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Differential modulation of stress-inflammation responses by plant polyphenols in cultured normal human keratinocytes and immortalized HaCaT cells

Saveria Pastore [a], Daniela Lulli [a], Alla I. Potapovich [a,b], Paolo Fidanza [a], Vladimir A. Kostyuk [a,b], Elena Dellambra [a], Chiara De Luca [a], Riccardo Maurelli [a], Liudmila G. Korkina [a,*]

[a] Lab. Tissue Engineering and Skin Pathophysiology, Dermatology Institute (Istituto Dermopatico dell'Immacolata, IDI IRCCS), Rome 00167, Italy
[b] Biochemistry Department, Byelorussian State University, Minsk 220050, Byelorussia

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ABSTRACT

Background: Environmental and endogenous stresses to skin are considered causative reasons for skin cancers, premature ageing, and chronic inflammation. Screening of substances with preventive and/or curative properties is currently based on mechanistic studies of their effects towards stress-induced responses in skin cell cultures.
Objective: We compared effects of plant polyphenols (PPs) on the constitutive, UVA-, LPS-, or TNF-alpha-induced inflammatory responses in cultured normal human epidermal keratinocytes (NHEK) and immortalized HaCaT cells.
Methods: Representatives of three classes of PPs, flavonoids, stilbenoids, and phenylpropanoids were studied. Their effects on mRNA were determined by qRT-PCR; protein expression was assayed by Western blot and bioplexed ELISA; phosphorylation of Akt1, ERK1/2, EGFR, and NFkappaB was quantified by intracellular ELISA or Western blot.
Results: PPs or their combination with UVA or LPS induced strong up-regulation of stress responses in HaCaT but not in NHEK. In addition, compared to NHEK, HaCaT responded to TNF-alpha with higher synthesis of MCP-1, IP-10 and IL-8, concomitant with stronger NFkappaB activation. PPs down-regulated the chemokine release from both cell types, although with distinct effects on NFkappaB, Akt1, ERK, and EGFR activation.
Conclusion: Results of pharmacological screenings obtained by using HaCaT should be cautiously considered while extending them to primary keratinocytes from human epidermis.

1. Introduction

Acute inflammatory response in the skin is a local protective reaction to various stresses including injury, microbial invasion, solar irradiation, and environmental pollutants [1]. On the other hand, chronic or excessive environmental and endogenous stresses or altered skin structure/functions may lead to persistent inflammation, which is considered a causative reason for skin cancers and premature ageing [2,3]. Keratinocytes appear to be not only primary sensors of stressful conditions but also major players of the extremely complex immune response in the skin conducting an orchestrated recruitment and functions of other immune cells involved in a stress-inflammation skin reaction [4]. Therefore cultured keratinocytes have become a prototype model for screening of anti-inflammatory, photo-protective, and cancer preventive substances for topical application [5–8].

There is a steadily growing interest in the skin protection by plant polyphenols (PPs) although the mechanisms by which these natural compounds exert their beneficial effects are not fully understood [9–12]. The skin benefits of PPs have been largely attributed to their classical antioxidant activity. However, in the last 5–10 years, evidence from numerous *in vitro* skin cell studies suggests that PPs can influence cellular functions by multiple other mechanisms, such as direct interaction with several receptors, modulation of intracellular signal transduction and transcription of a number of genes as well as post-translational modulation of enzymatic activities [2,13–15]. The hypothesis has been recently formulated and first publications have appeared that PPs-related modulation may also depend on their interaction with epigenomic processes [9,16,17]. It should be noted that the majority of the molecular pathways targeted by PPs are redox-dependent although free radical scavenging and metal chelating properties

* Corresponding author at: Laboratory of Tissue Engineering and Skin Pathophysiology, Istituto Dermopatico dell'Immacolata (IDI IRCCS), Via Monti di Creta 104, Rome 00167, Italy. Tel.: +39 06 66464258; fax: +39 06 66464253.
E-mail address: l.korkina@idi.it (L.G. Korkina).

