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REPORT

Novel contributions by Transmission Electron Immunomicroscopy on the intracellular trafficking of EGFR, PCNA, FOXO1A-P and LC3B and their biological response in clinical samples of diabetic foot ulcers treated with Heberprot-P®

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ABSTRACT

Diabetic foot ulcers (UPDs) is a diabetes complication and the leading cause of non-traumatic amputations. It was shown that Heberprot-P®, a therapeutic product based on epidermal growth factor (EGF), enhances the healing response or healing of chronic wounds (RCH) in UPDs. However, a better understanding of the cellular and molecular mechanisms of EGF receptor (EGFR) activation with its natural ligand in vivo, its intracellular traffic and the biological response to treatment was required. A study with electron immunomicroscopy was carried out for the detection and quantification of EGFR, its related molecules and autophagy vacuoles in fibroblast-like cells (FLCs) from UPDs. Samples were analyzed before and after treatment with Heberprot-P®. Before treatment, little EGFR and proliferating cell nuclear antigen (PCNA) were detected, predominating the transcriptional factor of phosphorylated hairpin head O1 (FOXO1A-P) in the cell nucleus and the induction of autophagy in FLCs from UPDs, possibly associated with the impairment of the functions of the FLCs in the RCH. Heberprot-P® induced an early increase in the immunodetection of EGFR and PCNA in the cell nucleus (15-60 minutes) and later in mitochondria (6 and 24 h). The nuclear functions of FOXO1A-P and autophagy decreased. EGFR and PCNA were shown associated with exosome-like structures and their accumulation in the extracellular matrix. All these were relevant for the restoration of FLCs functions, facilitating skin RCH. This was the first in vivo report demonstrating a prolonged biological effect (24 h) of Heberprot-P® infiltration in FLCs from UPDs, related to RCH, and supporting the current therapeutic protocol.

Keywords: Heberprot-P®, epidermal growth factor receptor, diabetic foot ulcers, proliferating cell nuclear antigen, FOXO1A, LC3B

RESUMEN

Novedosas contribuciones, por Inmunomicroscopía Electrónica de Transmisión, al tráfico intracelular del EGFR, PCNA, FOXO1A-P y LC3B y su respuesta biológica en muestras clínicas de úlceras del pie diabético tratadas con el Heberprot-P®. Las úlceras del pie diabético (UPD) son una complicación de la diabetes y la causa principal de amputaciones no traumáticas, que, tratadas con el Heberprot-P® (factor de crecimiento epidérmico), aceleran la respuesta de curación o cicatrización de heridas (RCH) crónicas como las UPD. Era necesario comprender mejor los mecanismos celulares y moleculares de la activación del receptor del EGF (EGFR) con su ligando natural in vivo, su tráfico intracelular y la respuesta biológica en las UPDs al tratamiento. Se detectó y cuantificó mediante la técnica de inmunomicroscopía electrónica (IME) al EGFR, sus moléculas relacionadas, y las vacuolas autofágicas en células semejantes a fibroblastos (FLCs) de UPD. Antes del tratamiento se detectó poco al EGFR y el antígeno nuclear de proliferación celular (PCNA), con predominio del factor transcripcional de cabeza de horquilla O1 fosforilado (FOXO1A-P) en el núcleo y la inducción de autofagia en las FLCs. El tratamiento con el Heberprot-P® incrementó la inmunodetección del EGFR y el PCNA en el núcleo celular (de 15 a 60 minutos) y en las mitocondrias (6 y 24 horas), disminuyeron las funciones nucleares de FOXO1A-P y la autofagia. El EGFR y el PCNA se asociaron con estructuras semejantes a exosomas y estas se acumularon en la matriz extracelular, vinculado a la restauración de las funciones de las FLCs y la RCH cutánea. Este fue el primer informe in vivo del efecto biológico prolongado (24 horas) en las FLCs de la infiltración con el Heberprot-P® de las UPD, vinculado a la RCH y como sustento del protocolo terapéutico actual.

