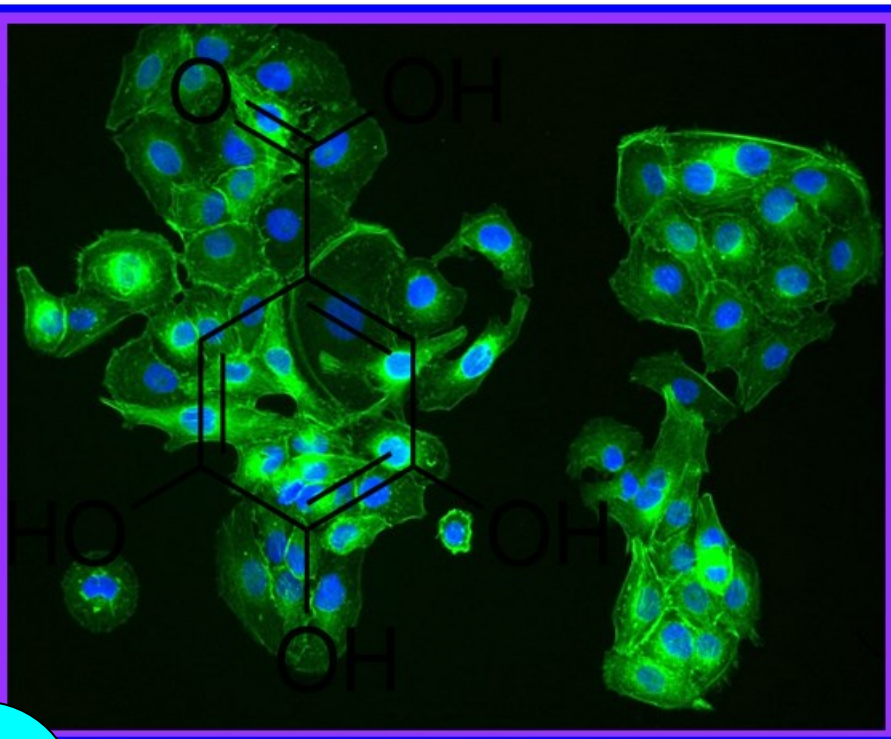

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Anti-inflammatory effect of gallic acid on HaCaT keratinocytes through the inhibition of MAPK-, NF-kB-, and Akt-dependent signaling pathways

Polly Ho-Ting Shiu[1], Chengwen Zheng[1], Panthakarn Rangsinth[1], Wen Wang[1], Jingjing Li[2], Renkai Li[1] and George Pak-Heng Leung[1]

[1]Department of Pharmacology and Pharmacy, The University of Hong Kong, Hong Kong SAR, China; [2]Department of Rehabilitation Sciences, Faculty of Health and Social Sciences, Hong Kong Polytechnic University, Hong Kong SAR, China.

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Abstract

The objectives of the present study were to evaluate the antioxidant and anti-inflammatory effects of gallic acid, a naturally occurring triphenolic compound, on HaCaT keratinocytes and study its mechanisms of action. The results showed that gallic acid at concentrations lower than 30 μM was non-toxic to HaCaT cells, reduced the intracellular level of reactive oxygen species, and suppressed the release of chemokines interleukin-8 and monocyte chemoattractant protein-1 in tumor necrosis factor-α- and interferon-γ-stimulated HaCaT keratinocytes. In addition, gallic acid reduced the total protein expression of NF-κB and inhibited the phosphorylation of ERK1/2, p38 MAPK, and Akt in stimulated HaCaT keratinocytes. In conclusion, our study revealed that gallic acid exhibited antioxidant and anti-inflammatory effects on keratinocytes, probably through the inhibition of MAPK-, NF-κB-, and Akt-dependent signaling pathways.

Introduction

Keratinocytes are one of the major cell types in the epidermis. In addition to their structural roles in preventing pathogens from getting into the body, keratinocytes play important immune functions by secreting cytokines and chemokines upon stimulation by allergens and microbiological agents, resulting in an inflammatory response.

Gallic acid is a naturally occurring tri-phenolic compound found in various plants, with a particularly high abundance in gallnuts, witch hazel, oak bark, tea leaves, areca nut, bearberry, and blackberry. Gallic acid exhibits antioxidant and anti-inflammatory properties (Kahkeshani et al., 2019). Because of these properties, its potential application in treating atopic dermatitis, a widely prevalent and chronically relapsing inflammatory skin disease, has been studied. Gallic acid has been reported to suppress the expression of intercellular adhesion molecule-1 and the release of chemokines and cytokines in KU812 basophilic cells (Liu et al., 2013). In addition, gallic acid can inhibit the release of the cytokine interleukin (IL)-12 and the expression of functional surface markers in monocyte-derived dendritic cells (Chan et al., 2015). Gallic acid also suppressed the release of cytokines and chemokines in an eosinophil-dermal fibroblast co-culture (Tsang et al., 2016). Furthermore, in a 4-dinitrochlorobenzene-induced atopic dermatitis-like mouse model, gallic acid alleviated skin inflammation through the immunomodulation of Th17 cells (Hu and Zhou, 2021).