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of PPs do not necessarily correlate with their anti-inflammatory, wound healing, or cancer preventive actions [2,5,12,13].

The immortalized human keratinocyte cell line HaCaT has been widely used in mechanistic and pharmacological studies of potential skin drugs, PPs among them [6,11,14,18,19], as an easy to handle substitutes for primary human keratinocytes. The cell line was established in 1988 as a spontaneously transformed immortal human epithelial cell culture from adult skin [20], which maintained normal keratinocyte morphology and epidermal differentiation capacity, remained non-tumorigenic, and underwent, like normal human keratinocytes, UV-induced apoptosis [10,21,22]. Although HaCaT cells displayed enhanced sensitivity to lethal doses of UV irradiation as compared to normal human epidermal keratinocytes (NHEK) due to mutations leading to the lack of intact p53 alleles [22,23] and a complete loss of p53 DNA-binding capacity [24], the model of UV-induced apoptosis in HaCaT has become classical in the comparative studies of PPs for skin photoprotection [6,10,18,19]. Further investigations on the molecular differences between HaCaT and NHEK have shown aberrant constitutive and UVB-induced NFκB activity/activation in the immortalized cells [25]. Since NFκB-dependent intracellular cell signalling pathways underlie inflammatory responses of keratinocytes to a variety of pro-inflammatory stimuli, such as pro-inflammatory cytokines (TNFα, IL-1, and IL-8), lipopolysaccharides (LPSs), lipid mediators of inflammation (platelet-activating factor, 4-hydroxy-2-nonenal (HNE), and leukotrienes), low molecular weight mediators of inflammation (ROS and RNS), and UV irradiation [1,15,26], alterations in the NFκB-driven molecular machinery may be crucial not only for a cellular reaction to external stresses but also for their modulation by potential anti-inflammatory substances.

In the present work, we sought to prove our working hypothesis that the use of HaCaT cells as a surrogate of NHEK could negatively affect results of the screening of anti-inflammatory and photoprotective substances. For this purpose, we challenged NHEK and HaCaT by non-lethal stresses known to induce NFκB-mediated responses, such as low dose UVA irradiation, bacterial LPS, and pro-inflammatory cytokine TNFα, and assessed their inflammatory responses at 3T (signal transduction, transcription, and translation) levels. To modulate stress-inflammation responses in cultured keratinocytes, we choose representatives of three major classes of PPs, flavonoids (quercetin (Qr) and its glycosylated form rutin (Rt)), phenylpropanoid glycosides (verbascoside (Vb)), and stilbenoids (resveratrol (Rv) and its glycosylated form polydatin (Pd)). All of them have been extensively studied for antioxidant, photo-protective, anti-inflammatory, wound healing, and skin cancer preventive properties (for review see [1,12,13,16,17]).

2. Materials and methods

2.1. Cell cultures

The spontaneously immortalized human keratinocyte cell line HaCaT was a kind gift from Dr. Norbert E. Fusenig, Deutsches Krebsforschungszentrum, Heidelberg, Germany. HaCaT cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS, PAA, Coelbe, Germany) and 1% penicillin–streptomycin (Biochrom KG, Berlin, Germany) at 37 °C in a 5% CO_2 humidified atmosphere. Primary cultures of keratinocytes (NHEK) were obtained from skin biopsies of healthy volunteers ($n = 5$) after their informed consent, as previously reported [27]. Briefly, the skin specimens were washed with PBS, minced, and trypsinized (0.05% trypsin/0.01% ethylene diamine tetraacetate, sodium salt) at 37 °C for 3 h. Cells were collected every 30 min and placed onto 6-well plates (final cell density $4 \times 10^4/cm^2$). Keratinocytes were grown in serum-free defined medium supplemented with hydrocortisone, transferrin, epinephrine, bovine pituitary extract, epidermal growth factor, insulin, and gentamicin/amphotericin B (KGM-Gold, Lonza, Walkersville, MD, USA). Both keratinocyte cultures were used for experiments at 60–80% of confluence.