Palabras clave: Heberprot-P®, receptor del factor de crecimiento epidérmico, úlceras del pie diabético, antígeno nuclear de células en proliferación, FOXO1A, LC3B

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*I*ntroduction

Diabetic foot ulcers (DFUs) are a diabetes complication and the main cause of non-traumatic amputations, which lead to disability, morbidity and a high rate of mortality in the five years, in average, following amputation [1]. In order to palliate its impact, the healing capacity of the epidermal tissue in diabetic patients has to be restored. One way is to exogenously administer the epidermal growth factor (EGF), which has to be available in the ulcer vicinity enough time for the controlled stimulation of cell receptors involved in the cicatrization process [2, 3]. Considering this, a procedure was developed to infiltrate EGF at supra-physiological concentrations in the wound bed and the edges of the DFUs for its successful closure, such procedure been successfully applied for over 18 years [2, 4-7].

In this setting, several studies showed that the deterioration of the signaling pathway mediated by the EGF and its receptor (EGFR) in peripheral tissues was a subjacent consequence of diabetes [8]. In fact, the stimulation with EGF induces the internalization of EGFR and its translocation into the cell nucleus to promote cell division and the phosphorylation of the PCNA transcriptional factor [9]. However, it was unknown the cellular compartmentalization and the biological response to the EGFR activation and its natural ligands in vivo, particularly under pathological conditions as DFUs and following EGF (Heberprot-P®) administration. Therefore, it was necessary to unraveled the mechanisms mediating the therapeutic effect of Heberprot-P® administration, specifically the distribution of molecules involved in EGFR signaling and its downstream cellular processes. In this work, it was established the functional recovery of the functional EGFR-mediated signaling affected in diabetic tissues, after the therapeutic administration of Heberprot-P®. This provided substantial scientific evidence for the therapeutic schedule followed for the administration of this product for DFUs cicatrization [10-13].

*M*aterials and methods

Population of the study and tissue biopsies

The study protocol was conducted following the ethical rules established by the Declaration of Helsinki in 1975 and the signed consent approval by all the patients enrolled in the study. The study was also approved by the ethics committees of the National Institute of Angiology and Vascular Surgery and the Center for the Integral Care of Diabetes, both in Havana, Cuba.

Up to 16 samples were collected, distributed among diabetes type 1 or 2 patients, 12 of them suffering from chronic neuropathic DFUs in their lower limbs and four control patients without ulcers. The 12 patients were infiltrated with Heberprot-P® at their DFUs at the same time point. Granulation tissue samples were then collected from DFUs at fixed timepoints: upon administration (t0), at 15, 45 and 60 min, and 6 and 24 h after treatment (T15, T45, T60, T6 and T24, respectively). Biopsies were analyzed by electron immunomicroscopy.using antibodies specific against EGFR, PCNA, FOXO1A-P and LC3 proteins, this last ivolved in autophagy.

The distribution of gold microparticles among fibroblasts' cellular compartments was compared at the different times after Heberprot-P® treatment through contingency analysis. The null hypothesis comprised the lack of differences on the distribution of gold particles in fibroblast at different timepoints following treatment.

Biopsy sampling wa reprsentative to the frequency, heterogeneity and clinical forms of diabetes mellitus and the ethnic multiplicity of populations worldwide affected by the disease.

The experimental design was logic and rational, schematized for transmission electron immunomicroscopy. All the results were conceptually and methodologically rigorous, according to the quality of the samples and its handling, as well as for the product administered and the adequate methods used. The information was rigorously acquired and supporting the scientific basis for the administration of the Heberprot-P®, having EGF as active pharmaceutical ingredient from the experimental point of view, both clinically and pharmacologically.

*R*esults

The therapy with Heberprot-P® increases the detection of EGFR and PCNA and its intracellular trafficking. Electron immunomicroscopy results showed poor detection of EGFR and PCNA at time 0 without treatment [1]. After treatment with Heberprot-P®, from 15 to 60 minutes later, the detection of EGFR receptor and PCNA increased in the cell nucleus, the rough endoplasmic reticulum (RER) and the Golgi complex (GC), in intracellular vesicles (V), multivesicular bodies (MVB), the extracellular matrix (ECM), in collagen fibers and exosomes. These two molecules were also detectable at the cell nucleus, RER, GC, mitochondria, MVB, the extracellular matrix, collagen fibers and exosomes at later time points after treatment (T6 and T24).

