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Original Article

Antagonism of P2Y11 receptor (P2Y11R) protects epidermal stem cells against UV-B irradiation

Yaguang Wu*, Lan Ge*, Song Li, Zhiqiang Song

*Department of Dermatology, Southwest Hospital, Army Military Medical University, Shapingba District, Chongqing 400000, China. *Equal contributors.*

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Abstract: Epidermal stem cells (ESCs) play essential roles in maintaining skin homeostasis and cell turnover of skin. Long-term exposure to UV-B irradiation induces a decrease in the population of ESCs and impairs the capacities of ESCs. The P2Y11 receptor (P2Y11R) is an important member of the P2 receptor family and plays a key role in mediating purinergic signaling and intracellular effects. In this study, we found that UV-B irradiation induced an increase in P2Y11R in ESCs. Antagonism of P2Y11R using NF157 ameliorated UV-B irradiation-induced oxidative stress by reducing reactive oxygen species (ROS) production and NADPH oxidase-4 (NOX-4) expression. Additionally, treatment with NF157 had a protective effect against UV-B irradiation-induced mitochondrial dysfunction by increasing mitochondrial membrane potential (MMP) and cytochrome c oxidase activity. Also, NF157 could mitigate lactate dehydrogenase (LDH) release and decreased the tumor necrosis factor-↑ (TNF-α), interleukin-6 (IL-6), and IL-8 secretion. Importantly, we found that treatment with NF157 attenuated UV-B irradiation-induced loss of ESCs capability by restoring the expression of integrin β1 and Krt19. Mechanistically, treatment with NF157 prevented UV-B irradiation-induced destruction of the Wnt/β-catenin signaling transduction pathway by increasing the expression of Wnt1, Wnt3a, c-Myc, and cyclin D1. These findings suggest a novel function of P2Y11R in regulating the capacities of ESCs upon UV-B irradiation.

Keywords: Epidermal stem cells, skin, P2Y11 receptor, inflammation, Wnt

Introduction

As the biggest organ of the human body, skin plays an essential role in protecting the body against various external stimuli, toxins, and pathogens [1]. Increasing evidence has shown that skin has a high frequency of cell turnover, which is mediated by stem cells [2]. Epidermal stem cells (ESCs) are a type of rare but pluripotent and multipotent stem cell that play a key role in maintaining skin homeostasis. On one hand, ESCs possess a self-renewal ability in order to maintain a certain population. On the other hand, ESCs can differentiate into all sets of cell lineages that comprise the skin [3]. The intracellular Wnt/β-catenin signaling pathway plays a central role in regulating the self-renewal and differentiation of ESCs [4]. Environmental insults such as ultraviolet (UV) irradiation have been reported to reduce the ESC population and impair the capacities of ESCs. UV irradiation comprises three components: UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (200-280 nm). Among them, UV-B can cross the stratospheric ozone layer and induce DNA damage in the epidermis [5]. UV-B irradiation causes damage due to oxidative stress in ESCs by increasing production of reactive oxygen species (ROS) and depleting antioxidants such as glutathione (GSH) in ESCs [6]. In recent decades, efforts have been made to explore the underlying mechanisms and develop new therapeutic agents to prevent UV irradiation-induced insults.

The P2Y11 receptor (P2Y11R), a metabotropic G protein-coupled receptor, is an important member of the P2 receptor family and plays a key role in mediating purinergic signaling and intracellular effects of extracellular adenosine triphosphate (ATP) [7]. A diversity of physiological functions of P2Y11R has been reported in previous studies. For example, P2Y11R expression is correlated with an increase in ATP-in-

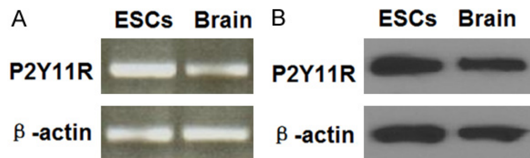

Figure 1. P2Y11 receptor (P2Y11R) is expressed in epidermal stem cells (ESCs). A. Reverse transcription PCR (RT-PCR) analysis revealed that P2Y11R is expressed in ESCs at the gene level with rabbit brain as a positive control; B. Western blot analysis revealed that P2Y11R is expressed in ESCs at the protein level. Experiments have been repeated for 3 times.

