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Olive-Oil-Derived Polyphenols Effectively Attenuate Inflammatory Responses of Human Keratinocytes by Interfering with the NF-κB Pathway

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Scope: Extra virgin olive oil (EVOO) is rich in phenolic compounds, including hydroxytyrosol (HTy) and hydroxytyrosyl acetate (HTy-Ac), which have presented multiple beneficial properties. Their impact on inflammatory responses in human keratinocytes and modes of action have not been addressed yet.

Methods and results: Primary human keratinocytes are pretreated with HTy-Ac or HTy for 30 min and stimulated with IL-1β or Toll-like receptor 3 ligand (TLR3-l). Thymic stromal lymphopoietin (TSLP), measured by ELISA, is attenuated by both polyphenols in a dose-dependent manner. The expression of several inflammation-related genes, including distinct TSLP isoforms and IL-8, are assessed by quantitative RT-PCR and likewise inhibited by HTy-Ac/HTy. Mechanistically, EVOO phenols counteracts IκB degradation and translocation of NF-κB to the nucleus, a transcription factor of essential significance to TSLP and IL-8 transcriptional activity; this is evidenced by immunoblotting. Accordingly, NF-κB recruitment to critical binding sites in the TSLP and IL-8 promoter is impeded in the presence of HTy-Ac/HTy, as demonstrated by chromatin immunoprecipitation. Promoter reporter assays finally reveal that the neutralizing effect on NF-κB induction has functional consequences, resulting in reduced NF-κB-directed transcription.

Conclusion: EVOO phenols afford protection from inflammation in human keratinocytes by interference with the NF-κB pathway.

1. Introduction

The skin is the largest organ and represent the first line defense against external irritants and pathogens of an organism. It is composed of the dermis and epidermis, with the latter forming the outer avascular segment that consists mostly of keratinocytes (>90%). Keratinocytes act as sentinels organizing immune responses during infection by production of cytokines like IL-1, IL-6, IL-8, and tumor necrosis factor (TNF).[1–3] When aberrantly regulated or not properly counter-balanced, keratinocyte-derived mediators can contribute to auto-inflammatory processes and a spectrum of skin diseases.

Thymic stromal lymphopoietin (TSLP), an influential keratinocyte-derived mediator, is triggered by physical, chemical, or biological disturbance of skin homeostasis.[4–6] It is directly associated with inflammatory and hypersensitivity responses occurring at barrier surfaces, as well as with Th2 immunity and chronic dermatoses like atopic dermatitis (AD) and psoriasis.[7–9] TSLP is expressed in two isoforms with different

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immunomodulatory roles: a short isoform, expressed in skin mainly under homeostatic conditions and a long isoform, which is exclusively expressed under inflammatory or otherwise pathogenic circumstances.[10,11] The two isoforms are controlled by different promoter usage.[12,13]

Extra virgin olive oil (EVOO), the major source of fatty acids in the traditional Mediterranean diet, is rich in phenolic compounds, such as secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, HTy) and hydroxytyrosyl acetate (HTy-Ac), which have a wide range of anti-inflammatory, anti-oxidant, and chemopreventive function in vitro.[14–17] These beneficial properties were confirmed in vivo in animal models of autoimmunity.[18–21]

The interest in dietary supplements and natural therapies in the management of skin disorders is increasing. However, despite favorable effects in inflammatory processes, the potential of EVOO or its derivatives in mitigating skin inflammation has not been addressed so far.

Here, we report that HTy-Ac and HTy interfere with the nuclear factor "kappa-light-chain-enhancer" of activated B-cells (NF-κB) pathway by reducing inhibitor of kB (IκB) degradation, NF-κB nuclear translocation, NF-κB promoter recruitment, and NF-κB-driven transcription. In doing so, EVOO polyphenols efficiently attenuate the expression of pro-inflammatory mediators such as TSLP, thereby restraining harmful processes set off by activated keratinocytes.

2. Experimental Section

2.1. Extraction and Chemical Characterization

HTy, obtained from olive mill wastewaters,[22] was used after purification by column chromatography (ethyl acetate (EtOAc)-cyclohexane 1:5 → 1:2). The synthesis of HTy-Ac was carried out from HTy according to a published protocol.[20]

2.2. Keratinocyte Isolation and Treatments

Human keratinocytes were isolated from foreskins and propagated according to a published protocol with several modifications.[23] The skin was obtained from circumcisions, with informed consent of the patients or their legal guardians and approval by the Charité University ethics committee. The experiments were conducted according to the Declaration of Helsinki principles. Briefly, cells were pretreated with HTy or HTy-Ac (12.5–100 µM) for 30 min and then stimulated with recombinant human (rh) IL-1β (10 ng mL^{-1}) (Immunotools, Friesoythe, Germany) or Toll-like receptor 3 ligand (TR3-l) (10 µg mL^{-1}) (Poly I:C) (Invivo Gen, Toulouse, France) for different experimental time points. The detailed method can be found in the Supporting Information.

2.3. Cell Viability Assay

Cell viability was measured by the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) cell viability kit (Cayman, Chemical Company, Ann Arbor, USA). The results are expressed in relation to the percentage of untreated control cells. The detailed method can be found in the Supporting Information.

2.4. ELISA

Cells (7.5×10^3cells) were used per well and treated as described in Section 2.2. Supernatants were collected after 24 h and measured by TSLP ELISA (eBioscience, San Diego, USA). Quantification was performed as per the manufacturer's instruction and the results were expressed in relation to the (IL-1β or TLR3-l) stimulated control.

