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Stress-Mediated Physiological Epinephrine Levels Reduce Imiquimod-Induced Psoriasis-Like Skin Inflammation

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Abstract

Psychological stress-mediated systemic hormones can aggravate psoriasis severity. However, some studies have shown that stress can also ameliorate this disease. We aimed to investigate the effects of a high physiological epinephrine dose, as observed in stressed individuals, in imiquimod (IMQ)-induced skin inflammation *ex vivo*. Normal human skin explants were treated *ex vivo* with 56 nM (a high physiological level) epinephrine, and then three days later, topically treated with IMQ for 6 consecutive days. Human keratinocytes were treated *in vitro* with IMQ or epinephrine. The administration of 56 nM epinephrine reduced the epidermal area and increased the protein levels of IL-10 in IMQ-treated human skin. The blockade of β_1- andβ_2-adrenergic receptors (β_1-/β_2-AR), the IL-10 receptor, and cAMP reversed the reduction in epidermal area induced by epinephrine levels (56 nM) in IMQ-treated skin. In human keratinocytes, a physiological epinephrine level and IMQ increased the protein levels of β_2-AR, cAMP, and IL-10. In conclusion, a high physiological level of epinephrine ameliorates IMQ-induced skin inflammation via cAMP and IL-10 production.

Keywords

Psoriasis, Psychological Stress, Epinephrine, Adrenergic Receptor, Imiquimod

1. Introduction

Psoriasis is a chronic inflammatory skin disease characterized by well-demarcated and erythematous plaques covered with scales that can be observed anywhere on the body [1]. Psoriasis is a painful and disfiguring disease that can lead to the develop-

ment of several comorbidities, such as cardiovascular diseases, arthritis, and psychiatric disorders [2]. The worldwide prevalence of this disease varies between 0.09% and 11.4%, which indicates that this disease is a serious health problem [2]. The Brazilian Society of Dermatology estimates that the prevalence of psoriasis in Brazil is 1.31% of the population and that 73.4% of Brazilian patients develop moderate to severe psoriasis [3]. Histologically, psoriatic plaques are characterized by thickening of the epidermis, epidermal hyperproliferation, increased dermal vascularization, and massive immune cell infiltration in the dermis and epidermis [1]. The activation of the interleukin-23 (IL-23) and T helper 17 (Th17) cell axes and the crosstalk between keratinocytes and immune cells are accepted as the primary mechanisms involved in the development and maintenance of the inflammatory response in psoriasis [4] [5]. Many environmental factors can trigger or aggravate psoriasis, one of which is psychological stress, even during therapy.

Psychological stress is defined as "the process through which environmental demands exceed an individual's perceived ability to cope, thereby resulting in affective, behavioral, and physiological changes" [6]. The prolonged and systemic activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system by stress induces excessive synthesis of glucocorticoids and catecholamines, which leads to relevant alterations in several processes, such as psoriasis [7] [8]. The majority of patients with psoriasis report that stressful events worsen the severity of their psoriatic skin lesions [9] [10]. In animal models, glucocorticoids and catecholamines induced by restraint stress aggravate psoriasiform dermatitis induced by imiquimod (IMQ) [11]-[13]. IMQ is a toll-like 7 and 8 agonist that induces psoriasis-like skin inflammation in mice with features similar to human psoriasis [14]. Nonetheless, some studies also suggest that exposure to a stressful event for a short period can activate neuroendocrine pathways that protect the body against injury [7] [8]. Catecholamines, such as epinephrine, can stimulate the activation of immune and inflammatory cells, but chronic exposure to high epinephrine levels can reduce the activity of inflammatory and immune cells [6] [8]. The topical application of isoprenaline, which is a β-adrenergic receptor (β-AR) agonist, reduces the scaling of psoriatic plaques in patients [15]. In addition, a chronic sensory stress model has been generated that is capable of ameliorating the development of psoriasis-like dermatitis in transgenic mice [16]. Therefore, the effect of stress on psoriasis is not completely understood. It has been proposed that the activation of β_2-AR by agonists can promote an anti-inflammatory response in immune cells of the bone marrow and spleen [17] [18]. Thus, the aim of this study was to understand the effects of high physiological dose of epinephrine, similar to that observed in stressed individuals, on the severity of psoriasis in an *ex vivo* model of psoriasis-like human skin inflammation induced by IMQ.

2. Material and Methods

2.1. Cell Culture

Human spontaneously transformed keratinocytes from normal skin (HaCaT

cells) (AddexBio Technologies, San Diego, CA, catalog number: T0020001) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Corporation, Grand Island, NY, USA, catalog number: 11965118) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, Brazil, catalog number: 0063), 50 units/mL penicillin and 50 μg/mL streptomycin (Life Technologies Corporation, catalog number: 15140130). The cells were cultured at 85% - 90% confluence and were maintained at 37˚C in an atmosphere of 5% CO_2.

To induce differentiation, HaCaT cells were incubated with 2 mM calcium chloride (Sigma-Aldrich, Inc., St. Louis, MO, USA, catalog number: C5670) for 4 hours [19] [20]. The cells were subsequently treated with 100 μM gardiquimod (Sigma-Aldrich, Inc.) in DMEM supplemented with 2% FBS for 15 minutes or 24 hours to promote psoriasis-like inflammation. The cells were also treated with 56 nM epinephrine hemitartrate (Hypofarma, Ribeirão das Neves, Brazil) in DMEM supplemented with 2% FBS for 15 minutes or 24 hours. Cells incubated in DMEM supplemented with 2% FBS served as the control group. The concentrations of epinephrine are similar to those of catecholamine found in chronically stressed humans or mice [21] [22]. Gardiquimod is a potent and specific agonist of human and mouse toll-like receptor 7 and stimulates a psoriasis-like inflammatory response, as observed after IMQ administration *in vitro* [11] [20]. Some cells were also treated with 3 nM epinephrine to mimic the physiological level of epinephrine observed in nonstressed individuals [23]-[26]. The experiments were repeated twice using triplicate samples.