Although an anti-inflammatory effect of gallic acid on dermatitis has been proposed, this was previously studied in white blood cells, such as basophils, eosino-

phils, and monocytes. To our knowledge, the effect of gallic acid on keratinocytes, which comprise 95% of the dermis and are involved in the inflammatory response of the skin, has never been investigated. Therefore, this study aimed to evaluate the antioxidant and anti-inflammatory effects of gallic acid on tumor necrosis factor (TNF)-α/interferon (IFN)γ-stimulated HaCaT keratinocytes. The mechanism of action underlying these effects was also explored.

Materials and Methods

Chemicals and reagents

RPMI 1640 medium, fetal bovine serum, penicillin-streptomycin, 0.25% trypsin, human TNF-α recombinant protein, and CM-H2DCFDA were purchased from Invitrogen (USA). RIPA lysis and extraction buffer, Halt™ protease inhibitor cocktail, and bicinchoninic acid protein assay kit were purchased from Thermo Scientific (USA). Gallic acid, thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide, and phenylmethylsulfonyl fluoride were purchased from Sigma-Aldrich (USA). The human CXCL8/IL-8 DuoSet, human CCL2/MCP-1 DuoSet, and human IL-1 beta/IL-1 F2 Duoset Enzyme-Linked Immunosorbent Assay (ELISA) kits and human IFN-γ recombinant protein were purchased from R&D Systems (USA). All antibodies used for western blotting were purchased from Cell Signaling Technology (USA). ECL select western blotting detection reagents were purchased from GE Healthcare Life Sciences (USA).

Cell culture and drug treatment

HaCaT keratinocytes (Thermo Fisher Scientific) were cultured in RPMI medium supplemented with 8% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin and cultured at 37°C in an incubator with a humidified atmosphere of 5% CO_2.

HaCaT keratinocytes were treated without (control) and with different concentrations of gallic acid in the presence of 10 ng/mL recombinant human TNF-α and 10 ng/mL IFN-γ for 24 hours. Thereafter, cell viability assay, reactive oxygen species (ROS) measurement, cytokine and chemokine release, and western blotting analysis were carried out as described below.

Cell viability assay

To study the toxicity of gallic acid in HaCaT keratinocytes, cell viability was assessed using the MTT assay (Bahuguna et al., 2017 for video). The cells were incubated in 0.4 mg/mL MTT for 4 hours at 37°C. Dimethyl sulfoxide was added to lyse the cells and dissolve the formazan crystals formed in the cells. The absorbance was measured at 560 nm using a microplate reader.

ROS measurement

The cells were washed with phosphate buffer solution and stained with 2 μM CM-H2DCFDA for 15 min. Unbound dye was removed by washing with PBS. The cells were then detached from the culture plates using 0.25% trypsin. The green fluorescent signal, which is an indicator of ROS, was detected and quantified using a flow cytometer.

ELISA

The cell suspension was centrifuged at 5,000 x *g* at 4°C for 10 min to remove cell debris. IL-1β, IL-8, and monocyte chemoattractant protein (MCP)-1 in the supernatants were detected using the human IL-1 beta/IL-1 F2 Duoset ELISA, human CXCL8/IL-8 DuoSet ELISA, and human CCL2/MCP-1 DuoSet ELISA kits, according to the manufacturer's instructions.

Western blotting analysis

The total protein from HaCaT keratinocytes was extracted using RIPA lysis buffer containing 1% phenylmethylsulfonyl fluoride and 1% Halt™ protease inhibitor cocktail. The cell lysate was centrifuged at 13,000 rpm for 10 min at 4°C to remove the unbroken cell debris and nuclei. The supernatant was collected, and its protein concentration was measured using the BCA assay according to the manufacturer's instructions. Protein samples (30 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and then electrotransferred onto phenylmethylsulfonyl fluoride membranes. The membranes were blocked with 5% non-fat milk for 1 hour at room temperature and then incubated with primary antibody (1:1000 dilution in 5% bovine serum albumin in tris-buffered saline) at 4°C overnight. After washing thrice with tris-buffered saline containing 0.1% tween-20 (TBST), the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:2500 dilution in 5% bovine serum albumin in tris-buffered saline) for 1 hour at room temperature. The membrane was washed with TBST, and the protein bands were visualized using ECL select western blotting detection reagents with a ChemiDoc XRS molecular imager (Bio-Rad Laboratories, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression was detected using a monoclonal mouse anti-actin antibody. The intensity of the different bands was normalized to that of the GAPDH bands for semi-quantitative analysis (Lim et al., 2021 for video).