2.5. Quantitative Reverse-Transcriptase Polymerase Chain Reaction

RNA was reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany) and quantitative reverse-transcriptase PCR (RT-qPCR) was performed with the Fast Start DNA Master SYBR Green I and LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Oligonucleotide primers (TIB Molbiol, Berlin, Germany), product sizes, and temperatures are specified in Table S1, Supporting Information. Expression levels of the target gene were quantified relative to the expression of the reference gene β-actin using the $2^{-\Delta\Delta CT}$ method. The relative expression values (i.e., TSLP normalized to β-actin for each cDNA individually) were expressed in relation to the stimulated control cells (IL-1β or TR3-l).

2.6. Immunoblottting Detection

Cells (1.2×10^5 cells per well in six-well plates) were treated as described in Section 2.2 and then stimulated for 5, 30, and 60 min. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Freiburg, Germany), blocked for 1 h with 10% casein and 5% sucrose/PBS, and incubated overnight at 4 °C with the specific primary antibody at 1:1000 (rabbit anti-IκBα or rabbit anti-p65; Cell Signaling, Danvers, MA) in 10% casein and 5% sucrose/PBS. After washing the membrane with PBS-Tween, HRP coupled secondary antibody goat anti-rabbit (Dako, Glostrup, Denmark; 1:10 000) was added for 2 h at room temperature. The immunosignals were captured using Fusion FX7 Spectra (VilberLourmat, Eberhardzell, Germany). The results were densitometrically analyzed by using freeware Image J (National Institutes of Health, Bethesda, MD). The detailed method can be found in the Supporting Information.

2.7. Chromatin Immunoprecipitation (ChIP) Assay

Cells (1.2×10^5 cells per well in six-well plates) were treated as described in Section 2.2 and then stimulated for 30 min.

After incubation chromatin was prepared using Chromatrap Enzymatic Shearing Kit (Chromatrap, Wrexham, UK) following manufacturer's instructions and was checked on 1% agarose gel for appropriate size of sheared chromatin. The chromatin was further processed using Abcam high sensitivity ChIP Kit (Abcam, Cambridge, UK) according to manufacturer's instructions and the immunoprecipitation was carried out using rabbit anti-human-NF-κB p65 antibody (Active Motif, La Hulpe, Belgium) and normal rabbit IgG (Cell signaling, Beverly). After purification, DNA was analyzed using Fast Start DNA Master SYBR Green I and the Light Cycler 1.5 device (Roche Diagnostics, Mannheim, Germany). ChIP DNA was normalized according to the input DNA. The sequence of primers and PCR conditions are given in Table S2, Supporting Information. The primers for NF-κB BS present in human IL-8 promoter were purchased from Qiagen (Hilden, Germany) Epitect ChIP qPCR assay.

2.8. Luciferase Reporter Assay and Cell Transfection

Cells (1.2×10^5 cells per well) were transfected with human IL-8 promoter construct (pGL4.15-basic vector encoding human IL-8 promoter; Promega, Madison, USA) or empty vector. Transient transfection of keratinocytes was performed using TransIT Keratinocyte transfection reagent (Mirus Bio LLC, Madison, USA) according to the manufacturer's instructions. To assess the IL-8 promoter luciferase activity, 1.5 µg of IL-8 promoter construct and 0.5 µg of Renilla luciferase reporter vector pRL-TK (Promega) were co-transfected in each well for 24 h. After 24 h, cells were washed twice with PBS and were lysed using lysis buffer. The luciferase activity was measured by the dual-luciferase assay system kit according to the manufacturer's protocol (Promega, Madison, USA) using a VictorTMX3 luminometer (PerkinElmer, Waltham, USA). All values were normalized to Renilla luciferase activity and expressed relative to the control (transfected non-stimulated cells).

2.9. Statistical Analysis

Values are expressed as arithmetic mean ± SEM. The data were analyzed with GraphPad Prism Version 5.01 software (San Diego, CA). Statistical significance was assessed by one-way analysis of variance using repeated measures test and a nonparametric test with Dunn's test as post hoc test. p-Values of <0.05 were considered statistically significant.

3. Results

3.1. EVOO Polyphenols do not Compromise Keratinocyte Survival

We first assessed the impact of HTy-Ac and HTy on keratinocyte viability by WST-8 survival assay. Viability was not compromised at concentrations from 0 to 100 µM of either HTy-Ac or HTy (Figure S1, Supporting Information). However, a concentration of 500 µM resulted in decreased viability. Based on this pattern, we decided to assay the compounds at concentrations from 12.5 to 100 µM in subsequent experiments.

3.2. EVOO Polyphenols Counter TSLP Induction in Human Keratinocytes

TSLP is associated with inflammatory processes of the skin and disorders like atopic dermatitis.[4,6,24] To induce TSLP production, keratinocytes were stimulated with IL-1β and TLR3-l based on previous studies from our laboratory.[4] To determine the effect of EVOO polyphenols, keratinocytes were pretreated for 30 min with HTy-Ac or HTy and stimulated for 24 h. Stimulation with IL-1β (Figure **1**A) or TLR3-l (Figure S2, Supporting Information) stimulated TSLP production. HTy-Ac or HTy prevented induction by IL-1β (Figure 1A). These effects were comparable when TLR3 ligation was used as stimulus (Figure S2, Supporting Information). A similar trend was noted for the low baseline TSLP production, indicating that both compounds broadly decreased TSLP production (data not shown).