2.2. *Ex Vivo* Model of IMQ-Induced Human Skin Inflammation

The experimental design is shown in **Figure 1(A)**. The *ex vivo* IMQ-induced human skin inflammation model is a reproducible model in which biochemical (IL-23/IL-17 pathway activation) and histological (epidermal thickness and hyperproliferation) alterations similar to those in psoriasis in humans are induced as a result of topical IMQ application in normal human skin explants for 6 consecutive days [27]. Explants of 6-mm normal human skin were obtained from fragments of human skin from patients subjected to otoplasty (day 0). The explants were cultured in DMEM (Life Technologies Corporation) supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. The explants were maintained at 37˚C in an atmosphere of 5% CO_2. After one day, the explants were treated daily with 3 nM or 56 nM epinephrine (Hypofarma, catalog number: 1203.0194-A) in DMEM supplemented with 10% FBS and antibiotics until the end of the experiment. Three days after the start of epinephrine administration, the explants were topically treated with 3 μL of Modik cream containing 5% IMQ (Germed Farmacêutica Ltda., Campinas, Brazil) for 6 consecutive days [27]. Other explants were topically treated with 3 μL of a nonionic cream base used in skin care formulations (Dermage, Rio de Janeiro, Brazil). Explants incubated in medium and topically treated with the cream base served as controls. Explants that did not receive topical treatment also served as controls. To block β_1- and β_2-AR, some ex-

plants were treated with 20 μM propranolol hydrochloride (Sigma-Aldrich, Inc., catalog number: PHR1308) dissolved in culture medium [21]. To inhibit cyclic adenosine 3,5-monophosphate (cAMP), other explants were treated with 50 μM Rp-cAMP (R&D Systems, Minneapolis, MN, catalog number: 1337/1) dissolved in culture medium [28]. To neutralize the interleukin-10 receptor (IL-10R), some explants were incubated with 40 μg/mL of an IL-10R polyclonal antibody (Invitrogen, Carlsbad, CA, catalog number: #PA5-47383) dissolved in culture medium. The level of medium was daily checked, ensuring that the epidermis was not submerged. The explants were harvested 0, 1, 3, 5, or 10 days later and some (n = 6 per group) were used in tissue viability assay (n = 6 per group), while others (n = 6 per group) were fixed in 10% formalin at pH 7.2 (Sigma-Aldrich, Inc.) for histological analysis. Others (n = 6 per group) were frozen at −80˚C for enzyme-linked immunosorbent assay (ELISA) and western blotting. This experiment was approved by the Ethics Committee for Human Studies of the University of Grande Rio (CAAE: 46799215.1.0000.5283). All patients (18 women and 10 men, 7 - 46 years of age) agreed to participate in the study and signed the consent form. In each experiment, biopsies from the same donor were used in each treatment group. Smokers and patients with diabetes and cutaneous diseases (such as psoriasis, dermatitis, and urticaria) were excluded from the study. This experiment was performed twice using triplicate samples.

2.3. Cell and Tissue Viability Assay

To assess the tissue viability, the explants were weighed and transferred to 24-well plates containing 400 μL of serum-free DMEM and 100 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at 5 mg/mL (Sigma–Aldrich, Inc., catalog number: M5655) [29]. The explants were subsequently incubated for 4 hours at 37˚C in an atmosphere of 5% CO_2. The explants were incubated in a mixture of isopropanol, 0.04 N hydrochloric acid, and Triton X-100 (Sigma-Aldrich, Inc., catalog numbers: I9516, 258148, and T8787) for 3 hours at room temperature to extract the formazan crystals from the explants. The extract of each explant was transferred to a 96-well plate, and the absorbance was read at 570 nm. The data were expressed as the optical density in nm per g of tissue. To test cell viability, the cells were incubated with MTT (Sigma-Aldrich, Inc.) for 4 hours. The media was subsequently discarded, and the cells were incubated with 100% isopropanol for 30 minutes after which the absorbance was read at 570 nm. The data are expressed as the absorbance at 570 nm.

2.4. Histological Analysis

Formalin-fixed human skin explants were processed, embedded in paraffin and sectioned. The sections (5 μm thick) were stained with hematoxylin and eosin and digitized using a Pannoramic Midi slide scanner (3DHistech Ltd., Budapest, Hungary). The epidermal area was measured in 5 random fields using Pannoramic Viewer software (3DHistech Ltd.). The area of the epidermis was considered from

the granular layer to the basal layer, and the edge of the image was used as the lateral limit [30]. The measurement of the epidermal area allows evaluation of the epidermal thickness and the elongation of rete ridges induced by topical IMQ application [27]. The whole epidermis of each human skin explant was analyzed.

2.5. Immunohistochemistry

Paraffin-embedded human skin sections (5 μm thick) were deparaffined in xylol, hydrated in ethanol and distilled water, and heated in citrate buffer (pH 6.0) at 70˚C for antigen retrieval. After washing in 1× phosphate-buffered saline (PBS), the sections were incubated with 3% hydrogen peroxide in methanol for peroxidase inhibition. The sections were subsequently incubated with an antibody against proliferating cellular nuclear antigen (PCNA) and an appropriate secondary antibody (**Table S1**). The antigen-antibody complexes were detected using a 3,3'-diaminobenzidine chromogen kit (DAKO, Carpinteria, CA, USA; catalog number: N-1939), and the nuclei were stained with hematoxylin. In each section, the number of PCNA-positive basal keratinocytes was counted in 10 random fields (total of 20 cells per field) using a Primo Star microscope (Carl Zeiss, Oberkochen, Germany) with a 40× objective [31]. The whole epidermis of each human skin explant was analyzed. The results are expressed as the percentage of PCNA-positive basal keratinocytes (number of positive cells plus 100 divided by 20).

