Original Research

The effect of neurotensin in human keratinocytes – implication on impaired wound healing in diabetes

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Abstract

Diabetic foot ulcers are an important complication of diabetes mellitus characterized by chronic, non-healing ulcers resulting from poor proliferation and migration of fibroblasts and keratinocytes, thus impairing a correct re-epithelialization of wounded tissues. This healing process can be modulated by neuropeptides released from peripheral nerves; however, little is known regarding the role of neurotensin (NT) as a modulator of human keratinocyte function under hyperglycemic conditions. Therefore, this work is focused on the effect of NT in human keratinocytes, under normal and hyperglycemic conditions at different functional levels, namely NT receptors, cytokine, and growth factor expression, as well as proliferation and migration. Human keratinocyte cells were maintained at either 10/30 mM glucose and treated with or without NT (10 nM). The results show that NT did not affect keratinocyte viability. In addition, NT and all NT receptor expression levels were significantly reduced by hyperglycemia; however, NT treatment stimulated expression of NT and neurotensin receptor 2 (NTR2) while neurotensin receptor 1 (NTR1) and neurotensin receptor 3 (NTR3) expression levels were unchanged. Keratinocyte proliferation was not affected by NT and hyperglycemia, while cell migration was reduced by NT treatment.

These results demonstrated that hyperglycemic conditions strongly impaired endogenous NT and NTR2 expression in keratinocytes. Despite the addition of exogenous NT to stimulate the endogenous NT and NTR2 expression, these changes do not translate into functional modifications on keratinocytes, particularly in terms of migration, proliferation, and production of cytokines or growth factors. These results suggest that NT production by keratinocytes may exert a paracrine effect on other skin cells, namely fibroblasts, macrophages, and dendritic cells for correct wound healing.

Keywords: Wound healing, keratinocytes, hyperglycemia, neuropeptides, neurotensin

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Introduction

Diabetes mellitus is one of the most complicated chronic diseases that affect millions of people worldwide.[1] Patients with diabetes are susceptible to develop complications such as chronic, non-healing diabetic foot ulcers (DFU) that cause pain, suffering, decrease in quality of life, and, in extreme cases, culminate with lower extremity amputations.[2,3]

An important phase during the wound-healing process is the re-epithelialization of wounded tissues. Correct re-epithelialization is an essential feature for the restoration of an intact epidermal layer and migration and proliferation of keratinocytes are critical steps in this process.[4,5] After injury, keratinocytes not only migrate and proliferate to cover the wound but also express cytokines and growth factors that regulate the wound-healing process.[6] Furthermore, the peripheral nervous system also plays an important role in the inflammatory, proliferative and reparative processes after skin injury. Neuropeptides act peripherally as paracrine and endocrine factors to regulate diverse physiologic processes and act as neurotransmitters or neuromodulators in the nervous system or on other skin cells, namely on macrophages and dendritic cells.[7,8] The interaction between peripheral nerves and the immune system is mediated by different types of cutaneous nerve fibers that release neuropeptides, such as substance P and neuropeptide Y, which in turn activate specific receptors on target cells in the skin, such as keratinocytes, mast cells, Langerhans cells, microvascular endothelial cells, fibroblasts, and macrophages.[8–11] In response to neuropeptides, these skin cells produce and release cytokines and growth factors.[10,12] These neuro–skin interactions influence a variety of physiologic and pathophysiologic functions

including cellular development, growth, differentiation, immunity, vasoregulation, leukocyte recruitment, and wound repair.[11,13]

Neurotensin (NT) is a bioactive trideca-neuropeptide that is widely distributed through the brain, cardiovascular system, and the gastrointestinal tract.[14–16] In addition, NT regulates inflammatory processes in the lung and gastric system.[16,17] NT functions are mediated through its binding to two G-protein coupled receptors: neurotensin receptor 1 (NTR1) and neurotensin receptor 2 (NTR2) (high and low affinity, respectively). The third receptor, the neurotensin receptor 3 (NTR3), is an intracellular, non G-protein coupled receptor[15,18] NT.

Since the effect of NT on keratinocytes has never been addressed before, this study aims to determine how NT modulates keratinocyte function under hyperglycemic conditions, namely exerting a paracrine effect on other skin cells, such as on macrophages and dendritic cells.