We explored whether the attenuation of TSLP protein was reflected at the transcript level. Indeed, while TSLP mRNA expression was remarkably induced following IL-1β or TLR3-l stimulation (Figure 1B; Figure S4, Supporting Information), HTy-Ac or HTy effectively countered this transcriptional induction (Figure 1B; Figure S4, Supporting Information). In humans, two TSLP isoforms are produced: a short isoform that mediates homeostatic functions and a long isoform weakly expressed at steady state and upregulated by tissue damage or inflammation.[10] We studied expression of the two isoforms separately. The long isoform was indeed increased to 100-fold over control by IL-1β stimulation (Figure 1C). Treatment with HTy-Ac or HTy significantly counteracted this induction (Figure 1C).

The expression of the short isoform was not significantly altered by IL-1β, and HTy-Ac and HTy did likewise not modulate the short TSLP transcript (Figure 1D). We conclude that HTy-Ac and HTy interfere with the induction of the long TSLP isoform.

3.3. EVOO Polyphenols Widely Modulate NF-κB Target Genes, But Have no Effect on Skin Structural Components

A major orchestrator of TSLP induction in inflammatory surroundings is NF-κB.[25–27] To assess whether EVOO phenols broadly interfere with NF-κB regulated genes in the skin, the expression of further candidates was quantitated by RT-qPCR. Indeed, TNF-α, IL-6, and IL-8 were induced by IL-1β or TLR3-l in keratinocytes, and HTy or HTy-Ac were found to effectively interfere with this induction (Figure **2**; Figure S4, Supporting Information).

Cyclooxygenase-2 (COX-2) is another downstream target of NF-κB and induced in skin inflammation.[28,29] Accordingly, COX-2 expression was upregulated by IL-1β and HTy-Ac as well as HTy counteracted this increase (Figure 2).

Our combined data implied that EVOO polyphenols exert their anti-inflammatory effects by interference with the NF-κB pathway in keratinocytes.

To assess whether inhibition was specific for inflammation-related genes, we extended our analysis to genes encoding

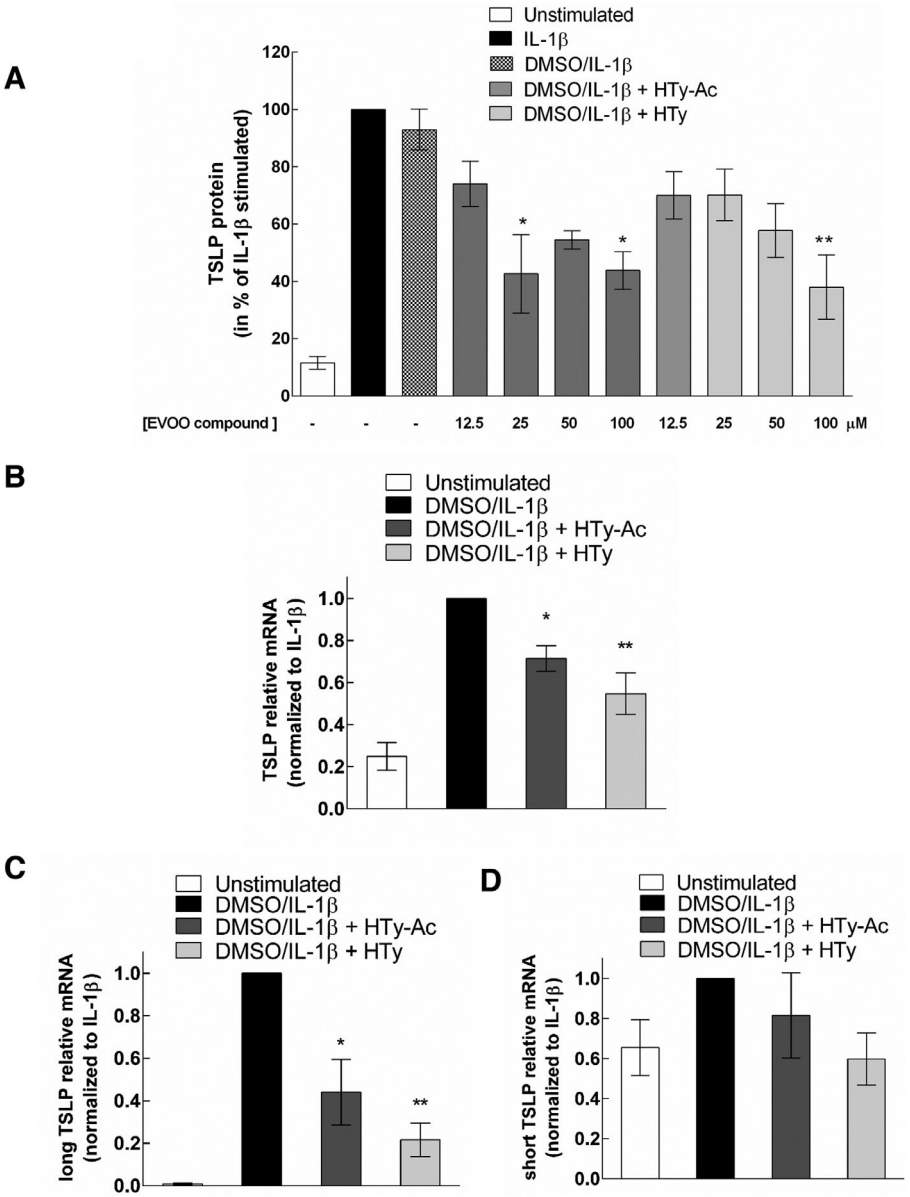

Figure 1. IL-1β induced TSLP production in human keratinocytes was inhibited by HTy and HTy-Ac. A) Levels of TLSP measured by ELISA in human keratinocytes stimulated with IL-1β (10 ng mL^{-1}) for 24 h. The data are given in percentage (%) of IL- 1β stimulated TSLP levels. Depending on the experiment, IL-1β yielded concentrations of TSLP between 40 and 270 pg mL^{-1}. B–D) Expression of TSLP (total), long and short TSLP isoforms by RT-qPCR after 4 h of stimulation with IL-1β (10 ng mL^{-1}), normalized to β –actin. Keratinocytes were pretreated with the compounds for 30 min before the addition of IL-1β. DMSO served as vehicle control. Each column represents the mean ± SEM of at least five independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ versus IL-1β stimulated cells.

