Prostaglandin E_2 is critical for the development of niacin-deficiency-induced photosensitivity via ROS production

Kazunari Sugita MD, PhD[1,2], Atsuko Ikenouchi-Sugita MD, PhD[3], Yasuko Nakayama[1], Haruna Yoshioka MD[1], Takashi Nomura MD, PhD[2], Jun-ichi Sakabe[4], Kyoko Nakahigashi MD, PhD[2], Etsushi Kuroda PhD[5], Satoshi Uematsu MD, PhD[6], Jun Nakamura MD, PhD[3], Shizuo Akira MD, PhD[7], Motonobu Nakamura MD, PhD[1], Shuh Narumiya MD, PhD[8], Yoshiki Miyachi MD, PhD[2], Yoshiki Tokura MD, PhD[4] & Kenji Kabashima MD, PhD[2*]

Departments of [1]Dermatology and [3]Psychiatry, University of Occupational and Environmental Health, Kitakyushu, Japan

Departments of [2]Dermatology and [8]Pharmacology, Kyoto University Graduate School of Medicine, Kyoto, Japan

[4]Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Japan

[5]Laboratory of Vaccine Science, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan

[6]Division of Innate Immune Regulation, International Research and Development Center for Mucosal Vaccines, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

[7]Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka

University, Osaka, Japan

*Corresponding author: Kenji Kabashima, MD, PhD or Kazunari Sugita, MD, PhD

Department of Dermatology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawara, Kyoto 606-8507, Japan

Phone: +81-75-751-3310

Fax: +81-75-761-3002

Email: kaba@kuhp.kyoto-u.ac.jp or k-sugita@med.uoeh-u.ac.jp (KS)

Methods

Clinical score. To assess the extent of colitis, body weight, stool consistency and occult blood in the stool were monitored. Diarrhea was scored as follows: 0, normal; 2, loose stools; 4, watery diarrhea. Hemoccult was scored as follows: 0, normal; 2, hemoccult positive; 4, gross bleeding.

Histology. For histological examination, tissues were fixed with 10% formalin in PBS at pH 7.4 and then embedded in paraffin. Sections of 5 μm thickness were prepared and subjected to staining with H&E. The histological findings were evaluated as reported previously[1]. In brief, samples were scored for severity and features of the inflammatory response using a subjective grading scale. Responses were graded as follows: 0, no response; 1, minimal response; 2, mild response; 3, moderate response; and 4, marked response. The total histology score was calculated as the sum of scores, including inflammation, neutrophil, mononuclear cells, edema and epithelial hyperplasia[1]. In addition, the histological degree of colitis was assessed by a histological scoring system as reported previously[2].

For human skin samples, paraffin-embedded sections were stained with PGE synthase (PTGES), a polyclonal rabbit antihuman antibody (dilution 1:200, Cayman Chemical, Ann Arbor, MI). As a negative control for PTGES, we used an isotype-matched control antibody. Non-specific binding was blocked by addition of 10% goat serum for 30 minutes at room temperature. Afterward, sections were incubated for 1 hour at room temperature with primary antibody followed by incubation with a species-specific

biotiynylated immunoglobulin for 30 minutes at room temperature. Thereafter, samples were incubated for 30 minutes using an avidine-biotin-peroxidase complex kit and visualized with 3,3'-diaminobenzidine. The numbers of immunoreactive cells and keratinocytes per high power field were enumerated at three locations (original magnification x200) per sample, and data were expressed as the number of PTGES positive cells per high power field and percentage of PTGES positive cells per keratinocytes. For experiments involving human subjects (including the use of tissue samples), we confirmed that informed consent was obtained from all subjects.

Measurement of NAD and NADH. Seven days after UVB irradiation, skin samples were quickly isolated and homogenized. Homogenates were separated by NAD/NADH lysis buffer and the products of NAD+ and NADH in the supernatant were analyzed by using fluorescent NAD/NADH detection kit (Cell Technology, CA).