Material and methods

Materials

NT was obtained from Bachem (Weil am Rhein, Germany). All primers were obtained from IDT (Ebersberg, Germany). The TRIzol reagent was purchased from Invitrogen (Barcelona, Spain). SYBR green was obtained from BioRAD (Hercules, CA, USA) and the High Capacity cDNA Reverse Transcription kit was obtained from Applied Biosystems (Carlsbad, CA, USA).

The antibodies against the NT receptors were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and the antibody against β-actin was purchased from the Millipore Corporation (Bedford, MA, USA).

Cell culture

The human keratinocyte cell line (HaCaT) was purchased from CLS (number 300493 Eppelheim, Germany) and was cultured in Dulbecco's Modified Eagle Medium (DMEM), pH 7.4, supplemented with 10% heat inactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified incubator containing 5% CO_2. Sub-culturing was performed according to CLS recommendations. The cells were maintained in either 10 mM (normal glucose) or 30 mM (high glucose) D-glucose, for more than 2 weeks, before starting the experiments.

MTT assay. HaCaT (4×10^4 cells/well) cells, at 30 mM glucose conditions, were plated in 48-well plates in 430 μL of DMEM. Cells were treated with 10, 50, or 100 nM of NT for 7days. After, either 1, 3, 5, or 7 days of incubation, 43 μL of MTT solution (5 mg/mL) were added to each well. The plates were further incubated at 37°C for 1 h, in a humidified incubator containing 5% CO_2. About 300 μL of acidic isopropanol (0.04 N HCl in isopropanol) was then added to each well and mixed in order to dissolve the dark blue crystals of formazan. Acidic isopropanol was collected in an enzyme-linked immunosorbent assay (ELISA) microplate and formazan quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

Table 1 Forward and reverse primers sequences used in RT-PCR

Primer	5′-3′Sequence (Forward; Reverse)
NT	For: GCATACATCAAAGATTAGT Rev: TAAAGCAGTAGGAAGTTT
NTR1	For: CCATCCACACTGCCACCGTCA Rev: TGAATGTGCTGTGCTCGCCC
NTR2	For: TCCAAGTCTTTATCCAGGTG Rev: TACGATGAAGCTGAGGAGAC
NTR3	For: TGGGTTGGAGATAGCACTGG Rev: ACGACTTCCTCCAGACACCT
IL-1β	For: GCTTGGTGATGTCTGGTC Rev: GCTGTAGAGTGGGCTTATC
IL-8	For: TTGGCAGCCTTCCTGATTTC Rev: AACTTCTCCACAACCCTCTG
EGF	For: AATCATGGCTGTACTCTTGGG Rev: CAGGACAGAAACATAAGGGAC
VEGF	For: CAGAATCATCACGAAGTG Rev: TCTGCATGGTGATGTTGGA
PDGF	For: CAGAAGCTAACCATCTCCTGG Rev: TCGGGAGTAATTTGGTGCTTC
HPRT1	For: TGACACTGGCAAAACAATG Rev: GGCTTATATCCAACACTTCG

Real-time polymerase chain reaction

HaCaT cells (5×10^5 cells/well) were seeded in 6-well plates and treated with 10 nM NT during either 6 h or 24 h. Total RNA were isolated from cells with the TRIzol reagent according to the manufacturer's instructions and concentration was determined by OD260 measurement using NanoDrop spectrophotometer (Thermo Scientific, St. Leon-Rot, Germany). The first cDNA strand was synthesized using High Capacity cDNA Reverse Transcription (Carlsbad, USA). Briefly, 2 μL of 10X RT buffer, 0.8 μL of 25X dNTP mix, 2 μL of 10X RT random primers, 1 μL of MultiscribeTM Reverse Transcriptase, and 4.2 μL of nuclease-free H_2O were added to 10 μL of RNA (1 μg) sample. Then, real-time polymerase chain reaction (RT-PCR) was performed in a Bio-Rad My Cycler iQ5. For each reaction 10 μL were used containing 2.5 μL cDNA, 5 μL 2X Syber Green Supermix, 1 μL of each primer (250 nM), and 0.5 μL of H_2O PCR grade. Primer sequences are given in Table 1. Gene expression changes were analyzed using iQ5Optical system software v2 (Amadora, Portugal). The results were normalized using a reference gene, hypoxanthine phosphoribosyltransferase 1 (HPRT-1), selected based on our previous results demonstrating that it does not change under these conditions. Quantitative RT-PCR results were analyzed through delta CT calculations.