structural proteins of the skin, which are not believed to require NF-κB activity. Filaggrin and Loricrin play important roles in the formation of the cornified layer of in the stratum corneum, and their decreased expression is related to inflammatory skin disease.[30] In our study, filaggrin and loricrin mRNA were not significantly impacted by IL-1β, and this was independent of the presence of HTy-Ac or HTy (Figure S5, Supporting Information). Collectively, while inflammatory mediators are potently reduced by the polyphenols, genes important to an ordered architecture of the skin remain unperturbed.

3.4. EVOO Polyphenols Restrain NF-κB Activation in Human Keratinocytes

To test our hypothesis that EVOO polyphenols suppress inflammatory mediators by impairing NF-κB activation in keratinocytes, we explored their impact on IκBα (the inhibitor of NF-κB) degradation by Western blot. Beforehand, we established that degradation of IκBα was elicited by IL-1β in our setting (Figure S3A, Supporting Information; degradation after 15 or 30 min of stimulation, followed by partial recovery at 60 min). The

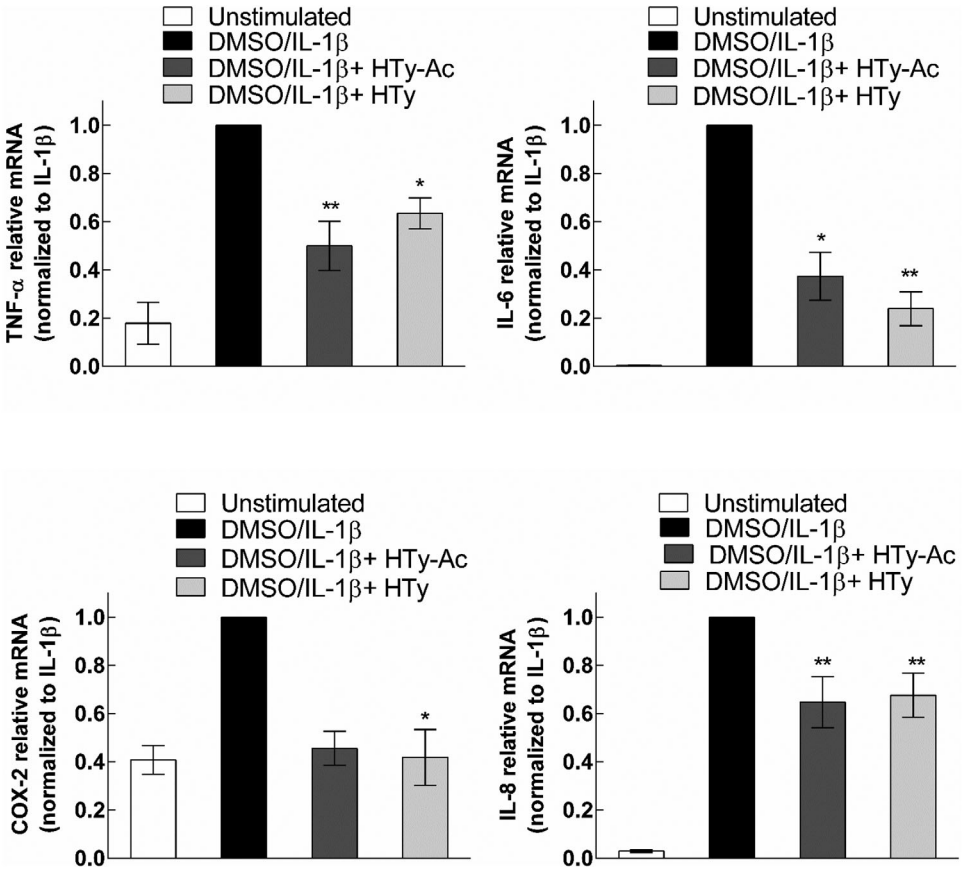

Figure 2. HTy and HTy-Ac counteracted the induction of NF-κB dependent genes. Expression of TNF-α, IL-6, COX-2, and IL-8 (normalized to β-actin) by RT-qPCR. Cells were pretreated with HTy or HTy-Ac for 30 min and then stimulated with IL-1β for 4 h. Each column represents the mean ± SEM of at least five independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ versus IL-1β stimulated cells.

disappearance of IκBα was associated with simultaneous translocation of the NF-κB member p65 to the nucleus (not shown, see also Figure 3B). In the presence of HTy and HTy-Ac, both the degradation of IκBα and nuclear translocation of NF-κB p65 were attenuated (Figure 3A,B; Figure S3B, Supporting Information). Consequently, higher levels of IκBα and lower nuclear levels of p65 were detected at both time points analyzed. We conclude that HTy and HTy-Ac counteract NF-κB activation by restraining IκBα degradation and subsequent p65 nuclear translocation.