2.6. Immunofluorescence

Paraffin-embedded human skin sections (5 μm thick) were deparaffinized, hydrated, and heated in citrate buffer (pH 6.0) at 70˚C for antigen retrieval. After washing with 1× PBS and distilled water, the sections were blocked with 10% normal goat serum diluted in 1% bovine serum albumin and 1× PBS at room temperature. The sections were subsequently incubated with primary antibodies against interleukin-10 (IL-10), CD3 and CD68 and appropriate secondary antibodies (Table S1). The nuclei were stained with 4',6-diamidino-2-phenylindole Fluoromount-G® (SouthernBiotech, Birmingham, AL, USA; catalog number: 0100-20). Photomicrographs were obtained with a fluorescence microscope (Observer A1, 40× objective) and an AxioCam Mrc 5 camera (Carl Zeiss).

2.7. Enzyme-Linked Immunosorbent Assay

Frozen human skin and HaCaT cells were lysed in a radioimmunoprecipitation assay buffer containing a 1% protease inhibitor cocktail for mammalian tissues and a phosphatase inhibitor cocktail (Sigma-Aldrich, Inc.). The total protein concentration was determined via a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, catalog number: 23227). The protein levels of IL-17A/F, IL-10, and cAMP were measured in tissue and cell lysates using commercial kits (R&D Systems, catalog numbers: DY5390-05, M1000, and KGE012B) according to the manufacturer's instructions.

2.8. Western Blot

Proteins (20 μg per well) from tissue or cell lysates were separated by electrophoresis on 10% polyacrylamide gels containing sodium dodecyl sulfate. The proteins were transferred to polyvinylidene fluoride membranes and blocked with 5% non-fat skim milk diluted in Tris-buffered saline containing 1% Tween-20. The membranes were subsequently incubated with primary antibody against β_2-AR and an appropriate secondary antibody (Table S1). The antigen-antibody complexes were detected using an Immobilon ECL Ultra Western HRP Substrate Kit (Merck Millipore, Burlington, MA, USA, catalog number: WBULS0100) and a ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The β-actin was used as loading control. The bands were measured using ImageJ software, and the results are expressed as arbitrary units.

2.9. Statistical Analysis

The results are expressed as means and standard deviations. The normality of the data was determined via the Shapiro-Wilk and Kolmogorov-Smirnov tests. Comparisons between groups were performed via one-way ANOVA with Bonferroni post hoc correction. The outliers were identified via ROUT (Q = 1%). GraphPad Prism version 10.1.1 (GraphPad Software, Boston, MA) was used for all data analyses. A p value less than 0.05 indicates statistical significance.

3. Results

3.1. A High Physiological Dose of Epinephrine Reverses IMQ-Induced Inflammation in Human Skin *Ex Vivo*

Initially, we evaluated whether a high physiological (56 nM) level of epinephrine, as observed in chronically stressed individuals, could cause histological (epidermal thickness and proliferation) and biochemical alterations (IL-17A/F protein levels) in an *ex vivo* model of IMQ-induced human skin inflammation, which are histological and biochemical features observed in psoriatic plaques. The MTT assay demonstrated that the administration of 3 nM or 56 nM, IMQ or cream base did not affect the viability of the human skin explants *ex vivo* at 0, 1, 3, 5 or 10 days (Figures S1(A)-(C)). In addition, the administration of 3 nM or 56 nM epinephrine did not affect the viability of human keratinocytes *in vitro* after 24 hours of treatment (Figure S1(D)). Macroscopically, there was no significant alteration in the human skin explants after topical IMQ application at 10 days (Figure 1(A)). Compared with the control, the topical application of IMQ increased the epidermal area of human skin explants at 10 days (Figure 1(B), Figure 1(C)). Surprisingly, the administration of 56 nM epinephrine reduced the epidermal area of human skin explants at 10 days after topical treatment with IMQ (Figure 1(B), Figure 1(C)). The results of the indirect immunohistochemical staining analysis demonstrated that, compared with the control, the topical application of IMQ increased epidermal proliferation in human skin explants at 10 days (Figure 1(D), Figure 1(E)). Interestingly, the administration of 56 nM epinephrine diminished epidermal proliferation in human skin

explants at 10 days after IMQ treatment (Figure 1(D), Figure 1(E)). ELISAs revealed that, compared with the control treatment, topical IMQ application increased the protein levels of IL-17A/F in human skin explants at 10 days (Figure 1(F)), while the administration of 56 nM epinephrine decreased the protein levels of IL-17A/F in human skin explants at 10 days after IMQ treatment (Figure 1(F)). In the normal skin groups, there was a reduction in the viability of skin explants when compared to days 0 and 10 (Figure S2(A)). Moreover, pyknotic nuclei and vacuoles inside the epidermal keratinocytes were observed in the skin explants without treatment (normal skin group) at 5 and 10 days (Figure S2(B)).