duced cell death in human T lymphocytes [8]. Additionally, strong purinergic stimulation and activation of P2Y11R suppresses tumor-derived endothelial cell migration and promotes pericyte attraction, resulting in vessel normalization [9]. P2Y11R agonist could activate adenylyl cyclase (AC) and stimulate cAMP release via calcium-dependent or G protein-coupled AC isoforms [10]. P2Y11R is widely distributed in a variety of human tissues and cells including myocardium, spleen, and immune cells [11]. ATP potentiates chemotactic response of neutrophils to N-formyl-methionyl-leucyl-phenylalanine (fMLP) via activation of P2Y11R, and this process plays a vital role in host defense and pathogenicity [12]. However, whether P2Y11R is expressed in ESCs and the biological functions of P2Y11R in ESCs have not been reported before.

Materials and methods

Cell isolation and treatment

All animal experiments performed were approved by the Institutional Animal Care and Use Committee at our institute. ESCs were isolated from skin on the backs of New Zealand white rabbits in accordance with the protocols as described previously [13]. Isolated ESCs were maintained in DMEM/F12 medium (Gibco, USA) supplemented with 15% embryonic stem cell screened FBS, 1% glutamine, 1% antibiotics (penicillin/streptomycin), and 4 ng/ml bFGF (Life Technologies, USA). To determine the expression of P2Y11R in ESCs, cells were exposed to UV-B irradiation (30, 60, 90 mJ/cm^2) for 12 h. NF157 was purchased from Tocris Bioscience (Cat. No. 2450/10), USA and dissolved in Dimethyl Sulfoxide (DMSO). To study the protective effects of NF157 against UV-B irradiation, ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h.

Measurement of reactive oxygen species (ROS)

After the necessary treatment, ROS levels in ESCs were measured via 2,7-dichlorofluorescin diacetate (DCFH-DA) staining assay. ESCs were washed three times with PBS and loaded with 1 μM DCFH-DA. After incubation for 20 min at 37°C in darkness, cells were washed 3 times with PBS. Fluorescent signals were visualized using an IBE2000 inverted fluorescence microscope (Zeiss, Germany).

Determination of mitochondrial membrane potential (MMP)

MMP in ESCs was determined using the dye tetramethylrhodamine methyl ester (TMRM) (Invitrogen, USA). Upon completion of the necessary treatment, ESCs were probed with 20 nmol/L TMRM for 30 min at 37°C in darkness. Cells were then washed 3 times with PBS, and fluorescence signals were visualized using an IBE2000 inverted fluorescence microscope (Zeiss, Germany).

Real-time polymerase chain reaction (PCR) analysis

Total intracellular RNA was extracted from ESCs using Qiazol (Qiagen, USA) in accordance with the manufacturer's instructions. RNA concentration was determined using a nanodrop microvolume spectrophotometer. RNA was used to synthesize the first-strand cDNA using a cDNA synthesis kit (Thermo Fisher Scientific, USA). Real-time PCR analysis was performed using SYBR Green Real-time PCR Master Mix (Bio-Rad, USA) on a StepOne Plus Real-Time PCR System (Applied Biosystems, USA) with melting curve analysis. Expression of target genes was normalized to GAPDH mRNA expression using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

ESCs were lysed with cell lysis buffer containing protease and phosphatase inhibitors. Extracted proteins were denatured at 100°C for 5 min. Samples of equal amounts were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfe-