3.5. Recruitment of NF-κB to Specific Promoters and NF-κB Transcriptional Activation are Curtailed by EVOO Polyphenols

Having established that the abundance of nuclear NF-κB was diminished by HTy or HTy-Ac treatment, we investigated the binding of this factor to the endogenous TSLP promoter by ChIP assay. As shown in Figure 4A, IL-1β de novo induced its recruitment to the essential NF-κB binding site located at –3.8 kb upstream of the transcription start site (TSS).[12,25,26] This interaction was markedly attenuated by HTy or HTy-Ac (Figure 4A), corroborating the findings for p65 nuclear translocation. To exclude nonspecific effects, an IgG isotype control was used instead of the anti-NF-κB antibody (Figure 4A). In addition, recruitment of RNA polymerase to the 5'-upstream regions of β-actin and GAPDH served as control for the quality of our ChIP technique. No binding of NF-κB to the TSLP promoter was detected in the absence of IL-1β, while Pol II binding to the regulatory regions of housekeeping receptors was unaffected by IL-1β (data not depicted). As expected, specific binding of RNA polymerase II to these promoter elements was comparable across conditions, in accordance with the housekeeping-like character of the genes (Figure 4B).

In addition to TSLP, many other cytokines and chemokines are regulated by NF-κB (compare also to Figure 2; Figure S4,). We next selected the IL-8 promoter, a chemokine predominantly regulated by NF-κB to extend the analysis to another key inflammatory mediator and well-defined NF-κB target.[31,32] As expected, we found strong recruitment of NF-κB to its binding site at –0.86 kb of the IL-8 gene. Again, pretreatment with HTy or HTy-Ac strongly inhibited this interaction (Figure 5A).

Furthermore, we used reporter assays to verify that reduced NF-κB binding had functional consequences to IL-8 promoter activity. To this end, we first optimized the transfection method for primary human keratinocytes. As shown in Figure 5B, the stimulation with IL-1β indeed resulted in ≈17-fold induction of reporter activity over baseline, validating the performance of our method. Meanwhile, the pretreatment with EVOO polyphenols

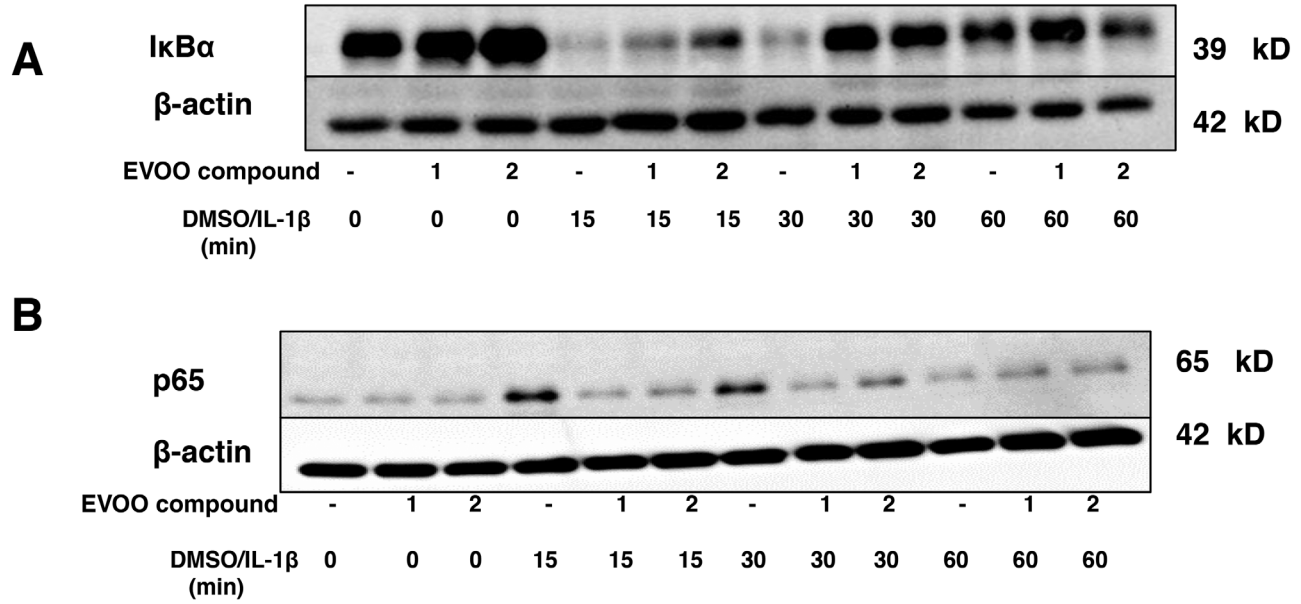

Figure 3. EVOO-compounds supressed IκBα degradation and NF-κB nuclear translocation. A) IκBα degradation. B) p65 nuclear translocation. Cells were untreated or treated with EVOO compounds (1 = HTy-Ac, 2 = HTy) for 30 min and then stimulated for 15, 30, and 60 min with IL-1β. β-actin served as loading control for normalization.

potently inhibited IL-8 promoter activation by IL-1β (Figure 5B), in accordance with our ChIP findings. Therefore, we conclude that the EVOO phenolic compounds HTy and HTy-Ac inhibit NF-κB mediated transcription of inflammation-associated genes.

4. Discussion

We demonstrate herein that two phenolic compounds derived from EVOO, namely HTy and HTy-Ac, attenuate inflammatory responses of human keratinocytes and suppress key epidermal cytokines, including TSLP. Favorable properties have been ascribed to EVOO in different contexts, and our study extends them to the environment of human skin.

