Change of Inducible Nitric Oxide Synthase Expression by Ultraviolet B Irradiation on the Skin of a Rat

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Background **: Nitric oxide synthase (NOS) is known to mediate ultraviolet B (UVB)-induced skin inflammation However, there is still ambiguity as to which NOS isotype mediates the process *in vivo*. Furthermore, contradictory results have been reported on which cell types respond to UVB irradiation *in vitro*.**

Objective **: This study was performed to evaluate the change of inducible NOS (iNOS) expression in vivo as a result of UVB radiation on the skin of a rat.**

Method **: To examine the time-course change in iNOS expression in the rat skin, the rats were exposed to 400 mJ/cm^2 of UVB radiation, and skin samples were taken at various time intervals up to 48 h. iNOS expression on the skin of a rat was evaluated by both Western blot analysis and immunohistochemical staining.**

Results **: From Western blot analysis, UVB irradiation induced inducible NOS (iNOS) expression in the epidermis at 12-48 h postirradiation with a peak expression at 24 h. Immunohistochemical staining revealed that UVB-induced iNOS expression was localized to the epidermis and infiltrating inflammatory cells in the upper dermis of the rat.**

Conclusion **: iNOS was induced by UVB irradiation on the skin of a rat, mainly in the epidermis. Therefore, iNOS is supposed to be one of the major mediators with regard to inducing an inflammatory response in UVB-irradiated rat skin in vivo.**

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Key Words : iNOS

Nitric oxide synthase (NOS), a key enzyme in synthesizing nitric oxide (NO) from L-arginine, has two distinct types based on different biological and biochemical characteristics. The Ca^{++}-calmodulin-dependent enzyme is constitutively expressed in several tissues (cNOS, constitutive NOS), and the Ca++-independent enzyme is induced by immunologic or inflammatory stimuli (iNOS, inducible NOS)[1]. iNOS was first described in macrophages, but in later studies, it was also shown to be found in several skin cell types including fibroblasts[2], Langerhans cells[3], and endothelial cells[4]. On the other hand, there have been other reports that iNOS mRNA and protein were constitutively present in the normal epidermis and epidermal keratinocytes without stimuli[5,6]. Human fibroblasts were also known to express the mRNAs and proteins of both cNOS and iNOS[7].

In terms of NOS expression and UV irradiation, contradictory results have been reported on the expression of the NOS isotypes that respond to UV irradiation. This has been partly due to the different cell types and experimental conditions used including the UV source and radiation dose. iNOS, which was not found in a non-stimulated

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state, was induced by UVB irradiation in the epidermal keratinocytes[8,9]. cNOS, which is known to be the major form present in the normal skin, can also be activated by UVB irradiation[10-12]. UVB irradiation induces cNOS expression in human keratinocytes[10], and also activates cNOS activity in the transformed keratinocytes, SCC-1311. Kang-Rotondo et al[12] demonstrated that brain NOS (bNOS) was the major isoform induced by UVB irradiation in human keratinocytes.

In view of previous studies on NOS expression, most have been performed with cultured cells of keratinocytes and fibroblasts in vitro. However, there is a limited amount of data available on NOS expression in normal and UVB-irradiated skin in vivo[6,13,14]. Furthermore, the available data is inconsistent in terms of NOS isotype(s) and cell type(s) in responding to UVB irradiation. In this study, we evaluate the change of iNOS expression on the skin of a rat by UVB irradiation in vivo.

MATERIALS AND METHODS

Animals

The rats used were male Sprague-Dawley rats (age; 4 wks, weight; 40g) and were purchased from the Dae Han Lab. Animal Research Center Co. (Seoul, Korea). The rats were housed in stainless steel isolation cages, and were fed with commercial mouse chow and tap water available ad libitum. The rats were kept in under standard temperature and light conditions. In the experiments, the rats were pretreated by shaving the hairs of the dorsal skin with a hair clipper.

UVB Source and UVB irradiation in vivo

A fluorescent UVB lamp (FSX72T 12/UVB-HO, National Biological Corp., OH, USA), which emits UV light at a wavelength of between 290-320 nm, with a peak intensity at 300 nm, was used. An IL 700 Research Lightmeter (International Light, MA, USA) fitted with a WN 320 filter, which is specific for UVB wavelengths, was used to measure the intensity of the UVB light. The intensity was maintained at 1.0 mW/cm^2/sec by adjusting the distance between the UVB lamps and the rats. A total UVB dose of 400 mJ/cm^2 was delivered once by adjusting the radiation time to 6 min 40 sec. During irradiation the rats were housed in a cage, moving freely as their shaved backs were constantly exposed to the lamp.