Western blotting

HaCaT (5×10^5 cells/well) cells were seeded in 6-well plates and treated with 10 nM of NT for 24 h. Cells were then washed twice with ice-cold PBS buffer and lysed

with RIPA buffer (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, proteases inhibitor cocktail, phosphatases inhibitor cocktail, and 1 mM DTT). Protein concentration was determined using the bicinchoninic acid method and the cell lysates were denatured at 95°C, for 5 min, in sample buffer (0.125 mM Tris pH 6.8; 2% w/v SDS; 100 mM DTT; 10% glycerol and bromophenol blue) for its use in western blot analysis. About 30 μg of total protein was resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) fat-free dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T), for 1 h, at room temperature. After blocking and washing, membranes were incubated overnight at 4°C with the primary antibodies against NT receptors (1:500). After incubation, membranes were washed and incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit antibody (1:5000), or alkaline phosphatase-conjugated anti-mouse antibody (1:5000). The membranes were exposed to the ECF reagent followed by scanning for blue excited fluorescence on the VersaDoc (Bio-Rad Laboratories, Amadora, Portugal). To test whether similar amounts of protein for each sample were loaded, the membranes were stripped and reprobed with an anti-actin antibody and blots were developed with an alkaline phosphatase-conjugated secondary antibody and visualized by enhanced chemifluorescence. The generated signals were analyzed using the Image-Quant TL software (Carnaxide, Portugal).

Proliferation

HaCaT (4×10^5 cells/well) cells were seeded in 6-well plates and treated with 10 nM of NT for 24 h. Cells were resuspended and 0.1 mL of 0.4% trypan blue stock solution in PBS was added to 1 mL of cells. The number of blue staining cells and the number of total cells were counted in a Zeiss Primo Vert Microscope (Carl Zeiss, Gottingen, Germany).

In vitro scratch assay – Migration

HaCaT (4×10^5) cells were resuspended in 3 mL of DMEM medium in μ-Dish$^{35\,mm,\,high}$ (Ibidi, Martinsried, Germany). After 24 h, a ''scratch'' was made in the cell monolayer, with a pipette tip, in a straight line to create an area without cells. The medium was removed and cells were washed two times with PBS 1X. Then, DMEM medium containing 2% of inactivated fetal calf serum was added to the cells to diminish cell proliferation. Photographs were captured with a coupled AxioCamMR3 camera with PALM reflector and 5X objective, using an inverted Axiovert 200 (Oberkochen, Germany). A specific number/letter marked area was chased to permit later recognition of the photographed area. HaCaT cells were then incubated with 10 nM of NT or maintained in DMEM medium containing 2% of inactivated fetal calf serum (control) and allowed to migrate during 24 h. After the incubation period, photographs in the same area were taken where the first photograph was taken. Photographs were analyzed and the number of cells in the scratched area was counted. For the analysis, the number of cells in the zero point was taken into account.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using either one-way analysis of variance or the unpaired student's t test by GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). *P* values less than 0.05 were considered statistically significant.

Results

All experiments were performed using HaCaT cells incubated with either 10 mM glucose (normal glucose conditions) or 30 mM glucose (high glucose conditions), for a period of 15 days.

Cell viability under hyperglycemic conditions

Different NT concentration treatments did not significantly affect the viability of keratinocytes under hyperglycemic conditions (Figure 1). Since no major differences were