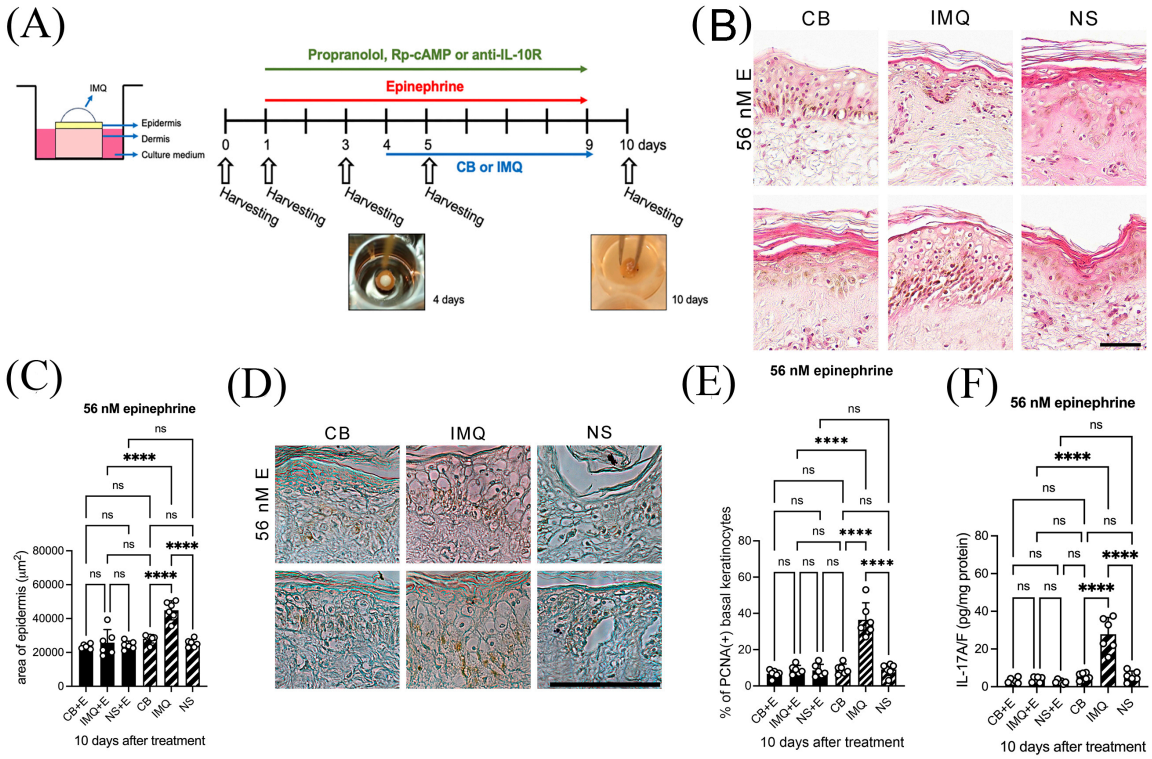
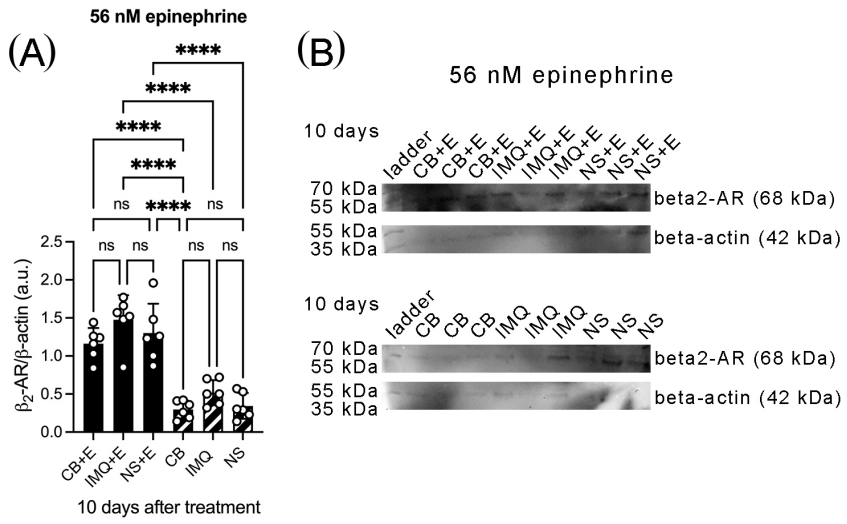


Figure 1. A low physiological dose of epinephrine reverses the imiquimod-induced psoriasis-like skin inflammation. (A) Experimental schema of a human skin explant topically treated with imiquimod (IMQ). Explants of human skin were skin were treated daily with 3 nM or 56 nM epinephrine in the medium for 9 days. Three days after the beginning of epinephrine administration, explants were topically treated with imiquimod or cream base for 6 days. Some explants did not receive any topical treatment (normal skin). Some explants were also treated with propranolol (an antagonist of β_1- and β_2-adrenergic receptors), Rp-cAMP (an inhibitor of cyclic adenosine 3',5'-monophosphate), and antibody against interleukin-10 receptor (IL-10R). The explants were harvested at 0, 1, 3, 5, or 10 days after treatment. (B) Representative images of epidermis in explants of human skin at 10 days after treatment. Sections were stained with hematoxylin and eosin. Scale bar = 50 µm. Those images were obtained from skin explants of different donors. (C) Measurement of area of epidermis expressed as μm^2 using a histological assay at 10 days after treatment. (D) Representative images of epidermis in explants of human skin immunostained for proliferating cellular nuclear antigen (PCNA) at 10 days after treatment. Scale bar = 100 µm. Those images were obtained from skin explants of different donors. (E) Evaluation of the percentage of PCNA-positive basal keratinocytes using a histological assay at 10 days after treatment. (F) Protein levels of interleukin-17A/F (IL-17A/F) in human skin explants as determined by ELISA at 10 days after treatment. Results (n = 6 patients per group, two independent experiments) were shown as mean ± standard deviation. Each dot represents one patient. One-way ANOVA with Bonferroni's post-test. CB: cream base; E: epinephrine; IMQ: imiquimod; NS: normal skin; ns: not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.2. Low-Dose Epinephrine Ameliorates IMQ-Induced Skin Inflammation in Human Skin *Ex Vivo* through IL-10 Production and β_2-AR Activation