Differences were found in the immune-staining of the EGFR and PCNA in different cellular compartments in fibroblasts at different times following the treatment with Heberprot-P® [14]. EGFR results showed that the main immune-staining variations were located at the cell nucleus, mitochondria, and RER plus GC. In the case of PCNA, major differences were found at the cell nucleus, mitochondria and cytoplasm (excluding mitochondria and RER and GC).

The same quantification methodology for determining the intracellular distribution was applied to study other molecules involved in the therapeutic effect of Heberprot-P® [15, 16]. In this sense, FOXO1A is a molecule relevant in signaling pathways mediated by the EGFR and it is involved in several physiological and pathological cellular processes. Therefore, the intracellular location of the phosphorylated form of FOXO1A (S319; FOXO1A-P) was studied in cells similar to the fibroblasts analyzed. The immunoelectron microscopy results showed that at start (T0), FOXO1A-P was detected mainly at the cell nucleus, GC and RER, mainly at the membranes surrounding mitochondria. Moreover, FOXO1A-P was detected very low at the nucleus, in the endosomal vesicles, the extracellular matrix, at the fibers similar to

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collagen and in exosome-like structures. Otherwise, at late phases of treatment (6 and 24 h after treatment, T6 and T24, respectively), FOXO1A-P was detected at RER, GC, in mitochondria and at lower levels in the cell nucleus.

Furthermore, the intracellular distribution of the LC3B protein was analyzed. That protein plays a significant role in autophagy. Therefore, it was shown that LC3B was located mainly at RER at T0, particularly near mitochondria and the cell nucleus. It was also found within RER-derived vesicles or near mitochondria, and less frequent in the cell nucleus. At T15-T60, LC3B was detected mainly at the RER membranes and near mitochondria. It was also found at GC, in vesicles near the RER, GC and mitochondria, and the cell nucleus. At later time points after treatment with Heberprot-P®, LC3B was mainly detected in RER and the cell nucleus. Furthermore, it was located in the rest of the cytoplasm and vesicles near the RER. Altogether, these results showed the role of key organelles for the pathogenesis of DFU and involved in the therapeutic effect of Heberprot-P® [17].

Discussion

The electron immunomicroscopy was proven to be a methodology useful for studying the activation mechanisms of membrane receptors and their intracellular location. In fact, this was the first quantitative study showing by this method the intracellular location of EGFR, PCNA, FOXO1A-P and LC3B in fibroblast-like cells, in response to Heberprot-P® treatment of DFU. Overall, this supports the pharmacological role of EGFR stimulation in the biology of the healing response in wounds under pathological conditions.

Particularly, in this work, the EGFR and PCNA were detected at low levels in fibroblast-like cells from DFUs analyzed prior to treatment with Heberprot-P®. This suggested an impair in the signaling pathway of EGFR in those cells, further confirming the evidences found in previous works and other experimental models.

Other findings in this study suggest the activation of different types of cellular stress responses at T0. These include damage to the normal physiology of mitochondria, RER and its expansion (RER stress). The morphological deterioration and the possible reduction in the normal functioning of mitochondria, together with the predominance of the nuclear functions of FOXO1A-P at T0, suggest the occurrence of oxidative stress in the fibroblast-like cells. Additionally, the possible inhibition of the EGFR signaling pathway and mitochondrial functions, the metabolism disruption typical of diabetes, the increase in FOXO1A-P levels, the decrease of LC3B at the nucleus and the higher levels of early and degradative autophagic vacuoles, altogether indicated the presence of an energy imbalance and authopagy induction process in the fibroblast-like cells. In one way, the activation of the RER stress response, the antioxidant activity of FOXO1A-P and the autophagy could favor the cellular adaptation and survival under pathological conditions in DFUs. On the other hand, the loss of proliferative and functional capacity of fibroblast-like cells, together with the pro-apoptotic and inflammatory effects of FOXO1A-P could inhibit the normal response of wound healing. Our results suggest that following treatment with Heberprot-P®, with EGF, EGFR became activated in the fibroblast-like cells in the biopsies analyzed. This is supported by the increase seen in the immunodetection of EGFR following treatment. Moreover, during the early response (T15-T60) there was an accumulation in the immunestaining for EGFR in the nucleus as compared to the late phase (T6-T24). These indicated the translocation of EGFR into the cell nucleus and the preponderance of its nuclear functions.

