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Involvement of NADPH oxidase 1 in UVB-induced cell signaling and cytotoxicity in human keratinocytes

Azela Glady[a,1], Manami Tanaka[a,1], Catharina Sagita Moniaga[a], Masato Yasui[a,b], Mariko Hara-Chikuma[a,*]

[a] Department of Pharmacology, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan
[b] Keio Advanced Research Center for Water Biology and Medicine, Keio University, Japan

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ABSTRACT

Members of NADPH oxidase (Nox) enzyme family are important sources of reactive oxygen species (ROS) and are known to be involved in several physiological functions in response to various stimuli including UV irradiation. UVB-induced ROS have been associated with inflammation, cytotoxicity, cell death, or DNA damage in human keratinocytes. However, the source and the role of UVB-induced ROS remain undefined.

Here, we show that Nox1 is involved in UVB-induced p38/MAPK activation and cytotoxicity via ROS generation in keratinocytes. Nox1 knockdown or inhibitor decreased UVB-induced ROS production in human keratinocytes. Nox1 knockdown impaired UVB-induced p38 activation, accompanied by reduced IL-6 levels and attenuated cell toxicity. Treatment of cells with N-acetyl-L-cysteine (NAC), a potent ROS scavenger, suppressed p38 activation as well as consequent IL-6 production and cytotoxicity in response to UVB exposure. p38 inhibitor also suppressed UVB-induced IL-6 production and cytotoxicity. Furthermore, the blockade of IL-6 production by IL-6 neutralizing antibody reduced UVB-induced cell toxicity.

In vivo assay using wild-type mice, the intradermal injection of lysates from UVB-irradiated control cells, but not from UVB-irradiated Nox1 knockdown cells, induced inflammatory swelling and IL-6 production in the skin of ears. Moreover, administration of Nox1 inhibitor suppressed UVB-induced increase in IL-6 mRNA expression in mice skin.

Collectively, these data suggest that Nox1-mediated ROS production is required for UVB-induced cytotoxicity and inflammation through p38 activation and inflammatory cytokine production, such as IL-6. Thus, our findings suggest Nox1 as a therapeutic target for cytotoxicity and inflammation in response to UVB exposure.

1. Introduction

Solar ultraviolet (UV) radiation is a primary risk factor for skin carcinogenesis, such as basal and squamous cells carcinomas [1–3]. Among three classified types of UV radiation (UVA, UVB, and UVC), UVB (280–320 nm) is the most energetic and leads to biological harm, including direct DNA damage, the activation of receptor-mediated signaling pathways, and the production of reactive oxygen species in high amounts (ROS; O_2^-, OH, and H_2O_2) [3,4]. The adverse effect of UVB-induced ROS in keratinocytes has been associated with the initiation of inflammation and cytotoxicity, followed by carcinogenesis, in which various molecular pathways are involved [1,5,6]. Previous studies have shown that UVB radiation activates several signaling pathways including nuclear factor-κB (NF-κB), the signal transducer and activator of transcription 3 (STAT3), p38/MAPK, or c-Jun N-terminal kinase (JNK) signaling, which affect apoptosis, cytotoxicity, or inflammation in human keratinocytes [1,3,6]. Among these pathways, the UVB-induced activation of p38 and JNK signaling has been reported to be partially ROS dependent in keratinocytes. However, mechanisms underlying the UVB-induced activation of these pathways remain undefined. In addition, the source of ROS generation in keratinocytes in response to UVB radiation has not been completely determined [1,7,8].

