 6 h (white bars), 30 h (black bars) (A), or simultaneously treated with 1 μg/ml of LPS and 10 nM of NT for 6 h (white bars), pre-treated with 10 nM of NT during 24 h and then incubated with LPS for 6 h (black bars) (B), at 37 °C, with 5% CO_2. Total RNA was isolated and retrotranscribed as indicated in "Materials and methods". The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relative to the control. Each value represents the mean ± S.D. from at least three independent experiments (*$p<0.05$, unpaired two-tailed Student's *t*-test relative to the corresponding control).

RT-PCR and WB, respectively. As a result, cells did not express the receptor nor the receptor was phosphorylated (data not shown), suggesting a possible paracrine function of this growth factor in other skin cells under these conditions.

4. Discussion

Neurotensin has been largely studied in the CNS, although poorly studied in the skin. In this work we demonstrated that skin dendritic cells express neurotensin receptors, namely NTR1 and NTR3, which were downregulated in inflammatory conditions. On the other hand, neurotensin was only produced in these cells under inflammatory conditions, suggesting that neurotensin expressed by FSDC may be important to other skin cells, exerting paracrine functions.

The activation of signaling pathways and gene expression was determined in the absence and in the presence of the inflammatory stimulus of LPS. LPS is a glycolipid component of the outer wall membrane of Gram-negative bacteria that has long been used as a potent inducer of DC maturation. It acts trough the toll-like receptor 4 (TLR4), triggering MyD88 and TRIF pathways that results in the phosphorylation of various intracellular kinases, including the IκB kinase (IKK), the ERK1/2, p38 MAPK and JNK1/2, implicated in the synthesis of inflammatory mediators, like some cytokines, chemokines and growth factors.

In this work we observed that neurotensin alone or under inflammatory conditions downregulated the activation of the signaling pathway NF-κB which is involved in diverse immune responses mediated by cells, increasing gene transcription of chemokines, cytokines, adhesion molecules, enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis [19]. The observed downregulated expression of the pro-inflammatory cytokines IL-6 and TNF-α might have been caused by the decrease observed in NF-κB activation, since the expression of these genes is strongly dependent of this transcription factor [18]. In addition to NF-κB, the inflammatory signaling pathway JNK was also downregulated by NT under inflammatory conditions. The anti-inflammatory cytokine IL-10 was

decreased by NT alone, suggesting that NT downregulated not only inflammatory cytokines, but also anti-inflammatory cytokines, decreasing the immunologic capacity of dendritic cells. On the other hand, the p38 MAPK signaling pathway was upregulated by neurotensin, being probably involved in the observed CCL5 increase, as described previously [18].

ERK can directly phosphorylate many transcription factors involved in the expression of important genes involved in cell cycle progression, like platelet-derived growth factor and heparin-binding epidermal growth factor (HB-EGF), in apoptosis prevention and in cytokine expression [20]. Therefore, the NT-induced increase of EGF expression observed in FSDC may be related to ERK activation by neurotensin. EGF may have a paracrine effect in other cells other than FSDC, promoting epidermal growth and keratinocyte proliferation, differentiation and migration, promoting epidermal thickness, important in the last phases of WH [21]. Furthermore, EGF treatment of the wound accelerates wound healing [22]. On the other hand, VEGF-A was decreased by NT at short-time incubation periods. VEGF is an important regulator of endothelial cell proliferation, migration and cell survival, inducing vascular hyperpermeability and angiogenesis, as well as, inducing monocyte/macrophage recruitment [23]. All these processes are involved in the first phases of WH but are irrelevant in the last phases of WH. Moreover, the most important results reported will be confirmed in human LC, isolated and differentiated from peripheral blood monocytes in the presence of IL-4, GM-CSF and TGF-beta, as previously reported by us [17].

Similar effects have been described for different neuropeptides in the skin. In fact, these effects seem to be enhanced when more than one neuropeptide act in the skin, evidencing a synergistic effect of some neuropeptides. For instance, CGRP and Substance P act together to increase blood flow, being potent and long lasting microvascular dilators in the surrounding of the site of injury, as well as α-MSH and ACTH peptides interact with MC receptors on melanocytes, keratinocytes, and Langerhans cells to modify their functional activity, to increase cutaneous melanin pigmentation and generate local anti-inflammatory and immunosuppressive effects [24,25]. Knowing the effect of neurotensin in the skin, its effects could be enhanced when acting together with other immunosuppressive neuropeptides, like α-MSH and ACTH. Moreover, NT effects in monocytes and PMN have not been determined to date, which along with dendritic cells play a major role in the inflammatory phase of WH. The effect of NT on monocytes and PMN may accurately clarify the possible NT function in the inflammatory phase of WH.

Translating these results obtained in skin dendritic cells to events occurring *in vivo* during wound healing we can hypothesize that if NT is released in the beginning of the inflammatory processes, it may induce pathological effects, diminishing the capacity of cells to respond to inflammation and probably promoting infections; however, if NT is released in the middle or at the end of the inflammatory phase, as well as, in the migration-remodeling phases of WH, NT may modulate de degree of inflammation, end inflammation and induce the progression to migration-remodeling phases of WH, promoting wound healing through EGF production.

In vitro pre-treatment (24 h) of cells with NT mimicked an exogenous and *in vivo* pre inflammatory treatment with NT. In this case, NT enhanced the inflammatory response evoked by LPS, probably provoked by the cumulative effects of LPS and NT. Indeed, this pro-inflammatory profile in FSDC may be important in the first phases of WH. Correlating these *in vitro* studies with those events occurring *in vivo*, if NT is applied immediately after injury in the wounds, previously to the start of the inflammatory process, NT can in fact upregulate the immune function of cells in the inflammatory phase of WH, being promissory in the treatment of normal WH. On the other hand, if NT is applied in the wounds after injury, when the inflammatory process has already started (*in vitro* cell stimulation with LPS and NT during 6 h), NT can negatively modulate the cells' immune responses. In these circumstances, delayed wounds known to have higher levels of NT, could possibly be treated with antibodies for NT that will decrease the NT concentration in the wounds, promoting WH. Moreover, once NT levels are determined in skin pathologies and if NT is then defective, a treatment with low doses of NT may promote WH. Further similar studies should be performed both in other skin cells and in *in vivo* conditions, in order to disclose the potential benefic therapeutic role of NT on skin pathological conditions.

Conflict of interest

No conflict of interest to declare.

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