Statistical analysis

All data are expressed as the mean ± SD of at least three independent experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was performed using GraphPad Prism 9 software (GraphPad Software Inc., USA) to determine the statistical significance within groups. Statistical significance was set at $p<0.05$.

Results

Effect of gallic acid on the viability of HaCaT keratinocytes

The cytotoxic effects of gallic acid were studied by incubating HaCaT keratinocytes with different concentrations of gallic acid for 24 hours. The results of the MTT assay showed that the viability of HaCaT keratinocytes was reduced by 18% at 100 µM but was not affected by lower concentrations (Figure 1). Therefore, gallic acid at concentrations below 30 µM was used in subsequent experiments.

Effect of gallic acid on intracellular level of ROS in HaCaT keratinocytes

The intracellular ROS levels in HaCaT keratinocytes were detected using the fluorescent probe CM-H2DCFDA. The ROS levels in HaCaT keratinocytes were elevated by 209% upon stimulation with TNF-α/IFN-γ. The TNF-α/IFN-γ-induced ROS levels were reduced by 17% and 9% by 30 µM and 10 µM gallic acid, respectively (Figure 1).

Effects of gallic acid on the release of cytokines and chemokines in HaCaT keratinocytes

The effects of gallic acid on the pro-inflammatory cytokine IL-1β and the chemokines IL-8 and MCP-1 were evaluated. TNF-α/IFN-γ increased the levels of IL-1β, IL-8, and MCP-1 released by HaCaT keratinocytes 39.2-, 11.3-, and 85.7-fold, respectively (Figure 2). Gallic acid at 30 µM inhibited TNF–α/IFN-γ-induced IL-8 and MCP-1 release by 29% and 46%, respectively, but did not affect IL-1β release. Lower concentrations of gallic acid did not affect Il-1β, IL-8, or MCP-1 release.

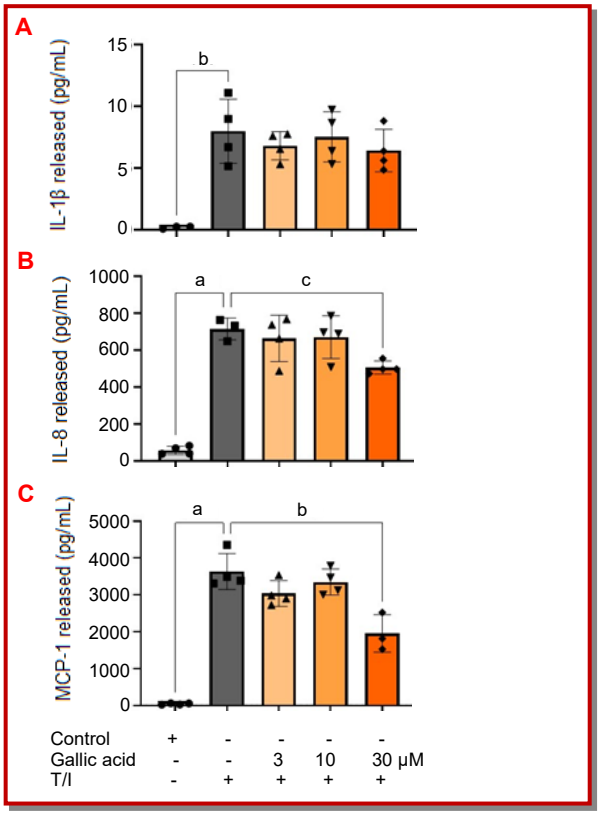

Figure 2: Effects of gallic acid on cytokine and chemokine release in HaCaT keratinocytes. HaCaT cells were treated without (control) and with different concentrations of gallic acid in the presence of 10 ng/mL recombinant human TNF-α and 10 ng/mL IFN-γ (T/I) for 24 hours. IL-1β, IL-8, and MCP-1 in the culture media were detected using ELISA. Data are presented as a percentage of control group values (mean ± SD of three to six independent experiments). [a]p<0.0001, [b]p<0.001, and [c]p<0.05 indicate a significant difference