Preparation of skin extracts from rat skin

To prepare skin extracts from rat skin, the excised skin tissue was trimmed by removing any adherent subcutis and immediately frozen in liquid nitrogen. To prepare the epidermal and dermal skin extracts, the whole excised skin was pretreated at 55oC for 30-60 sec, after which the epidermis was separated from the dermis with a scalpel[15]. The skin samples were pulverized in a mortar containing liquid nitrogen and further homogenized with 5 volumes of extraction buffer (50 mM potassium phosphate buffer, pH 7.4 including 0.1 M KCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) in a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 rpm for 15 min in a Sorvall SS-34 rotor at 4oC, and the supernatant fraction was taken to get skin extracts after removal of a surface lipid layer from the surface.

Western blot analysis of iNOS expression

The rat skin extracts (epidermal, and dermal) were used as protein samples for Western immunoblotting. Protein sample quantitation was performed by the method reported by Bradford[16] using bovine serum albumin as a standard. After boiling the samples for 5 min in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 10% -mercaptoethanol), 60 μg of each sample was separated on an 8% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane with the use of an electroblotter (Xcell II Mini-Cell, Novex, CA, USA). Blocking of the membrane was done in a 5% skim milk solution for 1 h at room temperature (RT), followed by washing with buffer solution (Tris-buffered saline with 0.1% tween-20; TBS-T) for 5 min 3 times. The membrane was incubated for 3 h at RT with a monoclonal antibody to the mouse macrophage (iNOS) diluted to 1:1000. The primary antibody was purchased from the Transduction Laboratories Co. (KY, USA). After washing, the membrane was incubated for 1 h at RT with the anti-mouse IgG antibody conjugated with horseradish peroxidase diluted 1:2000. Visualization of the immune complexes was performed with an enhanced chemiluminescence kit (Amersham, IL, USA).

The Western blot analysis was repeated at least 3 times for reproducibility.

Immunohistochemistry of iNOS in the rat skin

For immunohistochemical staining of the rat skin, excised dorsal skin was fixed in a 10% formalin solution overnight at RT. After which, the specimens were rinsed in absolute alcohol, rehydrated to 70% ethanol, then processed for paraffin embedding. The paraffin sections, 6μm in thickness, were deparaffinized by two washes with xylene. The samples were then preincubated with 3% hydrogen peroxide in distilled water for 10 min to block the endogenous peroxidase activity, and were incubated with a monoclonal antibody to iNOS diluted 1:200, for 1 h at RT. Subsequently, they were incubated with a biotinylated anti-rabbit secondary antibody to anti-IgG (LSAB kit, DAKO, Denmark), and further incubated with streptoavidin conjugated to horseradish peroxidase for 20 min at RT. The samples were then visualized with 3-amino-9-ethylcarbazole used as a chromogen. Between each step, the samples were washed with phosphate buffered saline 3 times. As a negative control, other samples were incubated with non-immunized serum instead of the primary antibody.

RESULTS

Western blot analysis of iNOS expression in UVB irradiated rat skin

To examine the time-course change of iNOS expression in the rat skin, the rats were exposed to 400 mJ/cm^2 of UVB dose, and skin samples were taken as various time intervals up to 48 h. iNOS expression was not identified in either the epidermal or dermal extracts of non-irradiated normal skin. However, UVB-irradiation induced iNOS expression, which was identified in the epidermal extract at 12-48 h post-irradiation, with a peak expression at 24 h (Fig. 1). The induced iNOS expression was not identified in the dermal extract (data not shown).

Immunohistochemistry of iNOS expression in UVB irradiated rat skin

After the 400 mJ/cm^2 of UVB dose was given to the rats, skin samples were taken at 12, 24, and 48 h after irradiation. In non-irradiated normal skin, positive staining for iNOS was not found in either

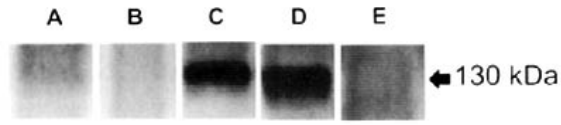

Fig. 1. Change of inducible nitric oxide synthase expression in the epidermal extract by ultraviolet B (UVB) irradiation. After the rats were exposed to 400 mJ/cm2 of UVB dose, skin samples were taken at various time intervals up to 48 h. Western blot analysis was performed as described in Materials and Methods. A; Non-irradiated control, B; 6 h post-UVB, C; 12 h post-UVB, D; 24 h post-UVB, and E; 48 h post-UVB.

the epidermis or the dermis except in the sebaceous glands (Fig. 2a). After 12 h post-irradiation, positive staining for iNOS was mainly localized to the infiltrating inflammatory cells in the upper dermis as a scattered pattern (Fig. 2b). At 24 h post-irradiation, positive staining for iNOS was localized to the epidermis and the inflammatory cells (Fig. 2c). The expression pattern of iNOS was normalized to that at 48 h post-irradiation (Fig. 2d). The negative control incubated with nonimmunized serum, did not show any specific staining in either the epidermis or the dermis.

