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Short Communication

DIFFERENTIAL EXPRESSION OF NITRIC OXIDE SYNTHASES IN THE SKIN TISSUE OF *P*-PHENYLENEDIAMINE-TREATED MICE

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ABSTRACT

Objective: *p*-Phenylenediamine (PPD) is a possible contact sensitizer in skin tissues; however, data are lacking regarding its specific effects on nitric oxide synthase (NOS) expression during the sensitization phase. The purpose of this study was to investigate nNOS and iNOS expression in the skin tissue of PPD-treated mice.

Methods: BALB/c mice were dermally exposed to PPD, at a dose of 10 or 50 mg/kg, on three occasions. The ear and dorsal skin tissues were then isolated from PPD-treated mice and vehicle-treated controls. Western blot analyses were performed on samples derived from the skin tissues.

Results: The dorsal skin tissues of PPD-treated BALB/c mice showed significantly increased levels of iNOS. However, nNOS expression in dorsal skin, and nNOS and iNOS expression in the ear, was not significantly altered in PPD-treated skin tissues compared to controls.

Conclusion: Because enhanced expression of iNOS may contribute to inflammation in allergic contact dermatitis, our data suggest that increased levels of iNOS may be involved in early immunological responses to PPD-induced pathogenesis in dorsal skin.

Keywords: Nitric oxide synthase, *p*-phenylenediamine, Dorsal skin, Ear, Sensitization.

p-Phenylenediamine (PPD) is an aromatic amine component of oxidative hair dyes, widely used as an active ingredient in such products in numerous countries [1]. PPD is estimated to be present in approximately 70% of hair dyes worldwide; these dyes represent the most common source of exposure to PPD [2, 3]. However, PPD is known to possess extreme sensitizing potency [4]. In one study, cases that had adverse reactions to hair dyes were all positive for PPD in patch tests [5]. Furthermore, PPD is a recognized, potent sensitizer that may lead to allergic contact dermatitis (ACD) [6, 7].

ACD is an immunologically mediated inflammatory dermatosis; nitric oxide (NO) is known to be involved in the pathogenesis of acute and chronic inflammatory conditions, including ACD [8, 9]. NO is produced from L-arginine in a reaction catalyzed by nitric oxide synthase (NOS) enzymes [10, 11]. There are three isomeric forms of NOS: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) types [12]. The calcium-dependent nNOS and eNOS enzymes produce low levels of NO, whereas iNOS generates markedly higher levels in a calcium-independent manner [13, 14]. All three NOS iso forms are expressed in various cell types located in the skin, including keratinocytes [15,16]. Among the NOS enzymes, iNOS is considered to be most-involved in inflammatory and pathological conditions [12]. Particularly in the skin, NO produced by iNOS mediates various physiological functions, ranging from the regulation of cutaneous blood flow to melanogenesis [17]; it also modulates antigen presentation [18]. Furthermore, a previous study has demonstrated that nNOS plays a role in autoimmune skin diseases such as pemphigus vulgaris [19]. Furthermore, NO generated by epidermal nNOS plays a significant role in cutaneous circulatory responses to mechanical stimulation [20].

Therefore, the present study was undertaken to determine whether topical application of PPD could change the expression of nNOS and iNOS in the ear and dorsal skin of mice. Using protein immuno blot methods, we investigated the effects of PPD on nNOS and iNOS expression.

BALB/c mice (n = 12, 9-w--old females; weight = 19.5±1.0 g) were divided into vehicle-treated control, 10 mg/kg PPD-treated, and 50 mg/kg PPD-treated groups. Animals in the control, 10 mg/kg PPD, and 50 mg/kg PPD treatment groups were contact-sensitized by topical application of acetone in olive oil (1:4), 10 mg/kg PPD, and 50 mg/kg PPD, respectively. Mice were sensitized on a shaved area of the ear and dorsal skin, once per day for 3 d. All animals were euthanized 5 h after the last treatment. The mice were purchased from Samtako (Osan, Korea); PPD (fig. 1) was purchased from Sigma (St Louis, MO, USA). Other reagents, of the highest available quality, were obtained from commercial sources. All experiments were approved by the Institutional Animal Care and Use Committee of Keimyung University, Korea. Experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Animals were housed in a specific pathogen-free (SPF) facility, with free access to food and water.

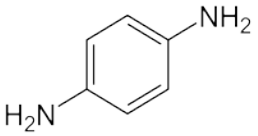

Fig. 1: The structure of *p*-phenylenediamine

Five hours after the treatment regimen ended, mice were euthanized by CO_2 asphyxiation, and the ear and dorsal skin tissues were removed rapidly on ice. Tissue samples were immediately placed into cryovials and stored at-70 °C until use. For Western blotting, tissues were homogenized in RIPA buffer (Sigma) containing 1% protease inhibitor and phosphatase inhibitor cocktails. The homogenate was centrifuged (14,000 rpm, 40 min) and the supernatant collected. The protein concentration was estimated using the Bradford method, with bovine serum albumin (BSA) used as the standard. An aliquot (5-µg protein) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fractionated proteins were then transferred to a nitrocellulose membrane. For immuno blotting, the following primary antibodies were used: mouse anti-nNOS monoclonal antibody (1:10,000 dilution; Santa Cruz Biotechnology, CA, USA) and mouse anti-iNOS monoclonal antibody (1:1000 dilution; Santa Cruz Biotechnology). After incubation with HRP-conjugated secondary antibodies (Amersham Biosciences, NJ, USA), the immunoreactive

bands were visualized using ECL Western blotting detection reagents (Amersham Biosciences) and X-ray film. Band intensity was measured using the ImageJ program (NIH, Bethesda, MD, USA). GAPDH was used as a control for immuno blotting.