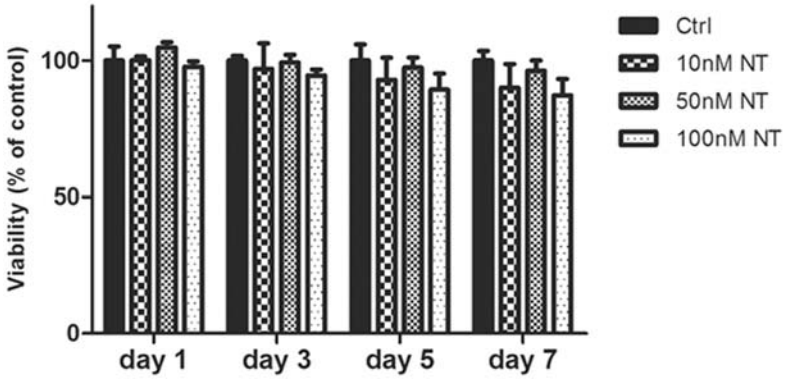

Figure 1 Viability of HaCaT cells, under either 10 or 30 mM glucose, by the MTT assay. HaCaT cells were plated at 4×10^4 /well and were treated with 10, 50, or 100 nM of NT for 7days. After, 1, 3, 5, or 7 days of incubation, the MTT assay was performed as described in Materials and methods section. Absorbance quantification was performed using a microplate reader at 570 nm, with a reference wavelength of 620 nm. Results are presented as mean ± SEM of three independent experiments

observed between the concentrations of NT used (10, 50, or 100 nM), the following experiments were performed with 10 nM of NT.

Expression of NT receptors in HaCaT cells under normal and hyperglycemic conditions

Gene expression results showed that in endogenous (non-treated) conditions, hyperglycemia strongly reduced the expression of NT and all NT receptors (Figure 2(a)–(d)). When stimulated with NT, total NT expression significantly increased ($P < 0.001$), either in normal or hyperglycemic conditions. However, in the same conditions, no differences were observed for NT receptors expression. Interestingly, only NTR2 expression showed a significant increase ($P < 0.05$) in response to NT treatment, under hyperglycemia.

However, at the protein level no differences were observed for all NT receptors after keratinocytes treatment with either 10 mM or 30 mM glucose (Figure 2(e)).

Proliferation and migration of HaCaT cells under normal or hyperglycemic conditions

After NT treatment, no statistical differences were observed in HaCaT cells proliferation under normal or hyperglycemic conditions, during 24 h (Figure 3(a)).

However, migration studies revealed that under 30 mM glucose, NT significantly decreased HaCaT migration when compared with 10 mM glucose treatment ($P < 0.05$) (Figure 3(b) and (c)).

Cytokine and growth factors expression by HaCaT cells under normal and hyperglycemic conditions

IL-8 and growth factors (VEGF, EGF, and PDGF) were not affected by NT treatment either in normo or hyperglycemic conditions (Figure 4(a)–(d)).

Discussion and conclusions

Skin is the outermost layer of the body, with a protective barrier against the external environment.[5,6] However, skin is susceptible to become injured and the healing process must be highly controlled and organized for correct repair.[2] Keratinocytes are important cells in the regulation of homeostasis and pathophysiological processes through proliferation, migration, and cytokines/growth factors secretion.[10] Keratinocytes from the wound edges are mainly responsible for the re-epithelialization phase of wound healing. They migrate across the wound site, proliferate in its edges, and differentiate to restore the functionality of the epidermis.[4] Alterations in this process are associated with chronic, non-healing ulcers, such as DFU. Neuropeptides are produced in the skin by peripheral nerves such as autonomic or sensory ones. In addition to neuronal cells, immunocompetent cells, as well as epithelial cells, such as keratinocytes are able to produce neuropeptides,[10,19,20] which can exert mitogenic actions and modulate the functions of other different cell types in the skin.[10] However, no studies have investigated the effects of NT in human keratinocytes under hyperglycemic conditions. Our results demonstrated that hyperglycemia significantly reduced NT and all NT receptors expression in human keratinocytes; however, it is not observed in protein expression.

The regulation of gene expression is a complex and highly controlled process. The steady state of RNA levels is dependent on transcription, processing, and stability processes. Gene expression is also affected by post-translational modification, alternative and differential splicing, and protein modifications.[21] Therefore, in most cases, mRNA and protein levels do not correlate, mostly due to these regulatory processes. In agreement with our results, previous studies have demonstrated that neuropeptides, such as substance P and neuropeptide Y expression are