DISCUSSION

The iNOS expression in the skin has been reported mainly in pathologic conditions such as atopic dermatitis, contact dermatitis, and psoriasis[4,17-20]. In psoriasis, iNOS expression is induced in the skin in vivo and in vitro[17-20]. In addition, normal epidermal keratinocytes can express iNOS, when they are stimulated by INF-γ, lipopolysaccaride (LPS), IL-1β and TNF-α[5,18]. However, there is a lot of data suggesting that iNOS is not expressed in the epidermis and epidermal keratinocytes, when they are not stimulated by cytokines or UV irradiation *in vivo* and in vitro[3,7,10,12-14]. In contrast, contradictory results suggest that iNOS can be expressed in normal keratinocytes without stimulation: iNOS mRNA was expressed in human keratinocytes *in vitro*[5]; and iNOS protein was expressed in epidermal keratinocytes and the outer root sheath of hair follicles[6]. Other reports have shown that fibroblasts could express iNOS in non-stimulated normal state[3,13]. In this study, iNOS expression, which was not identified in the epidermis and dermis in the nor-

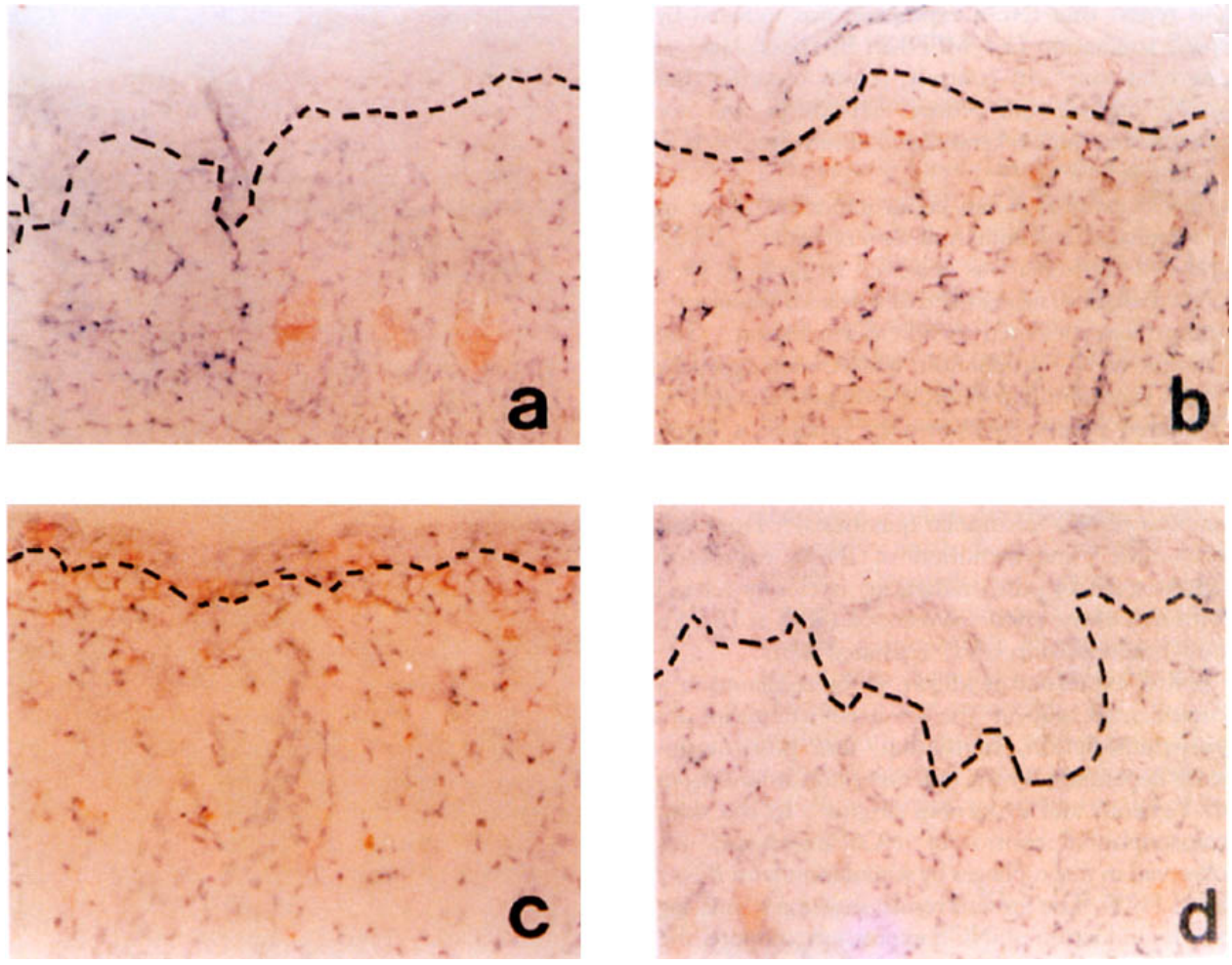

Fig 2. Time-course expression of inducible nitric oxide synthase in ultraviolet B (UVB)-irradiated rat skin a; Non-irradiated control, b; 12 hours post-UVB, c; 24 hours post-UVB, and d; 48 hours post-UVB (hematoxylin & eosin, x 200). A dotted line indecates the dermoepidermal junction of the skin.

mal condition, was induced by UVB irradiation in the epidermis.

Normal keratinocytes[10] and fibroblasts[2] have been reported to express cNOS without stimulation in vitro. We also identified the constitutive expression of cNOS, including bNOS and endothelial NOS (eNOS), in normal rat skin by Western blot analysis (data not shown). The accumulation of data indicates that cNOS (eNOS or bNOS) is also induced by UV irradiation. Up-regulation of NOS activity by UVB irradiation was observed in keratinocytes, and bNOS was the major isoform to be induced by UVB irradiation[11,12,21]. The mechanism by which UV radiations activate cNOS remains to be explained. It can be proposed that UV radiation increases one of cofactors involved in cNOS regulation, such as calcium, NADPH, or tetrahydro-biopterin (6-BH4), which is required for the enzymatic activity[10].

The induction of iNOS expression originated from the epidermis. From our Western immunoblot data, positive bands for iNOS were identified only in the epidermal extract. The result was commensurate with immunohistochemical data that showed positive signals for iNOS, which were mainly localized to the epidermis, besides infiltrating inflammatory cells in the upper dermis. The results are largely consistent with the previ-