Noteworthy, the detection of PCNA showed an intracellular distribution similar to that of EGFR. Besides, a time-dependent differential intracellular location was seen for EGFR following Heberprot-P® infiltration. In that scenario, EGFR predominated in mitochondria at T6-T24. Other molecules associated to EGF/EGFR signaling studied in this work (PCNA, FOXO1A-P and LC3B) also showed a time-dependent differential intracellular distribution after treatment. Furthermore, the infiltration of Heberprot-P® and the activation of the EGFR were related to the formation of exosome-like structures containing EGFR, PCNA or FOXO1A-P, which could favor wound healing. Autophagy was also found modified in the fibroblast-like cells, further suggesting the impair in the formation of degradative autophagy vacuoles at T-15 to T60 and the inhibition of the start of autophagy, or the biogenesis of early autophagy vacuoles at late phases. The predominance of the signaling pathways mediated by EGFR/AKT/mTORC1 and EGFR/RAS/MAPK, together with the restore of the mitochondrial and RER functions, the oxidative/antioxidiant balance, of ATP levels, the inhibition of FOXOA1 within the cell nucleus and the relocation of LC3B to the cell nucleus could contribute to decrease autophagy, further promoting the healing response (Figure).

Relevance of the study

In summary, the signaling mediated by the EGFR and PCNA is impaired in fibroblast-like cells from DFUs prior to treatment with Heberprot-P®, possibly related to the pathogenesis of the disease. Subsequently, the treatment with Heberprot-P® induced the intracellular relocation of EGFR and PCNA, and stimulated the production of exosome-like vesicles containing both molecules. Such a response could be related to its functions in promoting cellular proliferation, oxidative stress and DNA damage responses, the mitochondrial dynamics and ATP production. All these favor the healing response and the therapeutic effect of Heberprot-P®.

Furthermore, it was shown that the nuclear functions of FOXO1A were favored in fibroblast-like cells from DFUs prior to the treatment with Heberprot-P®. This could be related to its role in the antioxidant response for promoting the arrest of cell proliferation, the inflammatory response, autophagy and further affecting the fibroblast-like cells' physiology. In fact, it was demonstrated that those cells were licensed for autophagy induction prior to treatment with Heberprot-P®, further contributing to the impair of cicatrization. In that sense, the treatment with Heberprot-P® inhibited autophagy induction, possibly caused by the