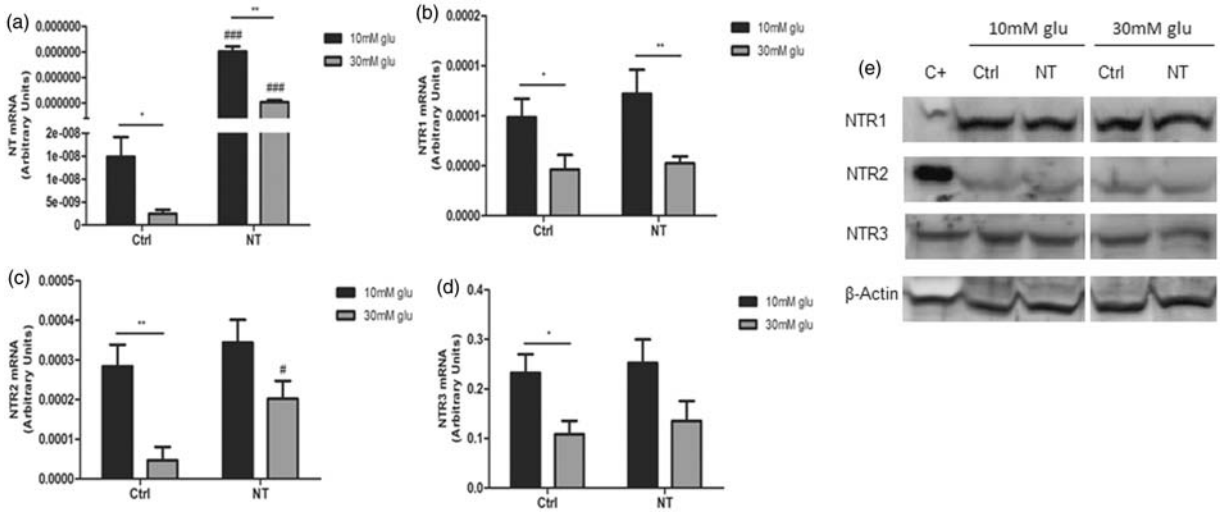

Figure 2 Expression of NT and NT receptors, NTR1, NTR2, and NTR3 in HaCaT cells under either 10 or 30 mM glucose, by real-time PCR (a–d) and Western Blot (e). Cells were plated at 5×10^5 / well and treated with 10 nM NT during 6 h or 24 h. Total RNA and cell lysates were performed as described in Materials and methods section. RT-PCR results are presented as mean ± SEM of six to nine independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; #$P < 0.05$; and ###$P < 0.001$ compared with respective control. In the Western Blots, cerebral cortex lysates (C+) were used as positive controls. Three independent experiments were performed for each antibody. (A clolor version of this figure is available in the online journal)

