Journal of Dermatological Science 78 (2015) 181–188

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Lipoxin A4 inhibits proliferation and inflammatory cytokine/chemokine production of human epidermal keratinocytes associated with the ERK1/2 and NF-κB pathways

Feng Hu [a,b,1], Xin-Xin Liu [b,1], Xin Wang [b], Mohammad Alashkar [b], Song Zhang [b], Jun-Tao Xu [b], Xue-Lian Zhong [b], Meng-Wen He [b], Ai-Ping Feng [b,*], Hong-Xiang Chen [b,*]

[a] Department of Dermatology, Wuhan No. 1 Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

ARTICLE INFO

Article history:
Received 9 October 2014
Received in revised form 3 March 2015
Accepted 9 March 2015

Keywords:
Lipoxin A4
Keratinocyte
Cell proliferation
Inflammation
Cell cycle

ABSTRACT

Background: Current *in vitro* studies show that lipoxin A4 (LXA4) has multiple biological functions including inhibiting cell proliferation and inflammatory cytokine production. Our previous studies showed LXA4 could inhibit the expression of IL-6 and IL-8 in normal human epidermal keratinocytes (NHEKs). However, more specific effects including regulation of cell proliferation and anti-inflammatory mechanisms of LXA4 in NHEKs have not been previously studied.
Objective: We proposed to investigate the effects of LXA4 on cell proliferation and inflammatory cytokine/chemokine production in NHEKs, and the possible molecular mechanisms of cell cycle and anti-inflammatory signal transduction pathway.
Methods: NHEKs were stimulated with LPS, with or without preincubation with LXA4. Cell proliferation and cell cycle of NHEKs were examined by WST-8, CFSE assay and DNA staining, respectively. The mRNA and protein levels of inflammatory cytokines were quantified by real-time quantitative PCR and ELISA. The expressions of signaling proteins cyclin D1, P16INK4A, ERK1/2 and NF-κB-p65 were analyzed using Western blotting.
Results: Cell proliferation and inflammatory cytokine/chemokine production of NHEKs were suppressed by LXA4, which caused G0/G1 phase cell cycle arrest in NHEKs. The expression of cyclin D1 was down-regulated by LXA4, contrary to the results of P16INK4A. The ERK1/2 phosphorylation and NF-κB-p65 nuclear translocation of NHEKs were both suppressed by LXA4.
Conclusion: Cell growth and inflammatory cytokine/chemokine production of NHEKs were inhibited by LXA4, and the inhibitory effects might be associated with the mechanisms of cyclin D1/P16INK4A, ERK1/2 and NF-κB signal transduction pathway.

1. Introduction

Lipoxin A4 (LXA4) is an endogenous lipoxygenase-derived eicosanoid mediator, synthesized by the sequential actions of lipoxygenase enzyme on arachidonic acid [1]. LXA4 displays potent dual pro-resolution and anti-inflammatory activities both *in vivo* and *in vitro* [2,3]. LXA4 inhibits leukocyte-mediated injury, promotes monocyte chemotaxis, recruitment, and phagocytosis of apoptotic neutrophils [4,5]. It also attenuates cell proliferation and pro-inflammatory cytokine/chemokine production induced by inflammatory cells and cytokines. Reports show that LXA4 inhibited the cell proliferation of human/rat mesangial cells and human lung fibroblasts induced by platelet-derived growth factor

Abbreviations: LXA4, lipoxin A4; IL-6, interleukin-6; IL-8, interleukin-8; NHEKs, normal human epidermal keratinocytes; LPS, lipopolysaccharide; CFSE, carboxyfluorescein diacetate succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase1/2; NF-κB-p65, nuclear factor kappa B p65.

* Corresponding authors at: Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Jiefang Avenue 1277#, Wuhan 430022, China. Tel.: +86 13871022932; fax: +86 2783694168.
E-mail addresses: arztfeng@126.com (A.-P. Feng), hongxiangchen@hotmail.com (H.-X. Chen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jdermsci.2015.03.009
0923-1811/

[6] and vascular endothelial growth factor, respectively, and that LXA4 antagonized IL-8 production in airway epithelial cells and IL-1β, IL-6 production in rat mesangial cell induced by TNF-α [7,8]. Some reports suggest LXA4 has an anti-inflammatory role that might involve the mechanism of signal transduction pathway of cell cycle proteins, MAPKs, PI3K/AKT, STAT3, and NF-κB signal transduction [8,9]. However, our previous study that reported the effects of LXA4 on normal human epidermal keratinocytes was the only study that suggested that LXA4 inhibited the release of IL-6 and IL-8 in NHEK. There is little research on the effects of LXA4 on NHEKs.

The epidermis is the most important medium between the host and the external environment. Keratinocytes constitute approximately 90% of the adult epidermis. Many reports have indicated that keratinocytes play an important role in inflammatory skin disease and cutaneous tumors [10,11]. In resting keratinocytes, immune factors are low. However, once triggered by various irritants, a significant increase of cell proliferation and production of inflammatory factors of keratinocyte could be detected both *in vitro* and *in vivo*. The inflammatory responses mediated by keratinocytes themselves and the cross-talk with other immune cells is the basis of pathology of many skin diseases [12].