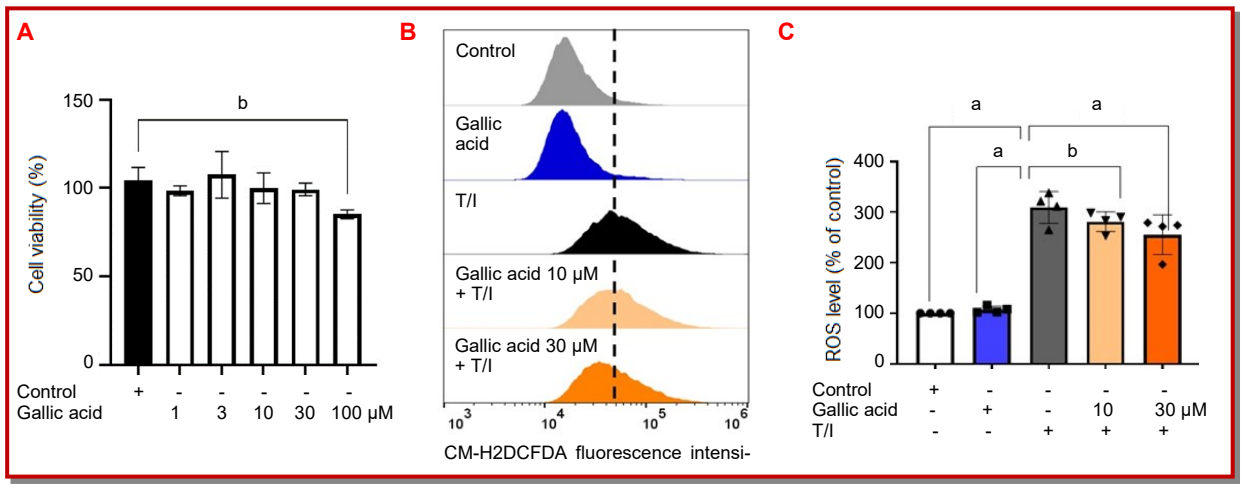

Figure 1: Effect of gallic acid on the viability of HaCaT keratinocytes (A) and ROS generation (B,C). HaCaT cells were treated with various concentrations of gallic acid or vehicle (control) for 24 hours. Cell viability was determined using the MTT assay. ROS generation in HaCaT cells was detected by CM-H2DCFDA staining and quantified via flow cytometry. HaCaT cells were treated without (control) and with different concentrations of gallic acid in the presence of 10 ng/mL recombinant human TNF-*a* and 10 ng/mL IFN-γ (T/I) for 24 hours. Data are presented as a percentage of control group values (mean ± SD of three independent experiments). [a]p<0.0001 and [b]p<0.05 indicate a significant difference