Abbreviations: UV, Ultraviolet; ROS, Reactive Oxygen Species; O_2-, Superoxide; OH, Hydroxyl radical; H_2O_2, Hydrogen peroxide; NOX, NADPH oxidase; PBS, Phosphate-buffered saline; DNA, Deoxyribonucleic acid; RNA, Ribonucleic acid; DPI, Diphenyleneiodonium; NAC, N-acetyl cysteine; H2DCFDA, Fluorescent 2',7'-dichlorofluorescein diacetate; LDH, Lactate dehydrogenase; IL-6, Interleukin-6; TNF-α, Tumor necrosis factor-alpha; GM-CSF, Granulocyte-macrophage colony-stimulating factor; NF-κB, Nuclear factor kappa B;; STAT3, Signal transducer and activator of transcription 3; MAPK, Mitogen-activated protein kinase; JNK, Jun N-terminal kinases;; Erk, Extracellular Signal-regulated kinase; MKK, MAP Kinase; MKP, MAPK phosphatase; ASK1, Apoptosis signal-regulating kinase 1; Bax, BCL2-associated X protein

* Corresponding author.
E-mail address: haramari@kuhp.kyoto-u.ac.jp (M. Hara-Chikuma).
[1] These authors contributed equally.

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Enzyme NADPH oxidase (NOX) is one of the most important sources of intracellular ROS in mammalian cells. NOX is activated by various cellular stresses, such as chemical factors, cellular stimuli, or UV exposure [9,10]. Members of NOX family (Nox1–5, Duox1, and 2) are involved in several physiological functions, including innate immunity, signal transduction, and biochemical reactions, and they show different tissue distribution with discrete activation mechanisms through ROS production [9,11]. Among the members of NOX family, Nox1 and 2 have been observed to generate ROS in response to UVA or UVB radiation in human keratinocytes [12–19]. In response to UVA, ROS production has been shown to be dependent on Nox1 and/or Nox2 in keratinocytes [13,14,17]. In contrast, though Ryu et al., have suggested the involvement of Nox1 in UVB-induced ROS production and apoptosis in keratinocytes [12], the source and the role of UVB-induced ROS are still undefined. Because ROS act as signaling molecules and regulate a variety of cellular responses [20,21], we postulated that Nox1-generated ROS might be implicated in some specific cell signaling pathways, leading to cell death or inflammation in response to UVB irradiation in keratinocytes.

The aim of this study was to determine the role of Nox1 in UVB-induced cell signaling, inflammation, or cytotoxicity via ROS production in keratinocytes. We utilized human keratinocyte cell lines HaCaT with Nox1 knockdown by siRNA. Here, we report that Nox1 is required for UVB-induced inflammation and cytotoxicity via a mechanism involving UVB-induced ROS production by Nox1 in p38/MAPK cell signaling pathway.

2. Materials and methods

2.1. Cell line and UVB irradiation

The human keratinocyte HaCaT cells (a kind gift of Dr Fusenig, German Cancer Research Center, Heidelberg, Germany) were cultured in Dulbecco's Modified Eagle's medium (DMEM, WAKO) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (5% CO_2, 37 °C). Cells were transfected with Nox1 or non-targeting-siRNA (ON-TARGET plus SMART pool, GE Healthcare) using Lipofectamine 2000™ (Invitrogen Life Technologies) according to the manufacturer's protocol [22]. After the cultures reached 90% confluency, the culture medium was replaced to phosphate-buffered saline (PBS), and cells were exposed to UVB radiation (15–100 mJ/cm^2, 302 nm peak, UV Bench Lamp, UVP) monitoring with a UV Light Meter. Cells were harvested at different time point as described later.

2.2. Cellular ROS analysis

Cellular ROS level was assayed using the CM-H2DCFDA reagent (Invitrogen Life Technologies) according to manufacturer's instructions using a microplate reader. Briefly, cells seeded in a 96-well plate were stained with CM-H2DCFDA reagent (20 μM in PBS, 10 min), and washed by PBS. The cellular fluorescence was assessed by a fluorescent microplate reader (SpectraMax Paradigm, Molecular Devices, Ex 492 nm, Em 525 nm). In some setting, cells were incubated with ML171 (10 μM), GKT137831 (100 μM), diphenyleneiodonium (DPI, 50 μM), NAC (5 mM) or vehicle (Veh) for 1 h, and followed by UVB irradiation.