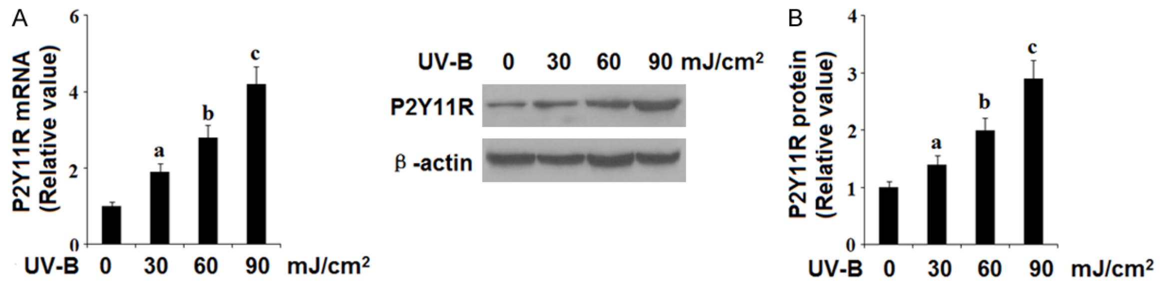

Figure 2. UV-B irradiation increased the expression of P2Y11R in epidermal stem cells (ESCs). ESCs were exposed to UV-B irradiation (30, 60, 90 mJ/cm^2) for 12 h. A. Real-time PCR analysis revealed that P2Y11R is increased in response to UV-B irradiation in a dose-dependent manner at the gene level; B. Western blot analysis revealed that P2Y11R is increased in response to UV-B irradiation in a dose-dependent manner at the protein level (a, b, c, P<0.01 vs. previous column group, n=5-6).

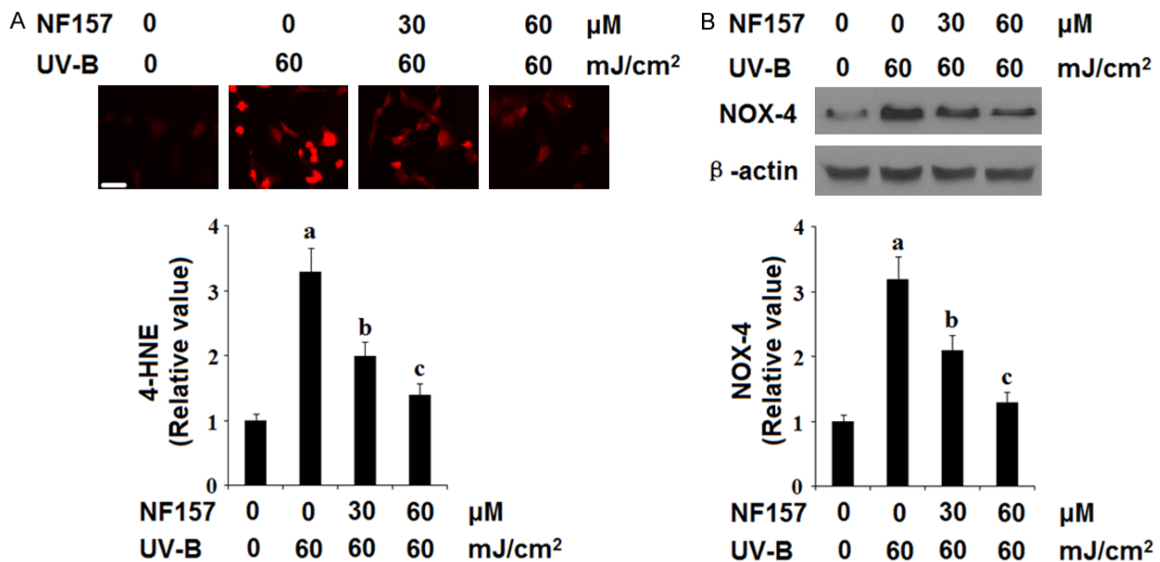

Figure 3. Antagonism of P2Y11R with NF157 protected epidermal stem cells (ESCs) against UV-B irradiation-induced oxidative stress. ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h. A. Intracellular ROS; Scale bars, 100 μm; B. NOX-4 expression determined by western blot analysis (a, b, c, P<0.01 vs. previous column group, n=5-6).

rred onto polyvinylidene difluoride (PVDF) membranes. Afterwards, membranes were kept in 5% non-fat milk blocking solution in Tris buffered saline with 0.01% Tween 20 (TBST), followed by incubation with primary antibodies overnight at 4°C. Membranes were then incubated with HRP-conjugated anti-rabbit or HRP-conjugated anti-mouse IgG secondary antibodies for 2 h at room temperature (RT). Specific bands were visualized using ImmunoStar ®LD (Wako).