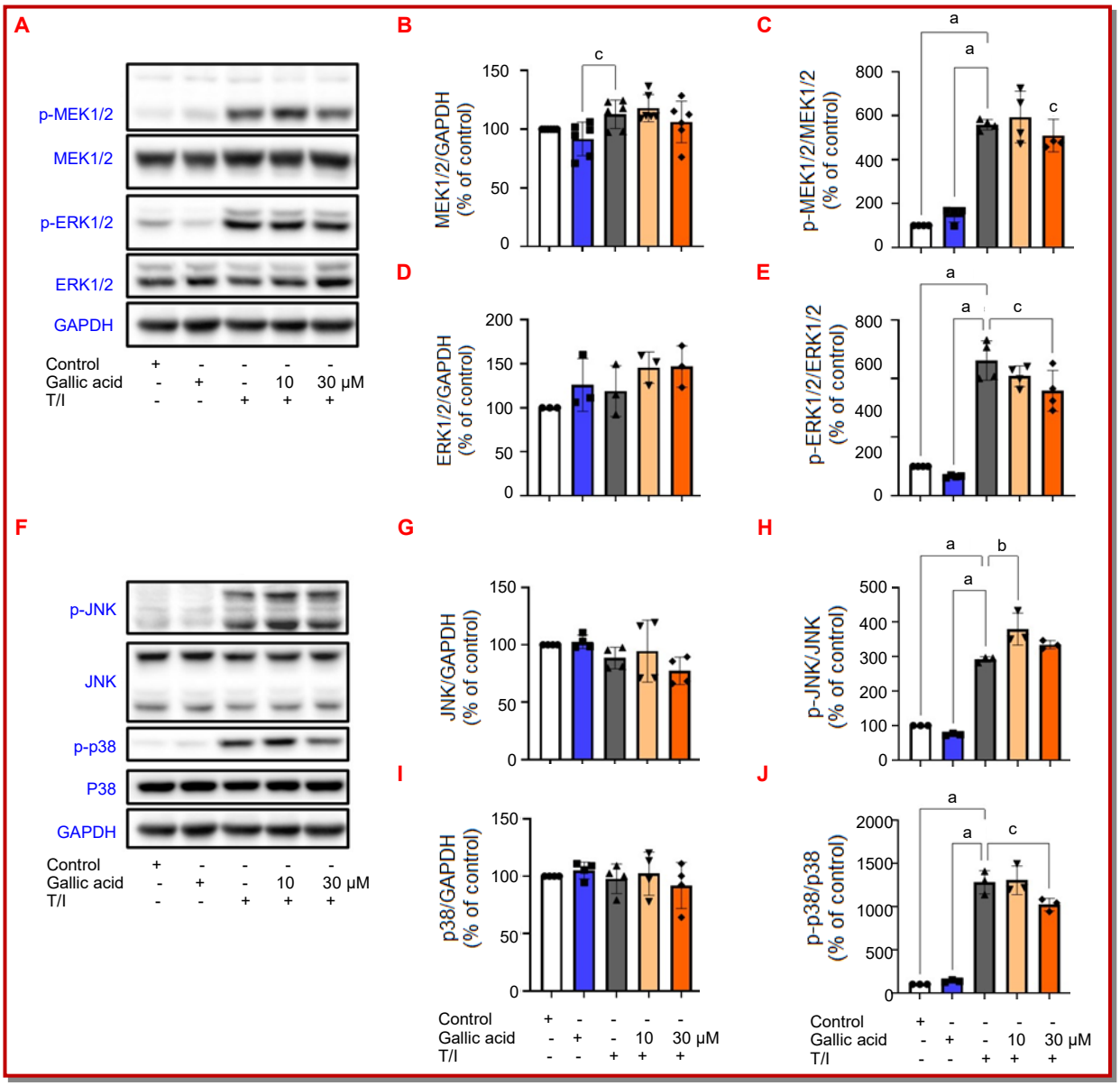

Figure 3: Effects of gallic acid on total and phosphorylated protein levels of MEK1/2, ERK1/2, JNK and p38 MAPK in HaCaT keratinocytes. HaCaT cells were treated without (control) and with different concentrations of gallic acid in the presence of 10 ng/mL recombinant human TNF-a and 10 ng/mL IFN-γ (T/I) for 24 hours. Protein expression levels of MEK1/2, p-MEK1/2, ERK1/2, p-ERK1/2, JNK, p-JNK, p38 MAPK, and p-p38 MAPK in HaCaT cells were determined using western blotting analysis. GAPDH was used as a reference and the amounts of different proteins were normalized to that of GAPDH. Data are presented as a percentage of control group values (mean ± SD of three to six independent experiments). $^{a}p<0.0001$, $^{b}p<0.01$, and $^{c}p<0.05$ indicate a significant difference

Effects of gallic acid on inflammation-related signaling pathways in HaCaT keratinocytes

Western blot analysis was performed to study the effects of gallic acid on inflammation-related signaling pathways, including mitogen-activated protein kinase (MAPK), NF-κB, and Akt-dependent pathways. TNF-α/IFN-γ did not affect the expression levels of total MAPK kinase (MEK)1/2 and extracellular signal-regulated kinase (ERK)1/2 but could increase their phosphorylation levels by 4.6- and 3.6-fold, respectively. Gallic acid (30 μM) did not affect the total and phosphorylated protein expression of MEK1/2 and ERK1/2 or the TNF-α/IFN-γ-induced phosphorylation of MEK1/2, but it reduced the TNF-α/IFN-γ-induced phosphorylation levels of ERK1/2 by 22% (Figure 3).

TNF-α/IFN-γ also did not affect the expression levels of total stress-responsive c-Jun N-terminal kinase (JNK) and p38 MAPK but increased their phosphorylation levels by 1.9- and 11.8-fold, respectively. Gallic acid (30 μM) did not affect the total and phosphorylated JNK