We subsequently investigated whether 56 nM epinephrine could ameliorate IMQ-induced inflammation in human skin *ex vivo* through the production of anti-inflammatory cytokines via β_2-AR. It has been reported that the binding of epinephrine to β_2-AR at low doses promotes the production of the anti-inflammatory cytokine IL-10 via an increase in the level of intracellular cAMP [18]. Compared with the controls, the administration of 56 nM epinephrine increased the protein levels of β_2-AR in human skin explants treated with the cream base and IMQ at 10 days (**Figure 2(A), Figure 2(B)**). No significant difference was observed in the protein levels of cAMP between the groups at 10 days, as determined by ELISA (**Figure 2(C)**). Moreover, the blockade of β_1-AR and β_2-AR with propranolol did not change the protein levels of cAMP in human skin explants at 10 days (**Figure 2(C)**). However, the blockade of β_1-AR and β_2-AR with propranolol reversed the effects of 56 nM epinephrine on the epidermal area of the human skin explants compared with the control treatment at 10 days (**Figure 2(D)**). In addition, the inhibition of cAMP with Rp-cAMP reversed the effect of 56 nM epinephrine on the epidermal area of the human skin explants compared with control explants at 10 days (**Figure 2(E)**). Compared with the control, the topical application of IMQ did not change the protein levels of IL-10 in human skin explants at 10 days (**Figure 3(A)**). However, the administration of 56 nM epinephrine increased the protein levels of IL-10 in human skin explants at 10 days after treatment with IMQ (**Figure 3(B)**). To determine the possible cells involved in IL-10 production in human skin explants treated with IMQ and 56 nM epinephrine, indirect double immunofluorescence staining was performed. We observed that CD3-positive T lymphocytes and CD68-positive macrophages expressed IL-10 in the dermis of human skin at 10 days after treatment with IMQ and 56 nM epinephrine (**Figure 3(B)**). Moreover, the neutralization of IL-10R reversed the increase in the epidermal area of human skin explants at 10 days after treatment with IMQ and 56 nM epinephrine (**Figure 3(C)**).



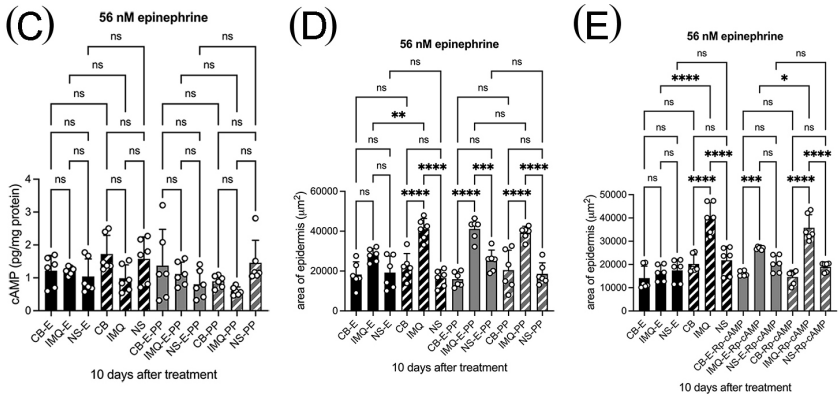


Figure 2. Expression of β_2-adrenergic receptor (β_2-AR) and cyclic adenosine 3',5'-monophosphate (cAMP) is involved in the beneficial effect of epinephrine dose on imiquimod-induced psoriasis-like skin inflammation. (A) The protein levels of β_2-AR in explants of human skin treated with epinephrine and/or imiquimod normalized to β-actin using western blot analysis, expressed as arbitrary units (a.u.). (B) Representative images of immunoblotting for β_2-AR and β-actin in explants of human skin at 10 days after treatment. (C) Protein levels of cAMP in human skin explants as determined by ELISA at 10 days after treatment. (D) Measurement of area of epidermis expressed as µm² in explants of human skin treated with propranolol (an β_1- and β_2-adrenergic receptor), epinephrine, and/or imiquimod. (E) Measurement of area of epidermis expressed as µm² in explants of human skin treated with Rp-cAMP (an cAMP inhibitor), epinephrine, and/or imiquimod. Results (n = 6 patients per group, two independent experiments) were shown as mean ± standard deviation. Each dot represents one patient. One-way ANOVA with Bonferroni's post-test. CB: cream base; E: epinephrine; IMQ: imiquimod; NS: normal skin; PP: propranolol; ns: not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.3. Activation of β_2-AR by Different Epinephrine Concentrations in Human Keratinocytes *in Vitro* Causes Differential Modulation of Inflammatory Responses Similar to Those in Psoriasis