Keratinocyte cell line and preparation of epidermal cell suspensions. PAM 212 cells, an epidermal cell line derived from BALB/c mice, were cultured in six-well culture plates in Dulbecco's minimal essential medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY). All experiments were performed at the semiconfluent stage. Skin sheets from mouse ear lobes were floated in 0.2% trypsin in PBS for 30 minutes at 37°C. The epidermis was

separated from the dermis in PBS supplemented with 10% FCS. The epidermal cells obtained were filtered through a 70 μm filter.

Quantitative PCR analysis. RNA was extracted from skin samples and PAM212 cells using RNeasy (Qiagen, Valencia, CA) and reverse-transcribed into cDNA with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Gene products were quantified using the TaqMan Gene Expression Assay in the ABI PRISM 7000 sequence detection system as follows: *Ptgs2* (COX-2) (Assay ID: Mm00478374_s1), *Ptges* (also known as mPGES-1) (Assay ID: Mm00452105_m1), *Ptges3* (cPGES) (Assay ID: Mm00727367_s1), *Ptgds* (PGDS) (Assay ID: Mm01330613_m1), *Ptgfs* (PGFS) (Assay ID: Mm00481612_m1), *Ptgis* (PGIS) (Assay ID: Mm00447271_m1), *Tbxas1* (TXS) (Assay ID: Mm00495553_m1), EP1 (Assay ID: Mm00443097_m1), EP2 (Assay ID: Mm00436051_m1), EP3 (Assay ID: Mm00441045_m1) and EP4 (Assay ID: Mm00436053_m1) (Applied Biosystems, Foster City, CA). As an endogenous reference, β-actin gene expression (*Actb*) was measured using the TaqMan rodent *Actb* control reagents (Applied Biosystems, Foster City, CA). The relative expression was calculated according to the $2_{T}^{-\delta\delta C}$ method.

Measurement of skin blood flow. The abdominal regions of mice were shaved and irradiated with UVB. Blood flow was assessed using a laser doppler flow meter (Advance Co., Ltd., Tokyo, Japan; model ALF21R). The probe was held to five spots on the abdominal area, and the mean of these five measurements was recorded.

Reactive oxygen species measurement. To examine the ROS production, we cultured primary keratinocytes from C57BL/6 mice in 6-well plates and exposed with UVB (30 mJ/cm^2) in the presence or absence of NAC (20 mM) and/or 6-AN (250 μM). ROS generation was measured using 2', 7'-dichlorodihydrofluorescin diacetate (Cell Biolabs, San Diego, CA), which was oxidized to fluorescent dichlorodihydrofluorescein in the presence of ROS. Cells were incubated with 10 mM of 2', 7'-dichlorodihydrofluorescin for 60 min, and the reaction was quenched via the addition of the cell lysis buffer provided with the kit. After incubation for 5 min, 150 μl of the mixture was transferred to a new microplate and the fluorescence was read with a fluorometric plate reader.

Statistical analysis. Data were analyzed using an unpaired two-tailed *t*-test. $P < 0.05$ was considered to be significant.

References

1. Nakajima, S., Honda, T., Sakata, D., Egawa, G., Tanizaki, H. *et al*. Prostaglandin I2-IP signaling promotes Th1 differentiation in a mouse model of contact hypersensitivity. *J Immunol* **184**, 5595-5603 (2010).

2. Kruschewski, M., Foitzik, T., Perez-Canto, A., Hubotter, A. & Buhr, HJ. Changes of colonic mucosal microcirculation and histology in two colitis models: an experimental study using intravital microscopy and a new histological scoring system. *Dig Dis Sci* **46**, 2336-2343 (2001).