Since benefits from natural compounds can potentially be achieved without the harmful or unwanted side effects of classical pharmacological drugs, this appreciation has encouraged shifts in biopharmaceutical research away from synthetic molecules to natural products. In fact, natural products have already revolutionized the management of several diseases in medical practice.[33,34] Polyphenols, compounds with at least one aromatic ring with a hydroxyl group attached to it, are active constituents among plant-derived natural products and exhibit important in vitro and in vivo properties, including anti-aging, antioxidant, anti-inflammatory, anti-diabetes, anti-hypercholesterolemia, and anticancer.[35–40]

EVOO polyphenols fit this picture, presenting qualities of medical interest including anti-inflammatory and immunemodulatory activities, only partially related to their antioxidant power, which could be useful in the management of skin diseases.[41–43] In this regard, a polyphenol-based cosmeceutical containing olive polyphenol extract, exhibited positive skin effects.[44] Along the same lines, an olive oil-based diet (10% of total calories) improved cutaneous wound healing of pressure injury in mice.[45] Besides, ex vivo topical administration of olive oil (0.01 mg mL^{-1}) was capable of attenuating the reduction in epidermis and dermis thickness and collagen fiber content induced by chronic stress in human skin.[46] The above reports suggest that olive oil polyphenols are capable of tipping the scales in the skin environment in vivo toward the maintenance of homeostasis and anti-inflammation.

HTy is the best studied EVOO-derived polyphenol and a good candidate for the development of drugs due to its prophylactic and preventive effects in cells and animal models of cancer, lupus, and ischemia-reperfusion among others.[20,47–50] Even though this is the first study to explore the impact of EVOO polyphenols on inflammation-associated pathways in primary keratinocytes of human origin, HTy was previously described to exhibit chemoprotective and antiaging effects against ultraviolet B (UVB) and blue-light-induced damage in human skin cells.[51,52] In a mouse model of AD, HTy had beneficial effects, reducing erythema intensity and dermatitis and decreasing T-helper cell cytokines in serum and skin, albeit only in the presence of hydrocortisone.[53,54] Recently, a study evaluated the safety of a cream with hydrocortisone and HTy, showing that long-term use had a favorable safety profile, a finding encouraging further clinical trials.[55]

HTy-Ac, found in EVOO at a proportion similar to HTy (depending on the variety of olive), has been less studied, but recently aroused interest among natural products. In this regard, HTy-Ac moderated the inflammatory response in LPS-treated macrophages by downregulating IκBα degradation and COX-2 expression.[56] It showed beneficial effects in models of immune-inflammatory disease, including systemic lupus erythematosus and acute colitis.[20,48] Interestingly, HTy-Ac reduced inflammatory markers in a model of murine arthritis, in which HTy had no effect, suggesting that (at least subtle) differences exist between HTy and HTy-Ac as for their mode of operation.[21] However, the