ous report that iNOS expression was induced by UVB irradiation (1.5 MED) in the basal layer of the epidermis[13]. In contrast, it was reported that iNOS mRNA and protein expression was not identified in human keratinocytes even after UVB irradiation[10].

iNOS expression can be an important event in UV-induced inflammation or erythema[8,9]. UVB-induced erythema was observed to peak at 18 h post-irradiation, suggesting that a delayed vascular response was mediated by iNOS expression[21]. The amount of NO production, which is mediated by NOS, is determined by NOS isotypes. The iNOS-mediated process is known to produce a larger amount of NO (nanomolar quantities), while the cNOS-mediated process produces a smaller amount of NO (picomolar quantities)[22]. From this study, the time evolution of iNOS expression (peak at 12-24 post-irradiation) is closely related with that of the erythema reaction (peak at 18-24 h post-irradiation) in UVB-irradiated skin[21].

iNOS expression is known to be regulated in a complicated way at the levels of transcription, post-transcription, translation, and post-translation, depending on the cell types and stimuli from endogenous and exogenous origins[23]. In the skin, transcriptional control of iNOS expression was observed in macrophages by stimulation with IFN-γ and LPS[24,25]. The present result, showing a time-lag for the induction of iNOS expression, supports the transcriptional regulation of iNOS expression in UVB-irradiated skin[9,13]. It is presumed that UVB up-regulates the production of various cytokines, which stimulate iNOS expression in the epidermis. In the previous report, UVB up-regulated IL-1 α, IL-6, IL-8, and TNF-α mRNA expression in human keratinocytes[26]. Factors that can stimulate iNOS include INF-γ, LPS, interleukins, TNF-α, and the granulocyte-monocyte colony-stimulating factor[27].

In summary, our study reveals that iNOS from the epidermis and inflammatory cells is the major isotype among NOS to be induced by UVB irradiation, and iNOS is one of mediators to induce an inflammatory response in UVB-irradiated rat skin. Further studies are warranted in order to develop iNOS inhibitors as a new anti-inflammatory agent for the UVB-induced sunburn reaction.

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