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Original Article

Inhibition of 5α-reductase of *de novo* Generation of Short Anti-oxidant Peptides

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This study aims to investigate the biological activities related to hair loss of short anti-oxidant peptides (DK peptides) 5α-reductase inhibition and anti-oxidation. The series of DK peptides were generated amphipathic helical properties using leucines, lysines and tryptophan residues. Cell viability and free radical scavenging activities were performed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, respectively. The DK peptides were investigated for inhibitory activity against 5α-reductase. Antioxidant activities were determined by means of, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays. All peptides could inhibit 5α-reductase in lipopolysaccharide-stimulated macrophage. In conclusion, DK peptides was suggested as the most attractive ingredients for improving hair loss, because of the high inhibitory against 5α-reductase inhibition.

Key Words: 5α-reductase, Anti-oxidant peptides, Hair loss

INTRODUCTION

Androgenetic alopecia is a significant issue that can occurs in all ages. Generally, it is caused hair loss, which depends on the presence of the androgenic hormones, including testosterone and dihydrotestosterone (Stough et al., 2005; Dawber 1987; Cotsarelis and Millar, 2001). In the human body, dihydrotestosterone is an enzymatic product converted from testosterone by the role of 5α-reductase. Since dihydrotestosterone is more active than testosterone, blocking the conversion of testosterone to DHT would reduce the androgenic effect. Thus, anti-androgenic drugs, which inhibit 5α-reductase or bind between dihydrotestosterone and androgen receptor, may be useful for protection from androgenetic alopecia (Matsuda et al., 2001). The hair follicle is a cutaneous organ that remodels itself during cyclical periods of active hair growth (anagen), apoptosis-driven involution (catagen), hair shedding (exogen), and relative rest (telogen) (Ahn et al., 2001). Beside the androgenic hormones, the miniaturization of hair follicle might be explained by a shorter anagen cycle (Whiting, 2001). The hair follicle size and the duration of anagen phase indicate the length and the size of hair shaft, respectively (Cotsarelis and Millar, 2001). The normal duration of anagen is around 2~6 years on average, and then it will turn to a short transitory period of catagen, in which the follicle will undergo apoptosis (Otberg et al., 2001). Free radicals, which are highly reactive molecules with unpaired electrons that can directly damage various cellular components, might be another factor affecting the hair loss in

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androgenetic alopecia. Since the oxidation process leads to progressive damage of cellular structures, the ageing phenotype of hair manifests as a decrease in hair production. It has been reported that lipid peroxides on hair follicles led to the early onset of the catagen in murine hair cycles (Wood et al., 2009). Therefore, antioxidant compounds might be used to prolong the anagen phase and reduce the hair loss. The series of DK peptides were generated amphipathic helical properties were conferred by using leucines, lysines and tryptophan residues were positioned at the critical amphipathic interface between the hydrophilic ending side and the hydrophobic starting side (Table 1).

Table 1. Amino acid sequence of DK peptides

Peptides	Amino acid sequence
DK-1	KWLLRKLRKWL
DK-2	KRLLRKLLKLL
DK-1-1	KWLLWK
DK-1-2	KWLLK
DK-1-3	KWLWK
DK-2-1	KLLKLKL
DK-2-2	KLKLL
DK-2-3	KLLKK

MATERIALS AND METHODS

The chemically synthesized model peptides were purchased as dry powders from the peptide manufacturer company A&PEP (Oksan, Korea). RAW 264.7 cells were cultured at 37℃ in 5 % CO_2 in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS (Hyclone, Logan, UT, USA) and antibiotics (Invitrogen). For viability assay, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used as described previously. Briefly, RAW 264.7 cells were plated onto 96 well plates and exposed to DK peptides. MTT was added to each well then incubated for additional 2 h in the dark at 37℃. The medium was then aspirated from the wells and the blue formazan product obtained was dissolved in DMSO. The plates were analyzed at 570 nm using a microplate reader (Tecan Trading AG, Switzerland). Each experiment was conducted in triplicate. Cell viability (%) was calculated as the ratio of the absorbance of sample to that of the non-treated sample, expressed as a percentage. In all other experiments, the cells were pre-treated with DK peptides with controls at indicated concentrations (10, 50, and 100 μg/mL) for 1 h before the addition of LPS (100 ng/mL, Sigma-Aldrich, St Louis, MO, USA) in serum free DMEM. An equal volume of sterile water was added to all control treatments. The radical scavenging activity of DK peptides was measured using a stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA). The scavenging effects were evaluated by employing a reaction mixture constituted with aliquots of the DK peptides and a DPPH methanolic solution as described previously (Nanjo et al., 1996). Briefly, a sample solution of 60 μL of each DK peptides, was added to 60 μL of DPPH (60 μM) in methanol. After mixing vigorously for 10 s, the mixture was then transferred into a 100 μL Teflon capillary tube and the scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer (Jeol Ltd, Tokyo, Japan). A spin adduct was measured on an ESR spectrometer exactly after 2 min. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5, and temperature, 298 K. Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent (Green et al, 1982). Briefly, RAW 264.7 cells (2×10^5 cells/mL) were seeded in 6-well plates in 500 μL complete culture medium and treated with the DK peptides at indicated concentrations (10, 50 and 100 μg/mL) for 1 h prior to stimulation with LPS (100 ng/ mL) for 2 h. Culture supernatant (50 μL) was reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4) in 96-well plates at room temperature in the dark. Nitrite concentrations were determined by using standard solutions of sodium nitrite prepared in the culture medium. The absorbance was determined at 540 nm using a microplate reader (Tecan). RAW 264.7 cells (1×10^5 cells/well) were cultured in 96 well plates and treated with the DK peptides at the indicated concentrations for 1 h and stimulated with LPS (100 ng/mL). At 4 h post LPS treatment, the cells