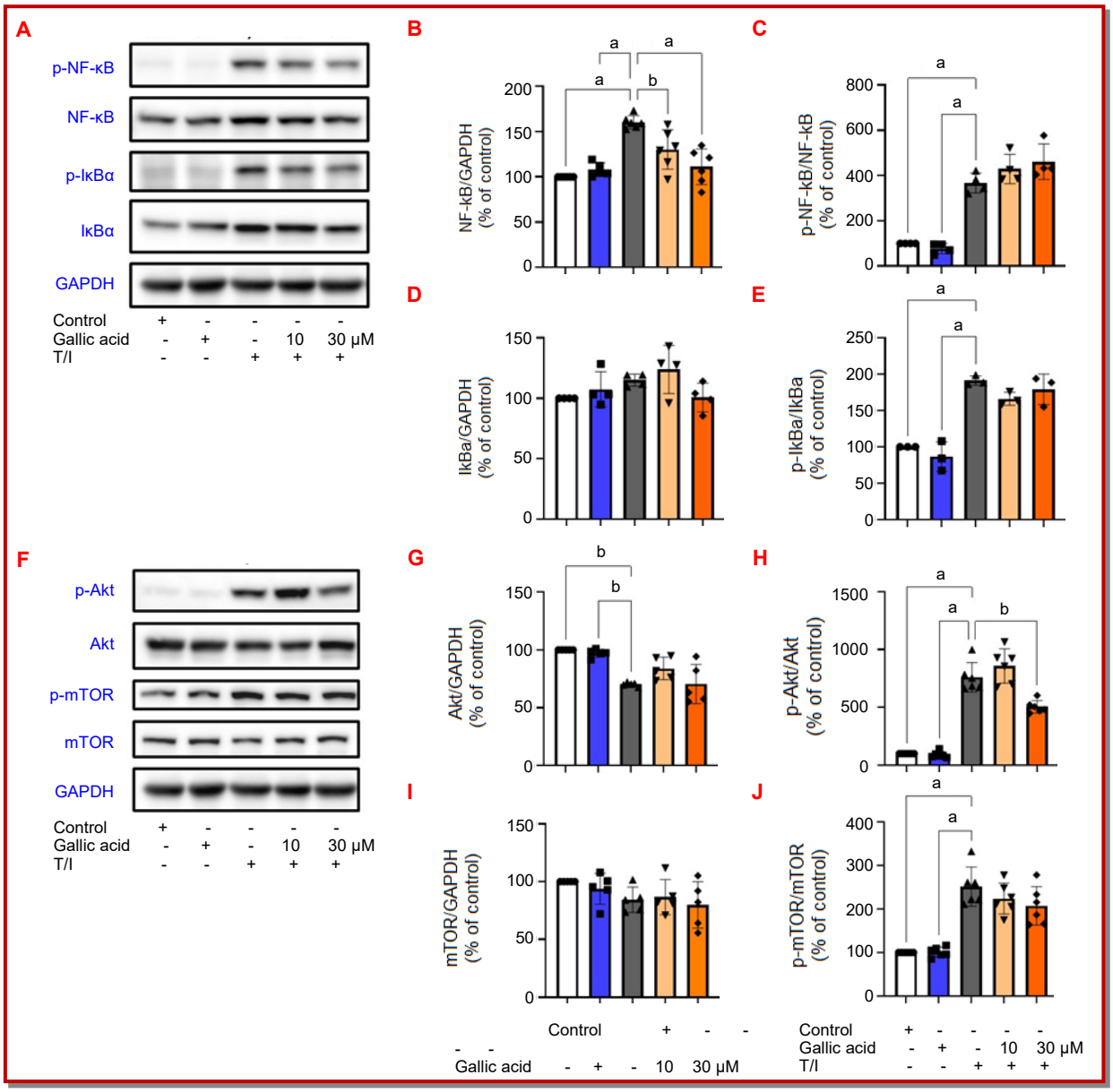

Figure 4: Effects of gallic acid on total and phosphorylated protein levels of IkBa, NF-kB, Akt and mTOR in HaCaT keratinocytes. HaCaT cells were treated without (control) and with different concentrations of gallic acid in the presence of 10 ng/mL recombinant human TNF-α and 10 ng/mL IFN-γ (T/I) for 24 hours. Protein expression levels of IκBa, p-IκBa, NFκB, p-NF-κB, Akt, p-Akt, mTOR, and p-mTOR in HaCaT cells were determined using western blotting analysis. GAPDH was used as a reference and the amounts of different proteins were normalized to that of GAPDH. Data are presented as a percentage of control group values (mean ± SD of three to six independent experiments). [a]p<0.0001 and [b]p<0.01 indicate a significant difference

and p38 MAPK protein levels. At a concentration of 30 μM, gallic acid reduced the TNF-α/IFN-γ-induced phosphorylation levels of p38 MAPK by 20%, but it did not effect the phosphorylation of JNK (Figure 3).

TNF-α/IFN-γ increased the expression level of total nuclear factor-kappa B (NF-κB) by 60% but did not affect the expression level of total nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBa). TNF-α/IFN-γ increased the phosphorylation levels of NF-κB and IκBa by 267% and 91%, respectively. Gallic acid (30 μM) alone did not affect the total and phosphorylated protein levels of NF-κB and IκBa and abolished the TNF-α-/IFN-γ-induced expression of total NF-κB. However, 30 μM gallic acid did not affect the phosphorylation of IκBa or NF-κB (Figure 4).