Enzyme-linked immunosorbent assay (ELISA) analysis

Secretions of TNF-α, IL-6, and IL-8 were measured using ELISA kits (R&D Systems, USA) in accordance with the manufacturer's protocols. ELISA plates were blocked with 1% BSA and 5% sucrose for 2 h at RT. After 3 washes, 100 μl culture supernatant was added to the ELISA plates and incubated overnight at 4°C. ELISA plates were washed and incubated with appropriate second antibody for 1 h at RT. Then, 100 μl tetramethylbenzidine substrate was added and incubated for 5 min. The reactions were stopped with 0.5 mol/L H_2SO_4. Absorbance measured at 450 nm was used to index the concentrations of TNF-α, IL-6, and IL-8.

Statistical analysis

Experimental data are displayed as means ± standard error (S.E.). Statistical analysis was

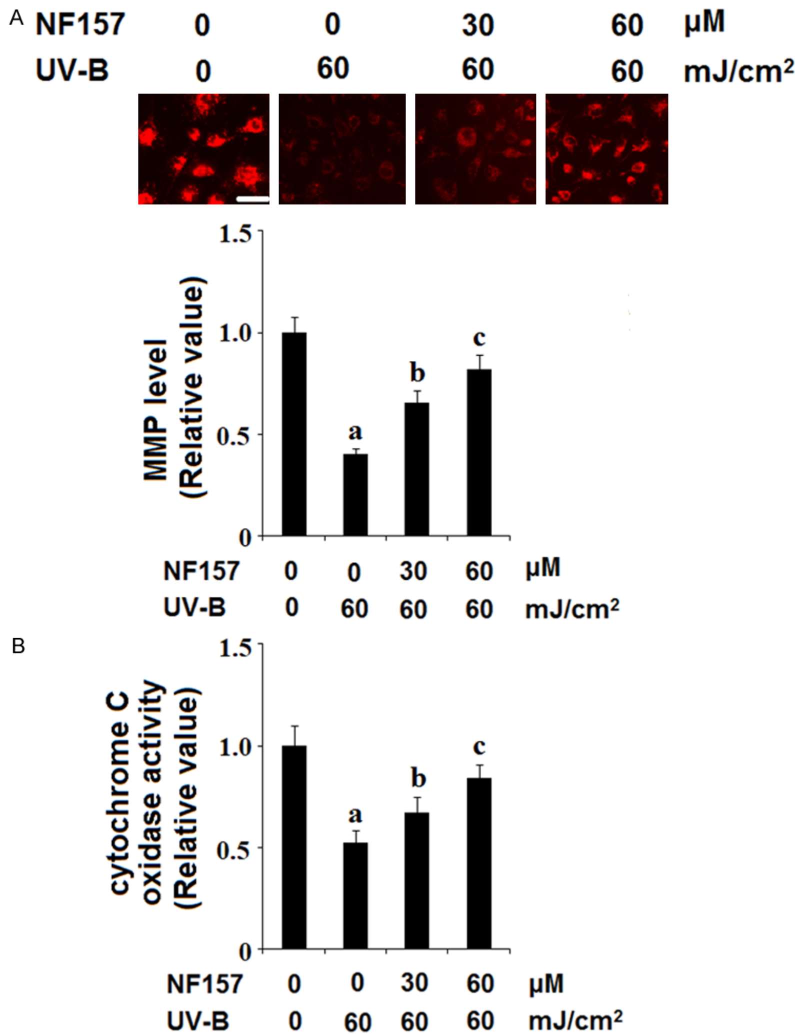

Figure 4. Antagonism of P2Y11R with NF157 protected epidermal stem cells (ESCs) against UV-B irradiation-induced mitochondrial dysfunction. ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h. A. MMP was measured by TMRM; Scale bars, 100 μm; B. Cytochrome c oxidase activity (a, b, c, P<0.01 vs. previous column group, n=5-6).

performed using the SPSS (Version 19.0) statistical software package. Results in multiple groups were compared using analysis of variance (ANOVA). A *P* value of <0.05 was the criterion for significance.