To assess the intracellular H_2O_2 level, cells were transfected with H_2O_2 sensitive probe pHyPer cDNA plasmid (Evorgen) with Lipofectamine 2000 (Invitrogen) according to a manufacture's instruction [23,24]. G418 resistant transfectants were isolated as pHyPer stably transfected cells. Cellular fluorescence was assessed by a fluorescent microplate reader (SpectraMax Paradigm, Molecular Devices, YFP: Ex 497 nm, Em 525 nm, CFP: Ex 434 nm, Em 525 nm). YFP to CFP excited (497/434) ratio was calculated as intracellular H_2O_2 level [23,25].

2.3. viability and cytotoxicity

The culture medium of HaCaT cells seeded in a 96-well plate was replaced to PBS, and cells were exposed to UVB radiation (15 or 30 mJ). Cells were cultured in DMEM medium containing 10% FBS for 24 h. To assess cell viability, cells were estimated with a Cell Count Reagent SF kit (Nacalai Tesque). For cytotoxicity assay, culture medium (DMEM) was replaced to PBS, and collected PBS after incubation for 2 h. Cytotoxicity was determined by Cytotoxicity LDH assay kit according to manufacturer's instructions (WAKO).

2.4. Real-time quantitative RT-PCR

HaCaT cells in a 24-well plate were irradiated with UVB (30 mJ/cm^2) and extracted mRNA at 6 h. Total RNA was extracted using TRIZOL (Invitrogen Life Technologies). The cDNA was reverse transcribed from total RNA using the Prime Script RT reagent kit (Takara Bio). Quantitative RT-PCR was performed using SYBR Green I (Takara Bio) and real-time PCR apparatus Step One Plus (Applied Biosystems).

2.5. Immunoblotting, ELISA assay, Immunofluorescence

Cells seeded in 60 mm culture dish were lysed with RIPA buffer (Cell Signaling Technology). The supernatant (10,000 g, 10 min, 4 °C) was performed for immunoblotting with antibodies against phospho-Erk, Erk, phosphor-p38, p38, phospho-Akt and Akt (Cell Signaling Technology, 1:1000), or β-actin (Sigma, 1:2000).

IL-6 amount in the culture supernatant (96-well plate) was quantified with IL-6 human ELISA Kit (Thermo Fisher Scientific) according to a manufacture's instruction.

For Nox1 immunostaining, cells were fixed with 10% formalin, and permeabilized with 0.2% saponin, and immunostained with anti-Nox1 (Santa Cruz), followed by anti-rabbit secondary antibody (FITC, Sigma).

For immunostaining with γH2AX, cells were fixed with 4% formalin, and permeabilized with 0.3% Triton X-100. Cells were stained with anti-phospho-Histone H2A.X (ser139, Cell Signaling Technology, 1:200), followed by anti-rabbit secondary antibody (cy3, Sigma). Cells were also stained with Alexa488-conjugated phalloidin (Invitrogen, Carlsbad, CA). Fluorescent images were obtained with a confocal microscopy LSM710 (Carl Zeiss).

2.6. Skin inflammation in vivo

C57BL/6 mice (8 weeks, female, JAPAN SLC) were administered intradermal ear injections of GKT137831 (200 μM in PBS, 50μl/ear) or vehicle (PBS, 50μl/ear). After 1 h, the mice were irradiated with UVB (100 mJ). The total RNA was extracted from harvested skin tissue at 24 h after UVB irradiation using TRIZOL (Invitrogen Life Technologies), and the mRNA level was analyzed by real-time PCR as described above.

HaCaT cells with control- or Nox1-siRNA were irradiated with UVB (30 mJ/cm^2). Cells were collected at 1 h and sonicated in PBS. C57BL/6 mice (8 weeks, female, JAPAN SLC) were administered intradermal ear injections of the cell lysate (50μl, 1 × 10^5 cells/ear). The ear thickness was measured using a micrometer at 24 h after injection. The mRNA level of harvested skin tissue at 24 h after injection was analyzed by real-time PCR.

All animal experiments were approved by the Committee on Animal Research of Keio University.

2.7. Statistical analysis

Unless specifically stated, statistical analysis was performed with the unpaired two-tailed Student's t-test.