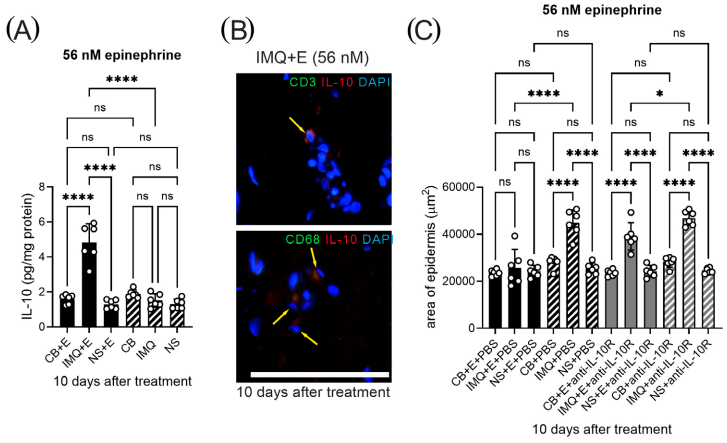


Figure 3. Production of interleukin-10 is associated with inhibitory effect of 56nM epinephrine on imiquimod-induced psoriasis-like skin inflammation. (A) The protein levels of IL-10 in human skin explants as determined by ELISA at 10 days after treatment. (B) Representative fluorescence microscopy images of CD3 (green), CD68 (green), and interleukin-10 (red) at 10 days after treatment. Nuclei were stained with DAPI (blue). Yellow arrows show T cells double-positive for CD3 and IL-10 (orange) and macrophages double-positive for CD68 and IL-10 (orange) in the dermis of human skin *ex vivo* treated with epinephrine and imiquimod at 10 days. Scale bar = 100 µm. (C) Neutralization of human IL-10 receptor (IL-10R) at 10 days after treatment. The area of the epidermis was evaluated using a histological assay. Results (n = 6 patients per group, two independent experiments) were shown as mean ± standard deviation. Each dot represents one patient. One-way ANOVA with Bonferroni's post-test. CB: cream base; E: epinephrine; IMQ: imiquimod; NS: normal skin; PBS: phosphate buffer saline; ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

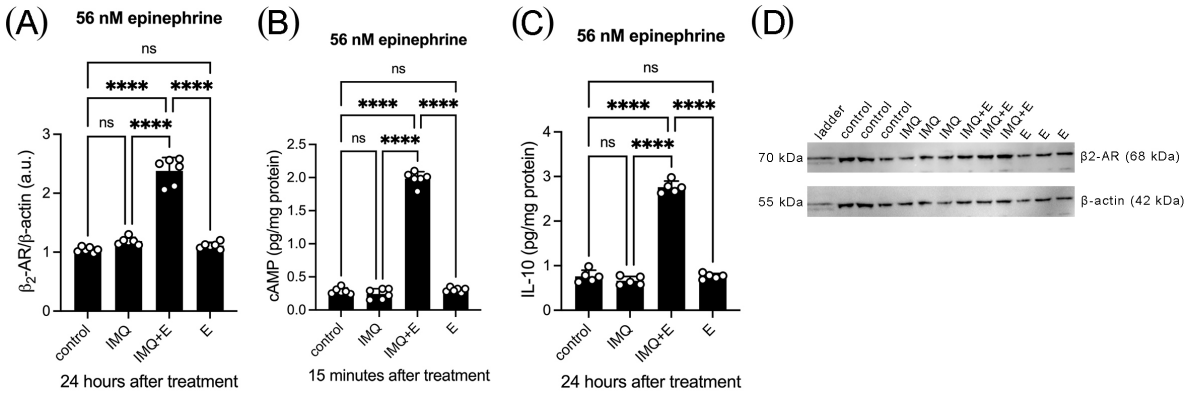


Figure 4. The dose of 56 nM epinephrine increases the protein levels of β_2-adrenergic receptor, cAMP and IL-10 in human keratinocytes treated with imiquimod *in vitro*. Human immortalized keratinocytes (HaCaT) were differentiated with 2 mM of calcium chloride in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) for 4 hours. Subsequently, cells were treated with 56 nM epinephrine and/or 100 μm gardiquimod for 15 minutes or 24 hours. Controls received only medium (DMEM with 2% FBS). (A) Densitometry of β_2-adrenergic receptor (β_2-AR) protein levels in cell lysate normalized to β-actin, expressed as arbitrary units (a.u.), at 24 hours using Western blot analysis. (B) Protein levels of cyclic adenosine 3',5'-monophosphate (cAMP) in cell lysate, expressed as ng per mg protein, at 15 minutes, using an ELISA assay. (C) Protein levels of interleukin-10 (IL-10) in cell lysate at 24 hours using an ELISA assay. (D) Representative images of immunoblotting for β_2-AR and β-actin at 24 hours. Results (n = 6, two independent experiments in triplicate) were shown as mean ± standard deviation. One-way ANOVA with Bonferroni's post-test. CB: cream base; E: epinephrine; IMQ: gardiquimod (TLR7 agonist similar to imiquimod); ns: not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

To understand the possible mechanisms involved in the effects of a high physiological (56 nM) level of epinephrine on IMQ-induced human skin inflammation *ex vivo*, differentiated HaCaT cells were treated with epinephrine and IMQ. Compared with control conditions, the administration of 56 nM epinephrine increased the protein levels of β_2-AR, cAMP, and IL-10 in HaCaT cells treated with IMQ for 15 minutes or 24 hours (**Figures 4(A)-(D)**).

4. Discussion

Several surveys and experimental studies have reported that psychological stress can exacerbate the severity of psoriasis, but others have reported opposite results in similar models [9]-[13]. Therefore, our research aimed to understand the effects of stress on the severity of this disease. Our hypothesis is that the activation of β_2-AR by low doses of catecholamines may improve the severity of psoriasis via stimulation of anti-inflammatory signaling pathways. To test this hypothesis, we used an *ex vivo* model of IMQ-induced human skin inflammation induced by IMQ that reproduces the histological (increased epidermal thickness and proliferation, T-cell activation) and the biochemical (augmented IL-17A/F production by T and Langerhans cells) alterations observed in psoriatic plaques in humans [27]. This model is also responsive to antipsoriatic drugs and maintains the complex architecture of human skin [27]. To mimic the stress-mediated increase in systemic epinephrine, human skin explants were treated with a high physiological (56 nM) dose of epinephrine added directly to the culture medium. The dose of 56 nM epinephrine is based on the plasma epinephrine concentration of patients

experiencing cardiac arrest, which is an acute maximal stressful event [22]. The circulating levels of epinephrine in patients with psoriasis who are not subjected to stressful events are similar to those in nonstressed, healthy individuals (0.05 - 3 nM of basal circulating epinephrine) [23]-[26]. Thus, the dose of 56 nM epinephrine used in this study was approximately 19 times higher than the basal concentration of epinephrine (3 nM) in nonstressed individuals with and without psoriasis. Nonetheless, both epinephrine doses (3 or 56 nM) did not change the tissue or cell viability.