Before all measurements/experiments, HaCaT cells were starved in DMEM without addition of FCS for 24 h, and NHEK were starved in serum-free defined medium without supplements for 24 h (starvation conditions). The starvation conditions were necessary to uniform the experiments and exclude any undesired interference of serum components with cell response to the stimulus.

2.2. Constitutive molecular characteristics of cell cultures

To characterize constitutive properties (cell cycle, differentiation, and activation of protein kinase B (Akt1), extracellular regulated kinases 1/2 (ERK1/2), and nuclear factor kappa B (NFκB) of primary and immortalized cultures, they were analyzed by Western blot or by intracellular ELISA. For immunoblots, HaCaT cells or NHEK were extracted on ice by lysis with Radio Immuno Precipitation Assay (RIPA) buffer. Western blot was performed using the following monoclonal (or polyclonal) antibodies: anti-p63 (4A4), anti-p53 (FL-393), anti GAPDH (FL-335) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-involucrin (SY5) from Sigma–Aldrich, Inc. (St. Louis, Missouri, USA); anti 14-3-3 σ (w55) from Primm, S. Raffaele Biomedical Science Park (Milan, Italy); anti 14-3-3 ζ was a kind gift of Alastair Aitken, (University of Edinburgh, UK) and anti-$p21^{Waf1}$ was a kind gift from Kristian Helin (University of Copenahagen, Denmark). 14-3-3 proteins belong to a large group of highly conserved and homologous dimeric proteins. 14-3-3 ζ is ubiquitously expressed and hence can serve as loading control, whereas 14-3-3 σ is abundantly expressed exclusively in the suprabasal layers of human epidermis.

Phosphorylation of NFκB constitutive protein (p65/RelA) at serine 536, Akt1, and ERK1/2 was quantified by modified ELISA assays without protein isolation from cultured cells using CASE™ Kit (SABiosciences Corporation, Frederick, MD, USA). These cell-based ELISA kits quantify the amount of phosphorylated protein relative to total protein. Results were expressed as percent of phosphorylated form of the protein relative to its total content.

2.3. Exposure to polyphenols and pro-inflammatory stresses

Qr dihydrate and Rt (98% purity; HPLC grade) were purchased from Sigma–Aldrich (Milan, Italy), Rv was from Biomol (Research Lab, Plymouth, MA, USA), Pd (95% purity; HPLC grade) was a kind gift from Prof. Gianpietro Ravagnan (University of Venice, Italy), and Vb (97% purity; HPLC grade) isolated from *Syringa vulgaris* plant cell cultures was a kind gift from Dr. Roberto Dal Toso (IRB S.r.l., Altavilla Vicentina, Italy). Chemical structures of tested PPs are given in Table 1. PPs were all added to cell cultures 1 h prior to exposure to any pro-inflammatory trigger in final concentration 50 μM unless otherwise specified in the figure. Equal volumes of DMSO (a vehicle for Rv, Pd, Qr, and Rt; final concentration of DMSO 0.25% (v/v)) or PBS (a vehicle for Vb, final concentration 0.25%) were added to the conditioned medium of control cultures.

In the experiments with UVA, cells suspended in PBS-glucose solution were exposed to standardized UVA irradiation without UVB admixture with the following characteristics: the light fluence rate on the cell monolayer 33 mW/cm^2, the exposure length 75 s, the total dose 2.5 J/cm^2, emission spectrum from 320 nm, and emission peak at 375 nm (Solar Simulator, Dermalight Vario with filter A1, Dr. Hoehnle AG, UV Technology, Planegg, Germany).

Bacterial LPS from *Escherichia coli* 055:B5 (Sigma Co., Milan, Italy) with the final concentration of 2.5 μg/ml or vehicle (PBS) were added to keratinocyte cultures for 4 h.