Densitometric measurements of nNOS and iNOS protein expression in PPD-treated animals were compared with those of controls using one-way analysis of variance, followed by a *post hoc* Duncan test. A P value<0.05 was taken to indicate statistical significance. Data are presented as means±SEM. All statistical analyses were conducted using the SAS software package (ver. 9.2; SAS Institute Inc., Cary, NC, USA). To elucidate the effect of PPD, nNOS and iNOS expression in the dorsal skin was compared between control and PPD-treated mice: PPD exposure increased iNOS expression in a dose-dependent manner; exposure to higher-dose PPD induced significant increases in iNOS expression (P<0.05). However, nNOS expression was not significantly altered following treatment with PPD (P = 0.6531, fig. 2).

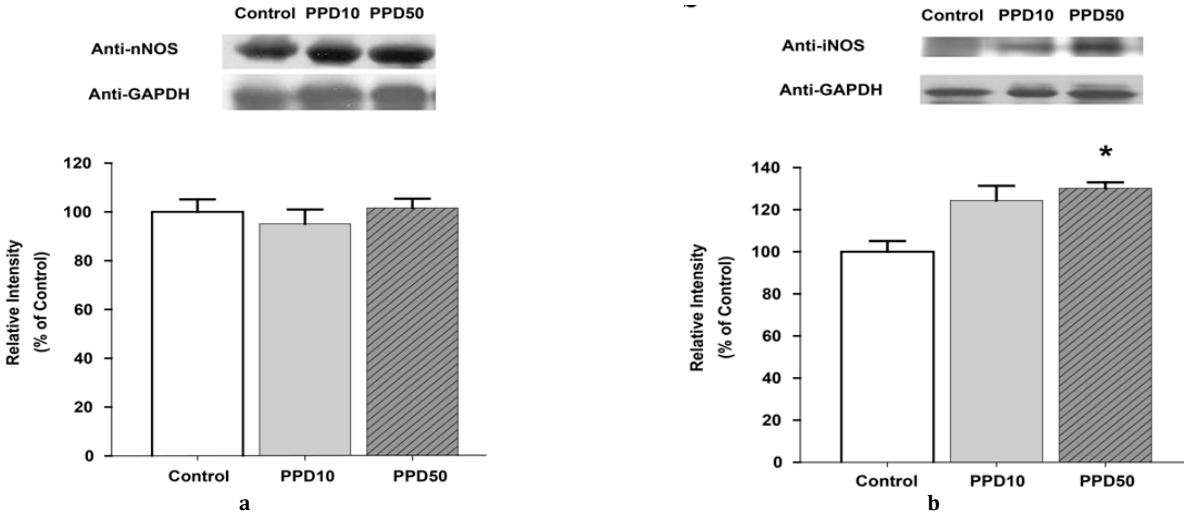

Fig. 2: Relative expression levels of nNOS (a) and iNOS (b) in the dorsal skin tissues of mice treated with 10 or 50 mg/kg PPD. Each panel shows a representative Western blot (top) and densitometric band analysis in the control and PPD-treated groups (bottom). Values represent means±SEM in each group (n = 4). *p<0.05, compared with the control group

nNOS and iNOS expression were also evaluated in PPD-treated ear tissues. Unlike iNOS expression in the dorsal skin, PPD had no significant overall effect on iNOS expression in the ear tissues of treated mice (P = 0.0531). Moreover, nNOS expression was not significantly altered by treatment with PPD (P = 0.3884, fig. 3).