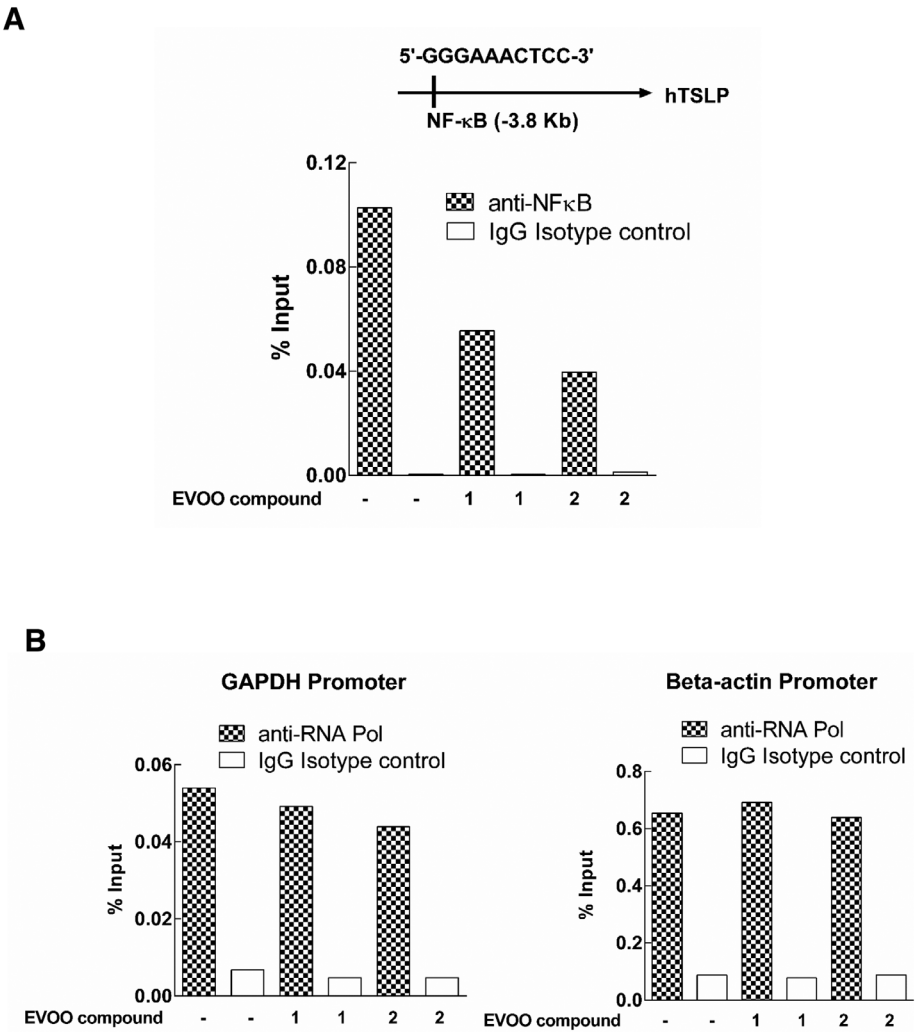

Figure 4. HTy and HTy-Ac prevented the recruitment of NF-κB to the TSLP promoter. A) IL-1β mediated recruitment of NF-κB at binding site located at –3.8 kb of TSLP promoter. B) β-actin and GAPDH promoter regions served to verify that the binding intensity of RNA polymerase II to housekeeping genes remained comparable across treatments. One representative experiment of three is shown. Cells were left untreated or treated with EVOO compounds (1 = HTy-Ac, 2 = HTy) for 30 min and then stimulated for 30 min with IL-1β.

effects specifically of HTy-Ac in the inflammatory process of the skin remained unexplored.

In our study, the two polyphenols prevented inflammatory responses in primary human keratinocytes with similar efficacy. Both substances countered IL-1β and TLR3-l induced generation of TSLP protein, and this was mirrored at the level of its transcript, where total TSLP mRNA and especially its long, inflammation-associated isoform were diminished. In addition, HTy and HTy-Ac dampened expression of diverse genes active in an inflammatory environment (IL-8, TNF-α, IL-6, and COX-2).

We succeeded in linking these effects to interference with NF-κB activation. NF-κB is a central orchestrator of inflammation, owing to its upstream and prominent position in the cascade controlling expression of a myriad of pro-inflammatory genes. Accordingly, NF-κB hyper-activation is associated with chronic diseases such as atherosclerosis, rheumatoid arthritis, and atopic dermatitis and it is therefore believed to be a primary target of anti-inflammatory therapeutics, including nutrients.[57]

In our study, we showed that HTy is able to modulate the NF-κB pathway, increasing the levels of nuclear p65 and decreasing the cytosolic IκB-α levels. IL-1 triggers a variety of concomitant and sequential signaling events that encompass MyD88, several IRAK members, TRAF6, MAP3K7, which then recruit and activate IKK, p38, and JNK, respectively, resulting among other in IκB degradation and p65 nuclear translocation.[58] The single events are interconnected by loops and feedback mechanisms, causing a high degree of complexity in the IL-1 signaling network. It will be of interest to delineate whether and which of the above signaling intermediates are directly bound by the polyphenols and thereby functionally modulated or attenuated.[58]

Another, not mutually exclusive possibility is through (a) different signaling cascades, that feed(s) into the NF-κB pathway. Interesting candidates in this regard are 5′ AMP-activated protein kinase (AMPK) and Sirtuin-1 (SIRT1), because previous studies have demonstrated HTy to be a potent AMPK-SIRT1 activator in different in vitro models.[59–63]