Results

Firstly, we investigated whether P2Y11R is expressed in ESCs. Both RT-PCR and western blot analysis revealed that P2Y11R could be detected in ESCs at both the gene and the protein levels (**Figure 1**). Rabbit brain cortex homogenate was used as a positive control. Interestingly, when ESCs were exposed to UV-B irradiation at 30, 60, and 90 mJ/cm^2 for 12 h, both real-time PCR (**Figure 2A**) and western blot analysis (**Figure 2B**) revealed that UV-B irradiation exposure increased the expression of P2Y11R in a dose-dependent manner. These findings suggest that P2Y11R may play a role in UV-B irradiation-induced damage to ESCs.

UV-B irradiation has been reported to induce intensive oxidative stress in ESCs. To investigate the possible involvement of P2Y11R in UV-B irradiation-induced damage to ESCs, the specific P2Y11R antagonist, NF157, was used. ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h. The DCFH-DA staining results in **Figure 3A** indicate that UV-B irradiation significantly increased the production of ROS in ESCs, which was ameliorated by NF157 in a dose-dependent manner. NADPH oxidase 4 (NOX-4) is an important member of the NOX family of NADPH oxidases. Here, we found that UV-B irradiation-induced increased NOX-4 expression was inhibited by NF157 in a dose-dependent manner (**Figure 3B**).

We then set out to investigate whether the presence of NF157 had an impact against UV-B irradiation-induced mitochondrial dysfunction in ESCs. The TMRM staining results in **Figure 4A** indicate that UV-B irradiation significantly reduced the level of intracellular MMP in ESCs, which was prevented by NF157 in a dose-dependent manner. Cytochrome c oxidase is an important enzyme located in mitochondria. Our data demonstrated that UV-B irradiation-in-

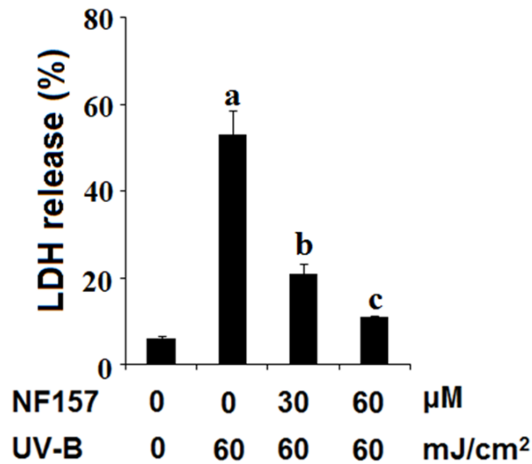

Figure 5. Antagonism of P2Y11R with NF157 protected epidermal stem cells (ESCs) against UV-B irradiation-induced release of lactate dehydrogenase (LDH). ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h. LDH release was measured using a commercial ELISA kit (a, b, c, P<0.01 vs. previous column group, n=6).

duced reduced cytochrome c oxidase activity was attenuated by NF157 (**Figure 4B**).

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is an indicator of cellular toxicity. Here, we found that UV-B irradiation significantly promoted the release of LDH from the cytoplasm into the cell culture medium. However, antagonism of P2Y11R by NF157 could reduce LDH release induced by UV-B irradiation in a dose-dependent manner (**Figure 5**). Excessive production of pro-inflammatory cytokines such as TNF-α and IL-6 is associated with UV-B irradiation-induced cytotoxicity. Here, we found that UV-B irradiation exposure significantly increased the expression (**Figure 6A**) and secretion (**Figure 6B**) of TNF-α, IL-6, and IL-8 in a dose-dependent manner, which was inhibited by NF157 in a dose-dependent manner.

ESCs are reported to have a self-renewal ability and can differentiate into all cell lineages that comprise the skin. The expression of ESC markers such as integrin β1 and Krt19 represents the differentiation ability of ESCs. Here, we found that the expression of integrin β1 and Krt19 in ESCs was reduced by exposure to UV-B irradiation. However, pre-incubation with NF157 prevented this process (**Figure 7**). Previous studies have shown that the Wnt/β-catenin pathway plays a critical role in maintaining the capacities of ESCs. Hence, we investigated the expression of members of the Wnt/β-catenin signaling pathway. Both real-time PCR and western blot analysis revealed that UV-B irradiation disturbed the Wnt/β-catenin signaling transduction pathway by reducing the expression of Wnt1, Wnt3a, c-Myc, and cyclin D1. However, blockage of P2Y11R with NF157 mitigated the reduction of these factors (**Figure 8**). These findings suggest that treatment with NF157 might maintain the capacities of ESCs by regulating the Wnt/β-catenin pathway.

