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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