The stimulation of β_2-AR by catecholamines promotes the activation of a canonical signaling pathway that involves the activation of G-coupled proteins and adenylate cyclase, the synthesis of cAMP, and protein kinase A (PKA) activation [32]. Traditionally, the activation of this pathway promotes a Th2-type immune response and the production of anti-inflammatory mediators (such as IL-10) that contribute to the suppression of inflammation [17] [18]. In our study, a high physiological dose of epinephrine (56 nM) could ameliorate the histological (epidermal thickness and proliferation) and biochemical (IL-17A/F production) parameters associated with psoriasis in humans. Moreover, stress-induced epinephrine at a high physiological dose (56 nM), which has antipsoriatic effects, has been shown to stimulate the production of IL-10 by T cells and macrophages in IMQ-induced human skin inflammation models [33]. The beneficial effects of a high physiological epinephrine dose on IMQ-induced human skin inflammation might be associated with activation of the canonical β_2-AR signaling pathway. The topical application of a β-AR agonist reduces the scaling of psoriatic skin lesions in patients [15]. Furthermore, the administration of a long-acting β_2-AR agonist mitigates IMQ-induced skin inflammation in mice through cAMP production and PKA activation [34]. In our study, increased protein levels of β_2-AR were observed in human skin samples exposed to IMQ and 56 nM epinephrine. The blockade of β_1- and β_2-AR and the neutralization of IL-10R reversed the positive effects of 56 nM epinephrine on psoriasiform dermatitis in human skin. Moreover, these data were confirmed by our *in vitro* assays using cultured human keratinocytes. Similar results have been observed in KRT-14-Vegfa-transgenic mice, which developed psoriasiform dermatitis in an IL-17-dependent manner in a repeated stress model [16]. In these animals, blockade of β_1-AR and β_2-AR suppresses the reduction in erythema and ear thickness induced by stress [16]. We also observed an increase in cAMP levels in human keratinocytes treated with IMQ and 56 nM epinephrine but not in the IMQ-induced human skin inflammation model. This might have occurred because the harvesting of human skin occurred after 10 days of treatment, while peak cAMP production was observed at 15 minutes [35]. Nevertheless, cAMP blockade inhibited the reduction in epidermal thickness induced by a high physiological epinephrine dose (56 nM) in IMQ-induced human skin inflammation. Thus, we propose that the activation of β_2-AR by a high physiological epinephrine dose (56 nM) promotes the production of cAMP and IL-10; this reduces epidermal thickening, epidermal proliferation, and the inflammatory re-

sponse in psoriasiform human lesions, which contributes to the amelioration of dermatitis.

The limitations of this study are associated mainly with the *ex vivo* model of IMQ-induced human skin inflammation. This model does not allow for the induction of some alterations observed in psoriasiform plaques, such as altered vascularization and the infiltration of new inflammatory and immune cells [27]. Moreover, the elongation of rete ridges is not frequently observed in this model, and some pyknotic nuclei and cellular vacuolization can be observed in explants after 6 days of topical IMQ application [27]. In addition, our model did not include other hormones associated with the action of stress on psoriasis (such as corticotropin-releasing hormone, adrenocorticotrophic hormone, corticoids, and norepinephrine).

In conclusion, a high physiological dose of epinephrine (56 nM) can ameliorate IMQ-induced skin inflammation, which contributes to a reduction in epidermal proliferation and thickening. This beneficial effect of a low epinephrine dose is related to the activation of the canonical β_2-AR signaling pathway, which leads to the production of cAMP and IL-10. These data indicate that the β_2-AR signaling pathway may be an important target in the development and severity of psoriasis and needs to be more studied in psoriatic patients.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary Material