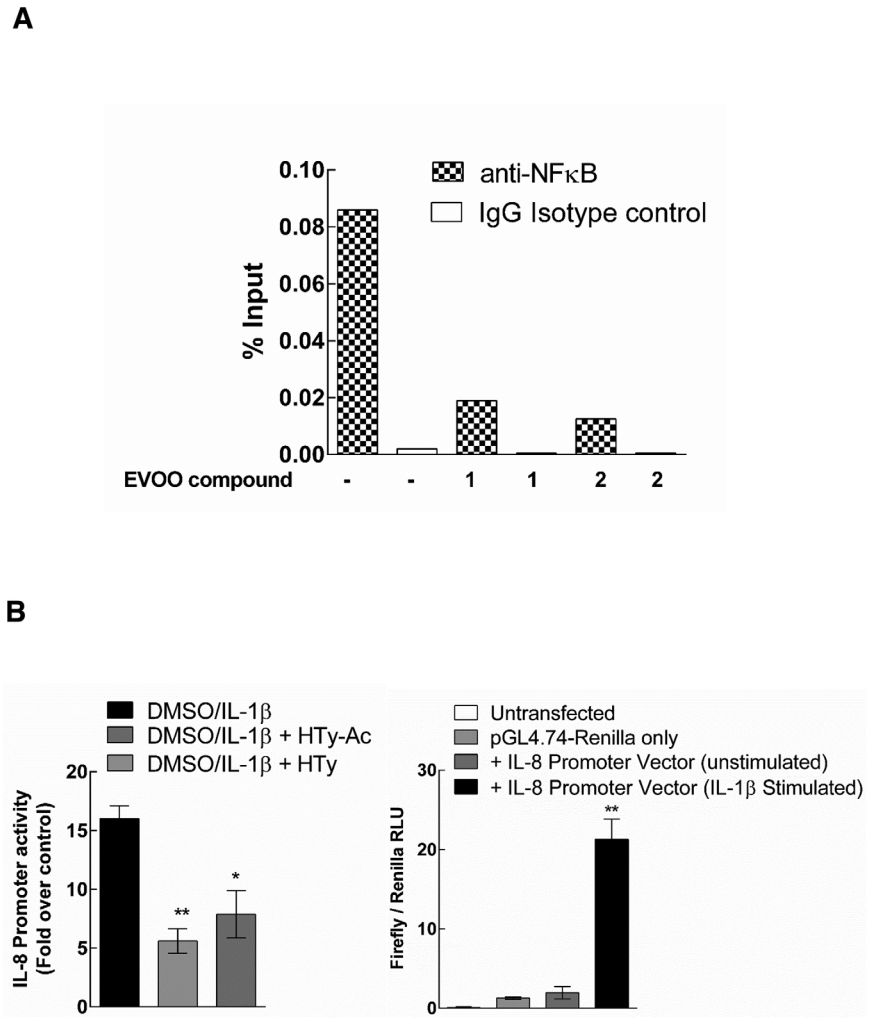

Figure 5. HTy and HTy-Ac interfered with IL-8 promoter activity. A) NF-κB recruitment to the NF-κB BS (at –0.86 kb) of the IL-8 promoter after IL-1β stimulation. pGL4.15-hIL-8 promoter (firefly)/pGL4.74 (renilla) and then normalized to control. One representative experiment of three is shown. B) Induction of IL-8 promoter activity by IL-1β. Each value represents the mean ± SEM of at least five independent experiments. Cells were treated with EVOO compounds (1 = HTy-Ac, 2 = HTy) (or vehicle control) for 30 min and then stimulated for 24 h with IL-1β. $^{*}p < 0.05$; $^{**}p < 0.01$ versus IL-1β stimulated cells.

Indeed, it has been reported that SIRT1 can be an inhibitor of NF-κB mediated transcription[64] and AMPK enhances SIRT1 activity,[65] and is likewise a modulator of the NF-κB pathway on its own.[65–67] However, there is so far only some first evidence of this connection in keratinocytes. For example, Barroso et al[65] reported that TNF-α mediated activation of the NF-κB pathway can lead to TSLP mRNA expression in the HaCaT keratinocyte cell line but that a peroxisome proliferator-activated receptor (PPAR-β/δ) agonist (GW501516) inhibited this process by activation of PPARβ/δ, AMPK, and SIRT1 pathways, respectively. While it is not possible to extrapolate the described effects to our study due to the different cells and models investigated by the previous reports, it will be appealing to delineate the precise targets of EVOO within the IL-1 and/or the AMPK cascades. EVOO phenolic compounds might in fact act by a number of different effects in keratinocytes, as it is well appreciated that different pathways orchestrate the regulation of NF-κB in a cell/stimulus specific manner.

We need to emphasize, however, that EVOO polyphenols have the power to potently interfere with NF-κB in keratinocytes. We were also able to kinetically resolve the mechanism of operation. Both EVOO polyphenols acted by blocking IκBα degradation and thereby prevented p65 nuclear translocation, leading to reduced presence of NF-κB in the nucleus. The TSLP promoter contains several NF-κB binding sites (BS), of which the BS located at –3.8 kb is biologically active and responsive to stimulation by IL-1.[12,25] While stimulation with IL-1β recruited NF-κB to this site also in our study, both HTy and HTy-Ac were able to efficiently inhibit this recruitment, demonstrating that reduced nuclear presence of NF-κB had an effect at this promoter. To verify whether this applies to other genes, as well, we selected the IL-8 promoter.

In agreement with previous studies, we found robust recruitment of NF-κB to the IL-8 promoter upon IL-1β stimulation.[31,68,69] This recruitment was potently blocked upon pretreatment with both HTy and HTy-Ac. Interference at the IL-8 promoter was even more pronounced than for TSLP,

emphasizing that the consequences can differ across NF-κB binding sites even when NF-κB reduction in the nucleus is equal. We were finally interested to delineate whether the reduced binding of NF-κB to the IL-8 promoter had functional consequences to the transcriptional activity of this promoter. We found that this was indeed the case. Using reporter constructs in primary keratinocytes, we verified that IL-1-induced IL-8 promoter activity was potently inhibited by HTy or HTy-Ac, complementing the attenuated binding of NF-κB to the IL-8 promoter, as revealed by ChIP.

By demonstrating that EVOO phenols exert anti-inflammatory effects on human keratinocytes, our study not only extends these previous findings to also encompass HTy-Ac, but it shows that human keratinocytes are direct targets of EVOO phenols, which appears translationally interesting in the context of inflammatory skin disorders, as also encouraged by the mentioned studies investigating the potential use of olive oil-derived compounds in the field of dermatology.[35–46,51–55]

Taken together, we present evidence that EVOO polyphenols counter inflammation initiated by human keratinocytes through the attenuation of key inflammatory mediators commonly deregulated in the inflamed skin, including TSLP, and may provide new avenues for the prophylaxis or treatment of inflammatory dermatoses.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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M.A.-S. and D.R. contributed equally to this study. M.W. and M.B. are shared senior authors. M.A.S. and D.R. performed keratinocyte cell cultures. M.A.S. performed cytokine measurements, RT-qPCR, and Western blot experiments. D.R. performed ChIP and luciferase assays. M.A.S., D.R., and M.B. wrote the main manuscript. J.F.B. provided the EVOO polyphenols. M.W., M.B., M.S.H., and C.A. designed and supervised the project and revised the paper. All authors discussed the results and implications and commented the manuscript at all stages. This study was supported by a grant from the Deutsche Forschungsgemeinschaft to M.W. (WO-541/13-1) and by a grant from the Spanish Ministerio de Economía y Competitividad to C.A. (AGL 2011–26949). M.A.S. gratefully acknowledges support from the Postgraduate National Program of FPU fellowship and financial sponsorship from the Spanish Ministerio de Educación, Cultura y Deporte and the "V Own Research Plan" from the University of Seville (Spain). D.R. was supported by a fellowship from Charité-Universitätsmedizin Berlin, Germany. The authors acknowledge support from the Charité-Universitätsmedizin Berlin publication fund for coverage of the open access publishing expenses of this publication as per the agreement between Charité-Universitätsmedizin Berlin and Wiley.

Conflict of Interest

The authors declare no conflict of interest.

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