Discussion

ESCs play a key role in the maintenance and repair of adult skin by producing various differentiated cells, which can replace aging cells and repair skin [14]. However, human ESCs tend to be sensitive to ionizing radiation such as UV-B-induced damage. Long-term exposure to UV-B irradiation causes DNA damage, which results in genetic instability of ESCs [15]. DNA damage caused by UV-B irradiation leads to a significant reduction in the colony-forming potential and viability of ESCs [16]. DNA damage can also regulate the expression of genes involved in skin stem cell homeostasis, including c-Myc and its target genes integrin β1 and Krt19 [17]. However, the underlying mechanisms remain to be elucidated. In the current study, for the first time, we report that P2Y11R is expressed in ESCs. Importantly, UV-B irradiation increased the expression of P2Y11R in an intensity-dependent manner, suggesting the potential involvement of this receptor in UV-B irradiation-induced damage in ESCs. Additionally, we found that antagonism of P2Y11R could maintain the capacities of ESCs by retaining expression of the two ESC markers integrin β1 and Krt19 as well as members of the Wnt/β-catenin signaling pathway, such as Wnt1, c-Myc, and cyclin D1. The Wnt/β-catenin signaling pathway plays a key role in controlling the fate of ESCs [18]. Moreover, the Wnt/β-catenin signaling pathway has been linked with cell survival and growth. Here, our results demonstrate that NF157 can preserve the activity of the Wnt pathway, implicating a novel mechanism.

P2Y11R has been identified as a G protein-coupled receptor, which can be stimulated by endogenous purine nucleotides [19]. The expres-

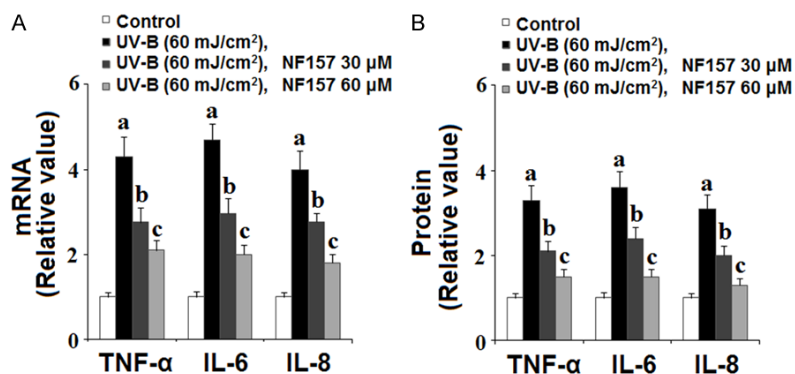

Figure 6. Antagonism of P2Y11R with NF157 protected epidermal stem cells (ESCs) against UV-B irradiation-induced expression and secretion of TNF-α, IL-6, and IL-8. ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h. A. Expression of TNF-α, IL-6, and IL-8 was determined by real-time PCR analysis; B. Expression of TNF-α, IL-6, and IL-8 was determined by ELISA (a, b, c, $P<0.01$ vs. previous column group, n=5-6).

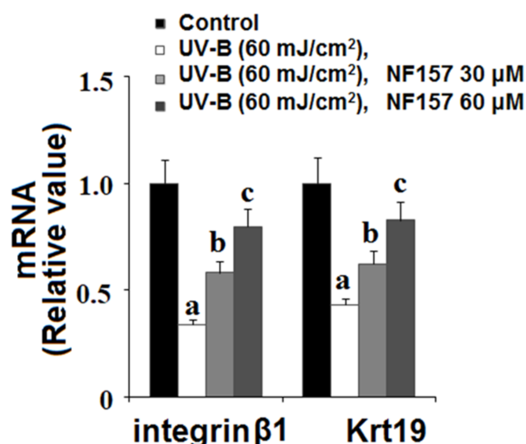

Figure 7. Antagonism of P2Y11R with NF157 protected epidermal stem cells (ESCs) against UV-B irradiation-induced impairment of the capacities of ESCs. ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h. Expression of integrin β1 and Krt19 was determined by real-time PCR analysis (a, b, c, $P<0.01$ vs. previous column group, n=5-6).