Lastly, TNF-α/IFN-γ did not affect the expression levels of protein kinase B (Akt) and the mammalian target of rapamycin (mTOR) but increased their phosphorylation levels by 6- and 1.5-fold, respectively.

Gallic acid (30 μM) alone did not effect total and phosphorylated protein levels of Akt and mTOR. Gallic acid (30 μM) reduced the TNF-α/IFN-γ-induced phosphorylation levels of Akt by 34% but did not effect the phosphorylation of mTOR (Figure 4).

Discussion

Gallic acid has been reported to suppress inflammatory responses in immune cells, such as monocytes, basophils, and eosinophils (Liu et al., 2013; Chan et al., 2015: Tsang et al., 2016). This study was designed to examine whether gallic acid exerts anti-inflammatory effects on keratinocytes. Primary culture of human keratinocytes is sometimes used to study the immunological and inflammatory responses of the skin. However, donor difference, instability between passages, and short culture lifetimes may affect data interpretation. Therefore, HaCaT cells were used in the present study. They are immortalized human keratinocyte lines that can differentiate and are similar to normal keratinocytes in terms of the release of inflammatory mediators in response to TNF-α and INF-γ (Cho et al., 2003; Han et al., 2021). They have been widely used as models to investigate anti-inflammatory pharmacology in skin diseases, including atopic dermatitis.

First, the cytotoxicity of gallic acid was examined. The results of the MTT assay showed that 30 μM gallic acid did not affect the viability of HaCaT keratinocytes; however, 100 μM gallic acid was slightly cytotoxic. These values were consistent with those of previous studies in which gallic acid was reported as safe and effective for most cells at lower concentrations and toxic at higher concentrations. For instance, 200 μM gallic acid had no effect on the viability of HepG2 cells (Su et al., 2013), while higher concentration was slightly cytotoxic to B16F10 and RAW 264.7 cells (Cheng et al., Tanaka et al., 2020). Gallic acid concentrations higher than 100 μM were cytotoxic to BV-2 and Nerve-2A cells; however, concentration less than 50 μM was safe to the cells (Kim et al., 2011). Gallic acid also produced significant cytotoxicity in Caco2, L929, and U937 cells at concentrations above 200 μM (Truzzi et al., 2020).

Oxidative stress is a major factor that contributes to inflammatory responses. ROS can activate NF-κB, which in turn regulates the gene expressions of TNF-α, IL-1, IL-6, IL-8, and inducible nitric oxide synthase (Allen and Tresini, 2000; Baeuerle and Henkel, 1994). Moreover, oxidative stress activates protein kinase C (Ricciarelli et al., 1998), which can increase the expression of TNF-α (Wang and Smart, 1999) and the produc -tion of cytokines (Chabot-Fletcher et al., 1994) in keratinocytes. Therefore, antioxidant compounds may attenuate skin inflammation (Portugal et al., 2007; Sardana and Sachdeva, 2022). The antioxidant effect of gallic acid is one of the most widely reported bioactivities (Cho et al., 2003; Serrano et al., 1998; Yoshino et al., 2002). The present study showed that gallic acid consistently reduced ROS production in TNF-α/IFN-γ-treated HaCaT keratinocytes. Gallic acid possesses comparable or even higher antioxidant activity than trolox, ascorbic acid, caffeic acid, sinapic acid, and vitamin E in scavenging DPPH (Badhani et al., 2015; Karamac et al., 2005; Nenadis et al., 2007; Sánchez-Moreno et al., 1998). The para-substituted OH group of gallic acid scavenges radicals through intramolecular hydrogen bonding. The efficient hydrogen donation ability of gallic acid is also due to the presence of an easily ionizable carboxylic group (Al Zahrani et al., 2020).