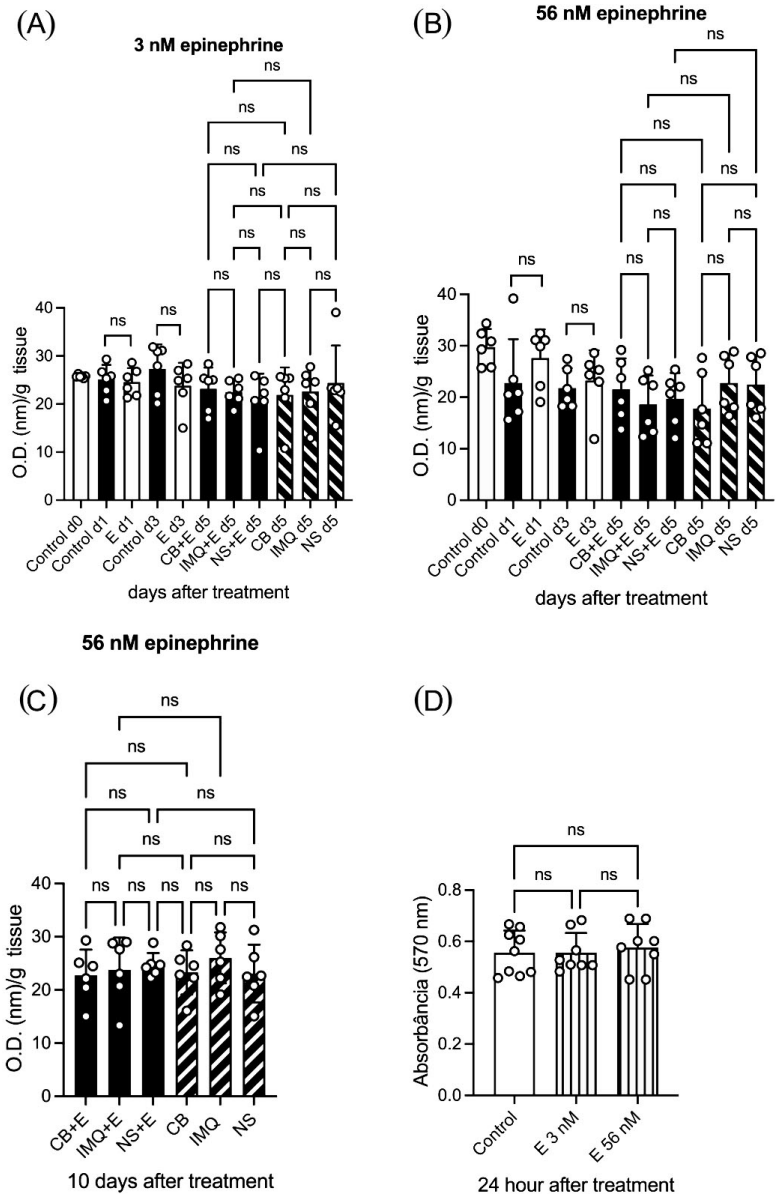

Figure S1. Tissue and cell viability. (A)-(C) Explants of human skin were skin were treated daily with 3 nM or 56 nM epinephrine in the medium. Three days after the beginning of epinephrine administration, some explants were topically treated with imiquimod or cream base for 1 day. Some explants did not receive any treatment (normal skin or control). (A)-(C) The tissue viability was evaluated using a colorimetric assay based on 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay at 0, 1, 3, 5 or 10 days after treatment. Results (n = 6 per group, two independent experiments) were shown as mean ± standard deviation. Each dot represents one patient. Student's t test (two tailed) or one-way ANOVA with Bonferroni's post-test. (D) Human immortalized keratinocytes (HaCaT) were treated with 3 nM or 56 nM epinephrine for 24 hours. Controls received only medium (DMEM with 2% FBS). Results (n = 9 per group, three independent experiments) were shown as mean ± standard deviation. One-way ANOVA with Bonferroni's post-test. CB: cream base; E: epinephrine; IMQ: imiquimod; NS: normal skin; ns: not significant; O.D.: optical density.

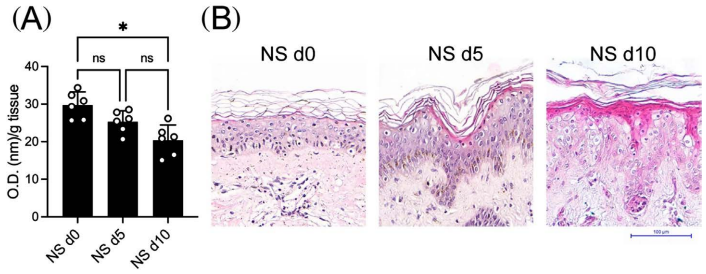

Figure S2. Tissue viability of normal skin group. (A)-(B) Explants of human skin did not receive any treatment (normal skin) and they were harvested at 0, 5 and 10 days. (A) The tissue viability was evaluated using a colorimetric assay based on 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay at 0, 5, or 10 days. Results (n = 6 per group, two independent experiments) were shown as mean ± standard deviation. Each dot represents one patient. Repeated measures one-way ANOVA with Holm-Sidák's multiple comparisons test. (B) Representative images of epidermis in explants of human skin without treatment (normal skin) at 0, 5 and 10 days. Sections were stained with haematoxylin and eosin. Scale bar = 100 μm. Those images were obtained from skin explants of different donors. NS: normal skin; ns: not significant; O.D.: optical density. *$p < 0.05$.

Table S1. Antibodies used in immunohistochemistry (IHC), immunofluorescence or western blot (WB).

Antibody	Target	Produced in	Dilution	Clone	Dye/ Enzyme	Company	Catalogue number
β-actin	Human Mouse Rat	Mouse	1:5000	AC-15	-	Sigma-Aldrich, Inc., St. Louis, MO, USA	A5441
β_2-Adrenergic receptor	Human Mouse Rat	Rabbit	1:200	EPR707(N)	-	Abcam, Waltham, MA, USA	ab182136
CD3	Human Rat Mouse	Rabbit	1:100	Polyclonal	-	Abcam, Waltham, MA, USA	ab5690
CD68	Rat	Mouse	1:100	ED1	-	Bio-Rad Laboratories, Hercules, CA, USA	MCA341R
Interleukin-10 (IL-10)	Mouse	Rat	1:50	JES5-2A5	-	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	sc-52561
Proliferating cell nuclear antigen (PCNA)	Human Rat	Mouse	1:50	PC10	-	Diagnostic BioSystems Inc, Pleasanton, CA, USA	Mob083
Secondary antibodies	Anti-rat	Goat	1:500	-	Texas Red-X	Invitrogen, Carlsbad, CA, USA	T6392
	Anti-rabbit	Goat	1:800	-	Alexa Flour™ 488	Invitrogen, Carlsbad, CA, USA	A11008
	Anti-rabbit	Goat	1:1000	-	Horseradish peroxidase	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	sc-2004
	Anti-mouse	EnVision+ System-HRP Labelled Polymer	Without dilution (IHC) or 1:200 (WB)	-	Horseradish peroxidase	DAKO Agilent, Santa Clara, CA, USA	K4001