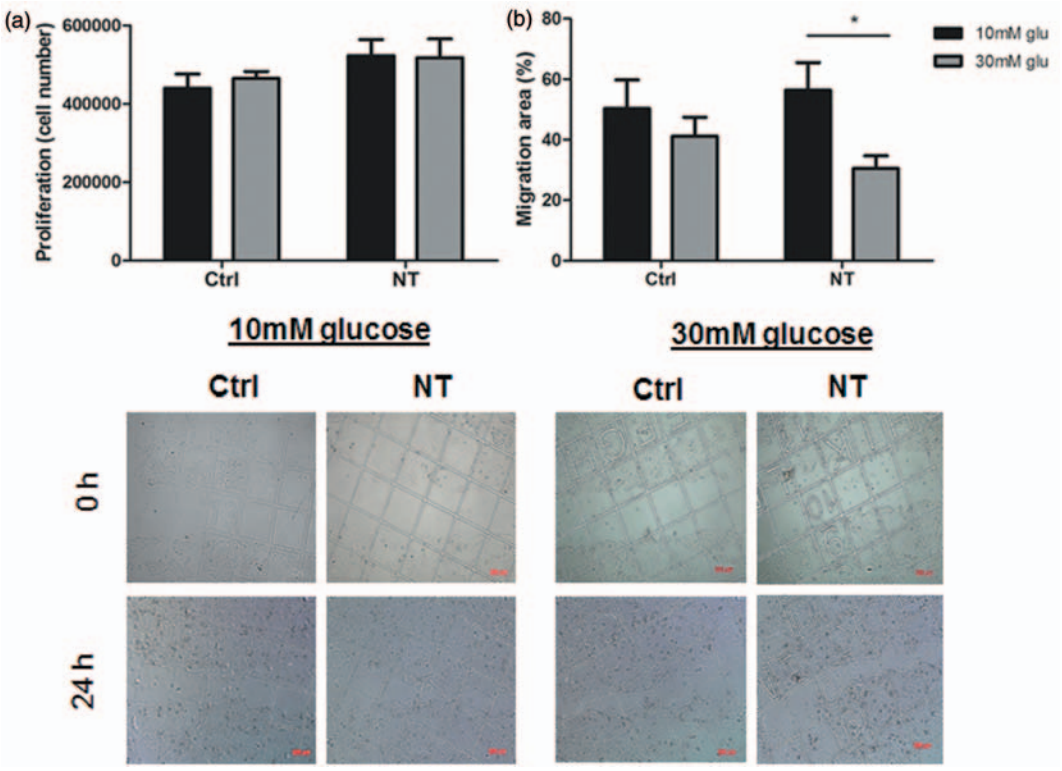

Figure 3 Proliferation of HaCaT cells under either 10 or 30 mM glucose, by the trypan blue assay (a). Migration of HaCaT cells under either 10 or 30 mM glucose, by the *in vitro* scratch assay (b). In both experiments, cells were plated at 4×10^5 / well and treated with 10 nM NT during 24 h. The experiments were performed as described in Material and methods section. The images were acquired by transmission microscopy and photographs were taken before cell treatment (0 h) and 24 h after treatments. Magnification used 40×. Results are presented as mean ± SEM of three independent experiments.*$P < 0.05$. (A color version of this figure is available in the online journal)

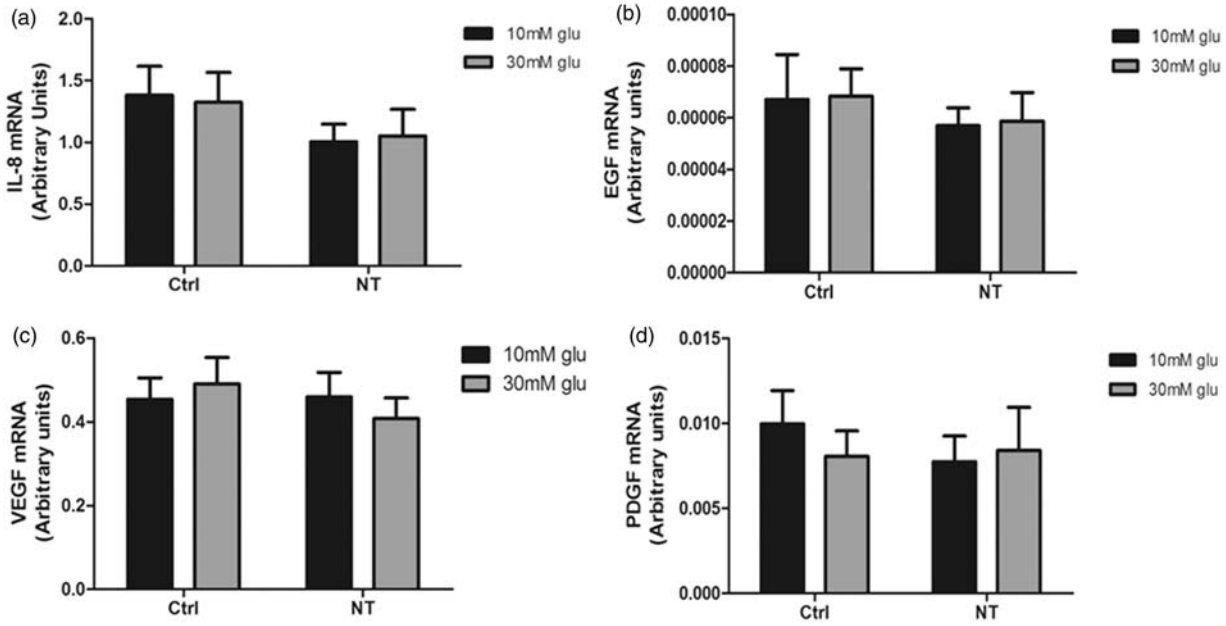

Figure 4 Expression of inflammatory cytokine, (a) IL-8, and (b–d) growth factors (EGF, VEGF, and PDGF) in HaCaT cells, under either 10 or 30 mM glucose, by real-time PCR. Cells were plated at 5×10^5 /well and treated with 10 nM NT during 6 h or 24 h. Total RNA was isolated as described in Materials and methods section. The relative gene expression is indicated as arbitrary units and was obtained after normalization with the HPRT gene. Results are presented as mean ± SEM of six to nine independent experiments. **$P < 0.01$