Supplementary Table S1

Scoring of histological findings of C57BL/6 mice treated with or without 6-AN

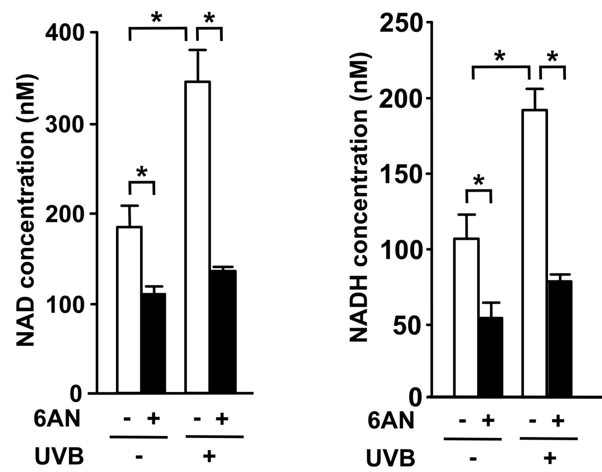
	6AN-	**6AN+**
Inflammation	1.67±0.44	2.75±0.38*
Neutrophils	1.33±0.32	3.5±0.5*
Mononuclear cells	0.67±0.17	1.92±0.36*
Edema	2.17±0.16	3.08±0.22*
Epithelial hyperplasia	1.83±0.43	2.08±0.30

*, $P<0.05$

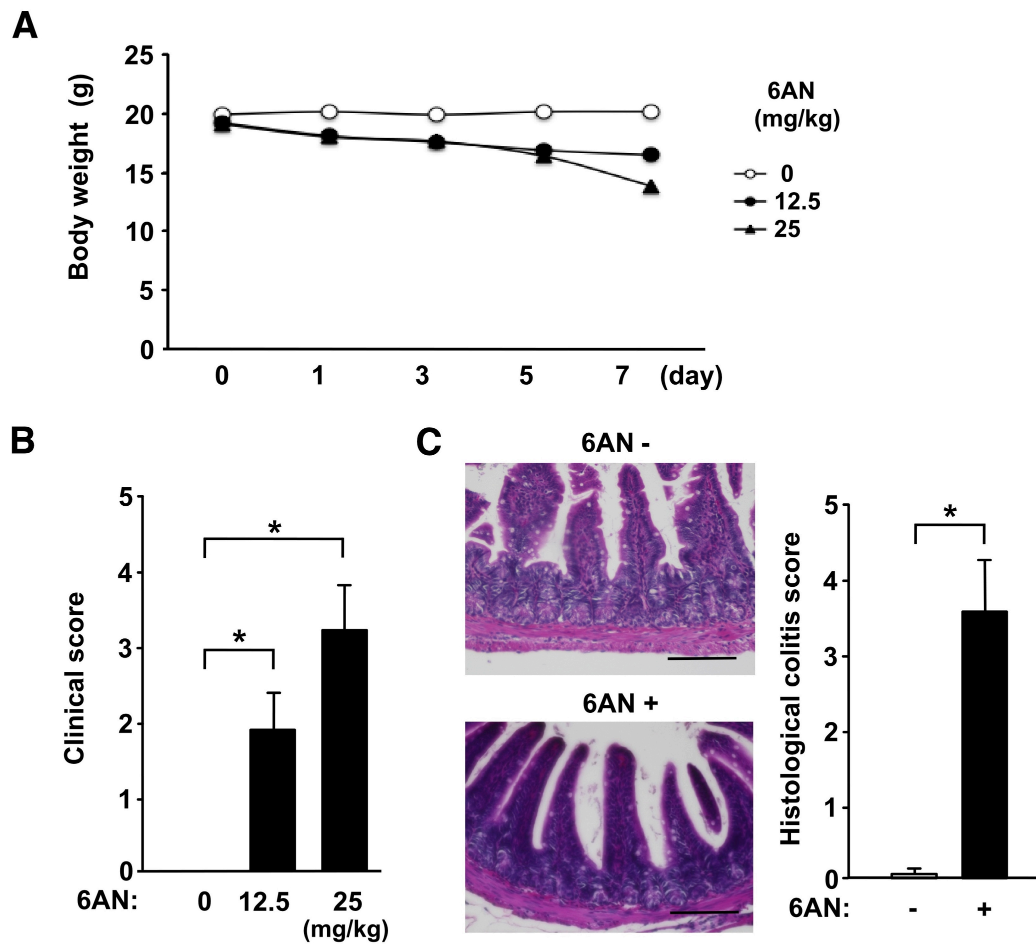
Supplementary Table S2

Scoring of histological findings in *Ptges*$^{-/-}$ mice treated with or without 6-AN