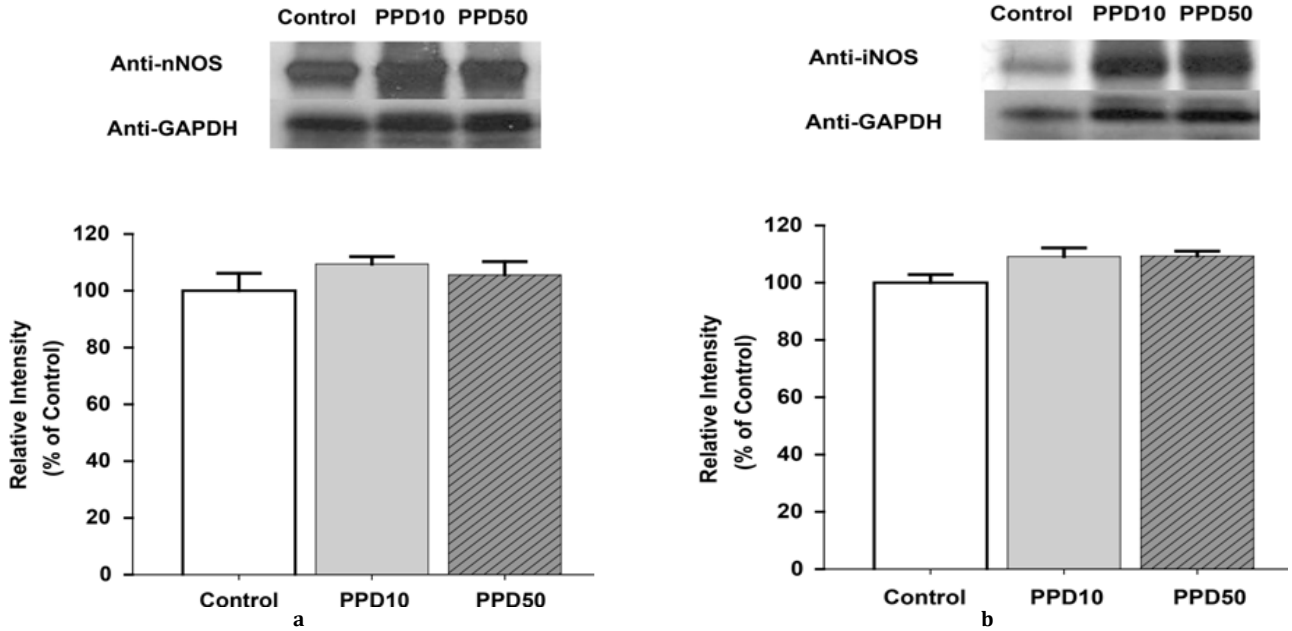

Fig. 3: Relative expression levels of nNOS (a) and iNOS (b) in the ear tissues of mice treated with 10 or 50 mg/kg PPD. Each panel shows a representative Western blot (top) and densitometric band analysis in the control and PPD-treated groups (bottom). Values represent means±SEM in each group (n = 4)

PPD exposure is known to cause hypersensitivity, which is characterized by a number of biochemical processes that result in contact dermatitis [21]. Although a wide range of effects on immunological signal transduction during hypersensitivity reactions have been reported, the specific effects of PPD on NOS expression are not fully understood.

Therefore, in this study we investigated the effects of PPD exposure on nNOS and iNOS expression in the ears and dorsal skin of mice.

NO is an important mediator in skin biology, and is also involved in inflammatory skin disorders including wound healing, burn injuries, ultraviolet light-induced sunburn erythema, psoriasis, and contact dermatitis [22–26]. Exposure of the skin to chemical irritants results in higher levels of NO synthesis and the production of pro-inflammatory cytokines [27]. NO is formed in a reaction catalyzed by NOS enzymes. The major cell types that comprise the skin, including keratinocytes, fibroblasts, melanocytes, and endothelial cells, all express NOS. Keratinocytes account for approximately 90–95 % of the cells in the epidermis that constitutively express nNOS [23, 25]. Keratinocytes are also known to express iNOS message and protein following exposure to inflammatory cytokines [23, 28].

Our data show that iNOS expression levels are increased significantly by exposure to 50 mg/kg PPD in dorsal skin. The expression of iNOS may influence several immunologic reactions in the skin; iNOS expression is observed in a number of inflammatory skin conditions including psoriasis, atopic dermatitis, irritant contact dermatitis and ACD [29, 30]. Moreover, high levels of iNOS expression have been observed in the skin of patients with Sjögren's syndrome, which is associated with photosensitivity [31]. Because prolonged exposure to PPD is known to cause ACD, our findings suggest a possible role of iNOS in the pathogenesis of ACD induced by PPD exposure.

Our data on iNOS demonstrate a difference in expression patterns between the ear and dorsal skin following PPD treatment. Therefore, the effect of PPD on NOS expression may differ according to skin type. One possible explanation for these differences among skin regions is that the rate of diffusion may play an important role in determining the level of iNOS expression. Differences in diffusion rates may be caused by skin tissue structure differences. In the ear, connective tissues under the dermis are tightly bound, and the elasticity of the skin is low. However, dorsal skin, which contains an abundance of fatty tissues within the subcutaneous connective tissues, is more loose and elastic [32]. Because of such differences, PPD administered to dorsal skin might be rapidly diffused between the subcutaneous tissues and muscle layers. Therefore, iNOS expression following PPD administration may be influenced by retention time differences between ear and dorsal skin.

In conclusion, PPD exposure induced differential iNOS expression patterns between the ear and dorsal skin, whereas nNOS expression remained unaltered in both ear and dorsal skin tissues. In the dorsal skin, iNOS expression was significantly increased in the high-dose PPD group. These data indicate that altered iNOS homeostasis in the dorsal skin may contribute to PPD-induced dermato toxicity. However, further studies, including measurements of NO and/or other signaling molecules, are required to fully address the effects of PPD on NO-mediated signal transduction and inflammation.

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CONFLICT OF INTERESTS

All authors have none to declare.

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