LPS has been commonly used to model proinflammatory signals including cell proliferation and proinflammatory cytokine production [13]. LXA4 exhibits its anti-inflammatory bioactions by binding to two key receptors including LXA4 receptor (ALXR) and the aryl hydrocarbon receptor (AhR) [4]. Substantial evidence during the past has indicated that keratinocytes express TLR-4, ALXR and AhR [14–16]. LPS could induce various pro-inflammatory cytokines and chemokines in NHEKs *via* TLR4-driven signaling, LXA4 down-regulated both endogenous and pathological IL-6, IL-8 synthesis in keratinocytes by bonding to ALXR, and it was found that deficiency or silencing of AhR made keratinocytes hyperresponsive to inflammatory cytokines. These studies showed that these receptors are necessary for LXA4 to perform its anti-inflammatory role in NHEKs. However, the effect of LXA4 on cell proliferation and anti-inflammatory molecular mechanisms in NHEKs is still uncertain.

In this present study, we investigate whether LXA4 could modulate the cell proliferation and multiple pro-inflammatory cytokines. First chemokine production of NHEKs was investigated, then the signaling pathways involved in these processes, including the expression levels of cyclin D1, p16INK4A, ERK1/2 and NF-κB were examined.

2. Materials and methods

2.1. Isolation and culture of normal human epidermal keratinocytes

NHEKs were prepared from healthy individuals undergoing circumcision after informed consent. After removal of the subcutaneous tissue and most of the reticular dermis, tissues were cut into strips and sequentially two-step enzyme digested with Dispase (grade II, Roche Diagnostics, Mannheim, Germany) and trypsin (0.25% trypsin with 0.53 mM EDTA; Sigma–Aldrich, St. Louis, MO, USA). After being neutralized with equal volumes of DMEM medium containing 10% fetal calf serum (Life Technologies, Gaithersburg, MD, USA), the cuticle debris was aspirated and filtered through nylon mesh to get a single-cell suspension. At a density of 4×10^4 cells/cm^2, epidermal cells were seeded in keratinocyte serum-free medium (K-SFM) supplemented with epidermal growth factor, bovine pituitary extract (Life Technologies, Gaithersburg, MD, USA) and antibiotic/antimycotic solution (HyClone, Logan, UT, USA), which is also known as keratinocyte growth medium (KGM). KGM that has no epidermal growth factor and bovine pituitary extract is called keratinocyte basal medium (KBM). Cells were cultured at 37 °C with a humidified atmosphere of 5% CO_2. Medium was changed in 2–3 days interval. The third to fifth subcultures of keratinocytes at 70–80% confluence were used. LXA4 and LPS (*Pseudomonas aeruginosa* 10) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Sigma Chemical Co., respectively. Product numbers were shown in the supplemental data.

2.2. Cell viability assay

The cell viability assay is according to the cleavage of the tetrazolium salt-WST-8 (Dojindo Laboratories, Tokyo, Japan), where in viable cells are dyed orange by highly water-soluble formazan. NHEKs were seeded into a 96-well culture plate at a density of 2500 cells/well. As the most minimum effective concentration through the concentration gradient experiment, 100 nM was chosen for the concentration of LXA4 (Supplemental Fig. S1). LPS and LXA4 doses were replenished every 2 days in the respective 100 μl KGM. After 7days, the growth of NHEKs was quantitated by the WST-8 assay. 10 μl of WST-8 solution was added to each well, the mixture was further incubated for 6 h. The absorbance of each well was measured at a wavelength of 450 nm, with background subtraction at 650 nm. Before making the assay, we examined the cytotoxicity of LXA4 by trypan blue dye exclusion test, after treatment with LXA4 (100 nmol/l) in KGM for 7 days, NHEKs were washed and added with fresh media, and then stained with trypan blue dye (0.4%, w/v). The number of viable and dead cells were scored under the light microscope with Neubauer' hemocytometer. The number of live (not stained) cells and dead (stained) cells were determined with a Neubauer' hemocytometer.

2.3. Cell proliferation assay

First, NHEKs were resuspended in prewarmed phosphate buffered saline (PBS; HyClone) at a final concentration of 1×10^5 cells/ml, then, cells were labeled with 5 μM Carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, Carlsbad, CA, USA) for 10 min, at 37 °C in the dark. Staining was ended by the addition of 5 volumes of ice-cold media. After incubated 5 min on ice and washed for three times in PBS, the CFSE labeled NHEKs were resuspended in KGM and stimulated in a 7-day culture. LPS and LXA4 doses in the respective medium were replenished every 2 days. Cell proliferation was quantified by flow cytometry using a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) at channel FL1-H and the geometric mean (Geom. mean) fluorescence intensity was calculated using FlowJo software (Tree Star Software, San Carlos, CA, USA). The value of Geom. mean is inversely proportional to the degree of cell proliferation.

2.4. Detection of cell cycle phase by DNA staining

NHEKs were seeded into 6-well plates at a density of 3×10^5 cells/well in KGM. After 24 h, the medium was changed to KBM for 24 h NHEKs starvation, then the synchronized NHEKs in the G0 phase of cell cycle were obtained. After different intervention for 24 h, the NHEKs were harvested by trypsinization, washed twice in pre-cold PBS, and fixed in 70% ice-cold ethanol for 48 h at 4 °C. Then the NHEKs were collected by centrifugation, after washed twice in pre-cold PBS, the nuclei were stained with 50 μg/ml propidium iodide (PI; Dojindo) in 0.1% Triton-X100/PBS containing 50 μg/ml DNase-free RNase for 30 min at 37 °C. The cell cycle phase was analyzed by gathering the signal at channel FL2-A and measuring the DNA content using a BD FACSCalibur flow cytometer. The percentage of cells in the G0/G1, S, and G2/M phases was determined by Modfit LT program (Verity Software House, Topsham, ME, USA).