downregulated in skin of diabetic rabbits, correlating with a suppression of a proper inflammatory response at the injury site.[22] These results highlight the crucial role of neuropeptides in wound healing and a dysfunctional expression of these molecules under hyperglycemic conditions could be correlated with the physiopathology of DFU. In addition, we verified that under hyperglycemic conditions, NT treatment significantly stimulated the expression of NT and NTR2, while NTR1 and NTR3 expression levels were unchanged. NTR1 and NTR2 are part of the high and low affinity G protein-coupled receptor family, respectively, while the NTR3 is a sortilin type I receptor with a single transmembrane domain.[23,24] NTR2 is internalized inside the cell after NT binding with a lower affinity (30–40%) compared with NTR1 (60%). In the end, NTR2 is efficiently recycled to the cell surface.[25] Our results may suggest that under hyperglycemia, these mechanisms could be modified. The NTR2 cellular coupling functions remain to be clarified. However, various studies referred its involvement in the analgesic effect of the neuropeptide.[25,26] In addition, NT fulfills the function of a growth factor in various human cancer cell lines; however, the trophic effect of NT on these cells has always been attributed to NTR1 and NTR3. Martin et al.[24] showed that the structurally different receptors NTR1 and NTR3 were co-expressed in several human cancer cells on which NT exerts proliferative effects. We may speculate that high glucose conditions also induce structural modifications in NTR2 rendering keratinocytes unresponsive to NT. Indeed, and excluding a slight effect on migration, exogenous addition of NT did not modulate keratinocytes function under hyperglycemia, despite the increase in NT and NTR2 expression. To understand the role of NT in the important process of re-epithelialization in diabetics, we performed proliferation and migration assays. We verified that neither proliferation nor migration was affected by NT. Only hyperglycemia decreased keratinocyte migration after NT treatment. Moreover, since neuropeptides can stimulate cytokine and growth factors expression, IL-8, EGF, VEGF, and PDGF were analyzed. All the analyzed cytokines and growth factors were not affected by the NT stimulus. Proliferation and migration are important steps in the re-epithelialization wound-healing process that need the recruitment of cytokines and growth factors, such as TNF-α, IL1, EGF, VEGF, and FGF.[4,12] As these factors were not affected either by high glucose or by the NT stimulus, no direct effect where observed in migration and proliferation.

We hypothesized that neuropeptides and specifically NT increase in keratinocytes under hyperglycemic conditions could have a paracrine effect on other skin cells, namely on macrophages and dendritic cells. In accordance, a previous study in our group demonstrated that NT promoted a pro-inflammatory status in a dendritic cell line (FSDC) under hyperglycemic conditions.[20] Furthermore, in a macrophage cell line (Raw 264.7), we showed that NT stimulates migration and inhibits the pro-inflammatory status of macrophages contributing to the resolution of inflammation and allowing the progression to the migration-remodeling phases of wound healing.[27] On the other hand, NT stimulated the phagocytic process in murine peritoneal macrophages.[28] In addition, Jain et al.[29] observed that hyperglycemia impairs dermal endothelial cell proliferation and tube formation, and these effects were mitigated by Substance P treatment.

Put together, our results demonstrate that NT and all receptors are decreased under hyperglycemia with little to no effect on keratinocyte function. Moreover, NT upregulates the expression of total NT and NT receptor 2 in human keratinocytes mostly under hyperglycemic conditions, which is detrimental to the re-epithelialization process once it reduces keratinocyte migration. However, NT did not affect proliferation and expression of inflammatory cytokines and growth factors in keratinocytes, under these conditions, reinforcing a potential paracrine effect of NT.

Further studies to analyze the function of NT and specifically NTR2 on human keratinocytes, under normal and high glucose conditions, are necessary to understand all the mechanisms involved.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; LM conducted the experiments and wrote the manuscript.

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