Keratinocytes play a critical role in the initiation and maintenance phases of skin inflammation. Keratinocytes respond to multiple triggers and release cytokines to stimulate the activation and maturation of myeloid dendritic cells. Once synergistically activated by pro-inflammatory cytokines, keratinocytes can produce chemokines to recruit leukocytes and other inflammatory mediators to amplify inflammation (Zhou et al., 2022). Gallic acid can eliminate and regulate inflammatory molecules (Tsang et al., 2016; Chao et al., 2020; Fan et al., 2019). It has been reported that gallic acid can suppress the levels of cytokines IL-1 and IL-6, chemokines MPC-1 and CCL-7, cyclooxygenase-2, and matrix metalloproteinase-9 in rheumatoid arthritis fibroblast-like synovial cells (Yoon et al., 2013). Gallic acid also decreased IL-4, IL-13, and IL-17 levels in nasal lavage fluid of a mouse model of allergic rhinitis (Fan et al., 2019). Interestingly, oral administration of gallic acid could reduce TNF-α, IL-4, IFN-γ, and IL-17 levels in the lymph nodes of a 4-dinitrochlorobenzene-induced mouse model of skin inflammation (hu and Zhou, 2021). In the same model, gallic acid activated regulatory T cells to suppress the immune response via the production of IL-10 and TGF-β (Hu and Zhou, 2021). Although the topical effect of gallic acid on skin cells in an *in vivo* model is not yet known, this systemic effect of gallic acid may shed light on its potential implications in inflammatory skin diseases. The results herein showed that gallic acid did not affect the production of cytokine IL-1β but reduced the release of chemokines IL -8 and MCP-1 in TNF-α/IFN-γ-stimulated keratinocytes. The present findings were different from those in other tissues because gallic acid mainly modulates the action of chemokines but not cytokines (Yoon et al., 2013; Hu and Zhou, 2021). The reason for the lack of an effect of gallic acid on IL-1β release is unknown, but it cannot exclude the possibility that IL-1β release in keratinocytes is relatively independent of ROS.

TNF-α/IFN-γ triggers the release of cytokines and chemokines in HaCaT cells by activating MAPKs, including ERK1/2, JNK, and p38 MAPK (Sung et al., 2012; Takada et al., 2004; Kyriakis and Avruch, 2001). These MAPKs are activated by MEK phosphorylation. For instance, ERK1/2 is activated by MEK1/2. In addition

to the MAPK signaling pathway, the NF-κB signaling pathway is also involved in inflammation by regulating the expression of genes, enzymes, and adhesion molecules (Lai et al., 2017). NF-κB is activated by IκBα phosphorylation. It has been reported that gallic acid mitigated the immunoglobulin E-induced systemic or local inflammatory reactions by inhibiting the activation of NF-κB and p38 MAPK-dependent pathways, resulting in the decreased expression of TNF-α and IL-6 (Dominque et al., 2020). Gallic acid also reduced the release of intercellular adhesion molecules, chemokines, and inflammatory factors in KU812 cells via the inhibition of p38 MAPK, JNK, and NF-κB (liu et al., 2013). Similarly, these results showed that gallic acid reduced the total protein expression of NF-κB and inhibited the phosphorylation (an indicator of activation) of ERK1/2, p38 MAPK in TNF-α/IFN-γ-stimulated HaCaT keratinocytes. Interestingly, gallic acid also inhibits Akt phosphorylation in HaCaT keratinocytes. There is no direct evidence that the anti-inflammatory effect of gallic acid is due to the inhibition of the Akt/mTOR-dependent pathway. However, consistent with the present findings, polyphenols extracted from mangoes, which contained mainly gallic acid, reduced the inflammatory response by Akt/mTOR-dependent mechanism, partly through up-regulation of miRNA-126 expression (Kim et al., 2017). Moreover, the inhibitory effect of gallic acid on the Akt/mTOR-depen-dent pathway has been reported in other cell types, such as bone marrow cells, in which the inhibition of the Akt/mTOR pathway by gallic acid could suppress mitochondrial respiration and induce apoptosis (Gu et al., 2018).

The findings in this study will provide valuable information for the research and development of novel therapeutic agents for inflammatory skin diseases, such as atopic dermatitis. Corticosteroids, commonly used in clinical practice, can cause skin atrophy if applied frequently and for prolonged periods. Gallic acid has few adverse effects. Interestingly, gallic acid could also lower the hydrocortisone concentration required to resolve the inflammatory response (Liu et al., 2013; Chan et al., 2015). Gallic acid not only acts on keratinocytes but also on other immune cells in the skin. Due to its low toxicity, gallic acid may be implicated in treating inflammatory skin diseases or at least may serve as a lead compound so that further modification of its chemical structure may result in a promising anti-inflammatory agent (Al Zahrani et al., 2020). Moreover, many plants have high amounts of gallic acid; therefore, the use of plant extracts to alleviate inflammatory skin problems is worth further investigation.

Conclusion

Gallic acid exerts antioxidant and anti-inflammatory effects in HaCaT keratinocytes, probably through the inhibition of MAPK-, NF-κB-, and Akt-dependent signaling pathways.

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Conflict of Interest

Authors declare no conflict of interest

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Author Info
George Pak-Heng Leung (Principal contact)
e-mail: gphleung@hku.hk