sion of human P2Y11R has been reported to play an important role in ATP-mediated cell death [20]. Here, we found that antagonism of P2Y11R ameliorated UV-B irradiation-induced release of LDH in ESCs, which is an indicator that NF157 may prevent cell death. We also found that NF157 attenuated UV-B irradiation-induced oxidative stress and improved mitochondrial dysfunction. Consistently, NF157 displayed a protective effect against human neutrophil apoptosis through a mitochondria-dependent pathway [21]. Interestingly, activation of P2Y11R has been associated with inflammatory response in skin inflammation induced by ionizing radiation. In the current study, we report that the presence of NF157 inhibited UV-B irradiation-induced expression and secretion of TNF-α, IL-6, and IL-8 in ESCs, which is consistent with a recent study showing that γ-irradiation-induced IL-6 expression and secretion was mediated by P2Y11 receptors in epidermal cells. Treatment with the specific P2Y11R antagonist NF157 inhibited IL-6 and IL-8 production in irradiated cells through suppressing the p38 mitogen-activated protein kinase (MAPK) and NF-κB signaling in keratinocytes [22, 23]. Importantly, another study reported that the administration of NF157 mitigates bacterial lipopolysaccharide (LPS)-induced systemic inflammatory response by suppressing the production of inflammatory cytokines such as IL-6, IL-1β, IL-12, and TNF-α and blocking macrophage M1 polarization [24].

Collectively, the results of the current study provide a new insight into the biological functions of P2Y11R in protecting ESCs against UV-B-induced damage and impairment of the capacities of ESCs. The underlying mechanisms suggest that antagonism of P2Y11R might become a possible therapeutic strategy against UV-induced skin damage.

Acknowledgements

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Disclosure of conflict of interest

None.

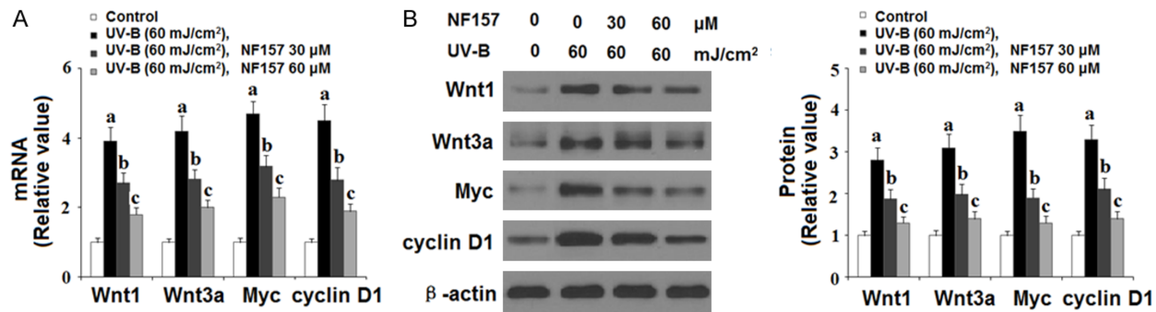

Figure 8. Antagonism of P2Y11R with NF157 protected epidermal stem cells (ESCs) against UV-B irradiation-induced reduced Wnt/β-catenin pathway proteins. ESCs were treated with NF157 at the concentrations of 30 and 60 μM for 12 h, followed by exposure to UV-B irradiation (60 mJ/cm^2) for another 12 h. A. Expression of Wnt1, Wnt3a, Myc, and cyclin D1 was determined by real-time PCR analysis at the gene level; B. Expression of Wnt1, Wnt3a, Myc, and cyclin D1 was determined by western blot analysis at the protein level (a, b, c, $P<0.01$ vs. previous column group, n=5-6).

Address correspondence to: Dr. Zhiqiang Song, Department of Dermatology, Southwest Hospital, Army Military Medical University, No. 30, Gaotanyanzheng Street, Shapingba District, Chongqing 400000, China. Tel: +86-023-68754000; E-mail: hail2121@163.com

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