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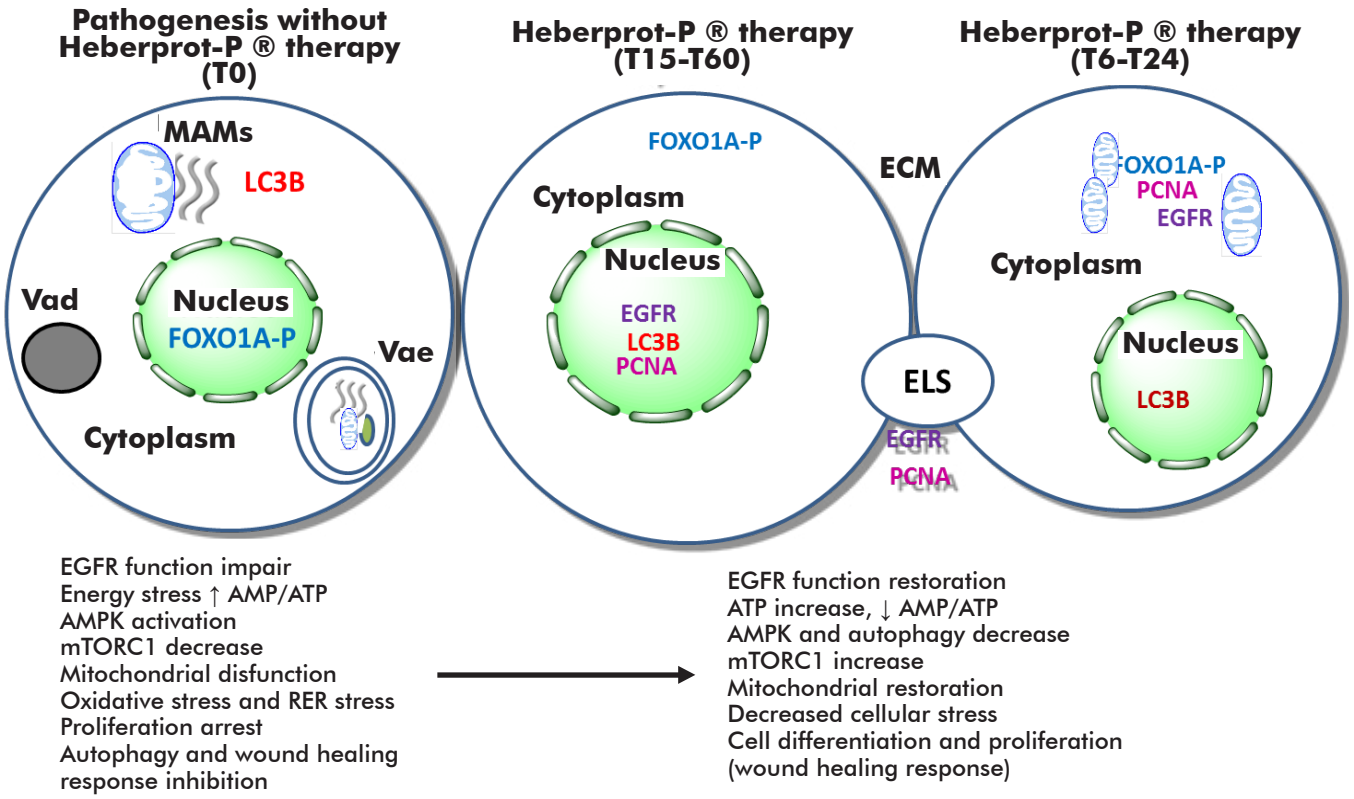

Figure. Integrative model of fibroblast-like cells before and after treatment with Heberprot-P®. Data taken from treatment at 0, 15, 60 minutes and 6 and 24 h (T0, T15, T60, T6 and T24, respectively). FOXO1A-P: transcriptional factor of phosphorylated hairpin head O1. mTORC1: mammalian target of rapamicin complex 1. RER: rough endoplasmic reticulum. EGFR: epidermal growth factor receptor. PCNA: proliferating cell nuclear antigen. ECM: extracellular matrix. ELS: exosome-like structures. MAMs: mitochondrial-associated membranes of the RER. Vad: autophagic late/degradative vacuoles. Vae: autophagic early/initial vacuoles.

regulatory effects of EGFR-related signaling, the inhibition of the nuclear functions of FOXO1A and the generation of exosome-like structures. All these could contribute to restoring the functions of fibroblast-like cells in the cicatrization response and in response to Heberprot-P® treatment, further suggesting the rationale of using autophagy inhibitors in combination with Heberprot-P® for a more successful wound healing.

Additionally, the production of extracellular vesicles following Heberprot-P® treatment implicated its possible use as prognosis biomarkers and also as therapeutic agents to enhance the therapeutic effect of Heberprot-P®.

Ultimately, it was demonstrated that Heberprot-P® induces a sustained biological effect up for 24 h in fibroblast-like cells at DFUs, which is compatible with the restoration of the wound healing response, further supporting the current therapeutic scheme.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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