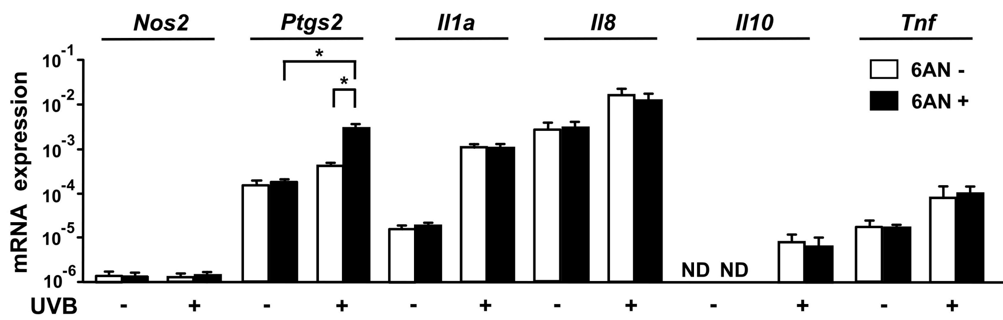
	6AN-	**6AN+**
Inflammation	0.83±0.23	1.25±0.27
Neutrophils	1.00±0.20	1.75±0.10
Mononuclear cells	0.83±0.24	1.32±0.17
Edema	0.67±0.31	0.82±0.10
Epithelial hyperplasia	1.58±0.30	1.77±0.09



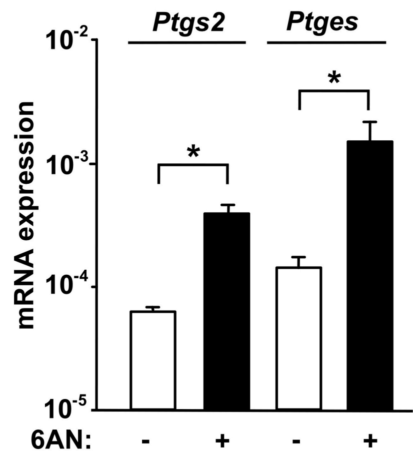
Supplementary Figure S1 Concentrations of total NAD and NADH in the UVB irradiated skin. Administration of the niacin antagonist (6-AN) significantly decreased NAD and NADH levels. Data are presented as means ± SEM. *, $P < 0.05$.



Supplementary Figure S2 Niacin antagonist (6-AN) induces the development of diarrhea.
(A) C57BL/6 mice treated with or without 6-AN were weighed at the indicated time points. (B) Clinical scores of mice treated with 6-AN. Data are presented as means ± SEM. *, *P*< 0.05. (C) Representative H&E staining of large intestine after 7 days of 6-AN treatment. Original magnification, x200. Scale bar 100 μm. Histological score of mice treated with 6-AN (12.5mg/kg). Data are presented as means ± SEM. *, *P*< 0.05.



Supplementary Figure S3 Quantitative PCR analysis of *Nos2*, *Ptgs2* and cytokine mRNA expression.
C57BL/6 mice were treated with or without 12.5 mg/kg 6-AN. Earlobes skin samples were collected three days after 5 kJ/m^2 UVB exposure and homogenized. The amounts of mRNA for *Nos2*, *Ptgs2* and the indicated cytokines were measured and expressed as relative quantities normalized to β-actin (*Actb*). Data are presented as means ± SEM.
*, P<0 .05



Supplementary Figure S4 Increased *Ptgs2* and *Ptges* levels in the large intestine of 6-AN treated mice.
Large intestines from C57BL/6 mice treated with or without 6-AN (25mg/kg) for 7 days after the treatment were examined