were collected and the supernatants were evaluated for 5α-reductase enzyme contents using a murine 5α-reductase ELISA kit from BD Biosciences, respectively (San Jose, CA, USA) according to the manufacturer's instructions. All data are represented as the mean ± S.E.M of at least three independent experiments. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA) using one-way analysis of variance, followed by Dunnett's multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Amino acid sequences of the tested DK peptides and their structural parameters calculated from the sequences are summarized in Fig. 1. As shown in Fig. 2, DK peptides showed significant DPPH radical scavenging activity in a concentration-dependent manner. The maximum scavenging activity was observed at 100 μg/mL of concentration ($P < 0.001$). However, DK peptides at concentration of 10 and 50 μg/mL also showed significant scavenging of DPPH radicals ($P < 0.05$ and $P < 0.01$ at 10 and 50 μg/mL, respectively). The concentration needed for 50% inhibition of DPPH radical by DK-1 and DK-2 (10 μg/mL) were 45.64 ± 1.64 μg/mL and 46.24 ± 1.87 μg/mL. Oxidation process is another pathway related to hair loss since free radicals could damage the hair follicle cellular structures and lead to a decrease in hair production (Wood et al., 2009). There are several methods to investigate the antioxidant activity of natural compounds, including DPPH assay. However, the most relevant method related to hair loss was oxidation assay since it has been reported that oxidation on hair follicles led to the early onset of the catagen which would lead to the hair loss (Naito et al., 2008). As shown in Fig. 3, treatment with LPS (100 ng/mL) with or without DK-1 and DK-2 peptides at various concentrations (10~100 μg/mL) did not affect the overall cell viability nor did they exhibit any cytotoxicity on RAW 264.7 cells. NO is one of the important inflammatory mediators produced by activated microglia. To study the effect of DK peptides on LPS-stimulated NO release, RAW 264.7 cells were treated with various concentrations of DK-peptides (10, 50 and 100 μg/mL) for 30 min prior to

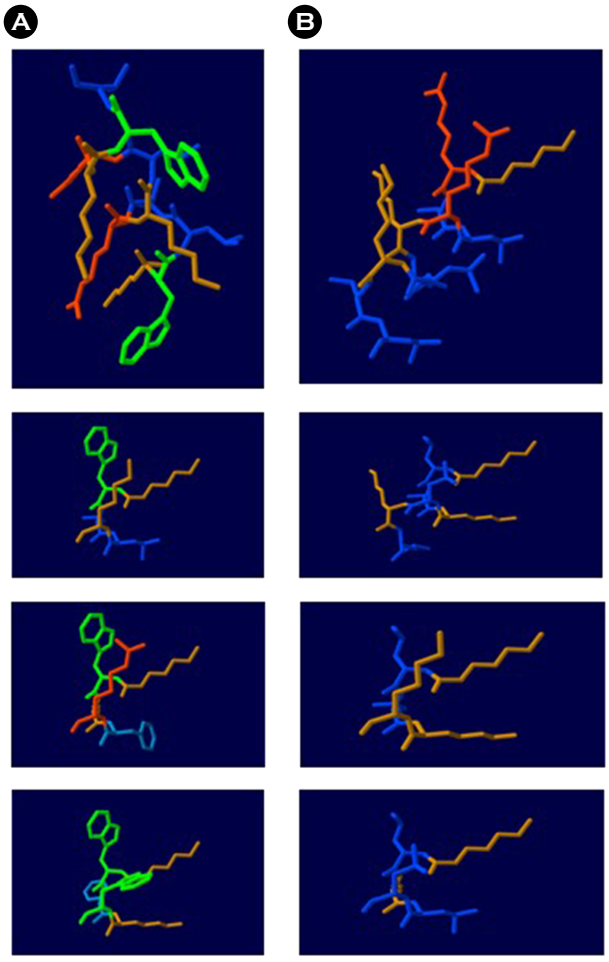

Fig. 1. 3D model of structures of DK-peptides, (A) DK-1, left upper panel; DK-1-1, left middle panel; DK-1-2, left second middle panel; DK-1-3, left lower panel, (B) DK-2 (right upper panel), DK-2-1, left middle panel; DK-2-2, left second middle panel; DK-2-3, left lower panel.

LPS (100 ng/mL) stimulation for 4 h. NO production by LPS-activated cells was found to be significantly inhibited by DK-peptides in a concentration-dependent manner (Fig. 4). As shown in Fig. 5, 5α-reductase levels were increased significantly after LPS treatment (1 μg/mL) when compared to those in untreated cells ($P < 0.001$). However, DK-peptides (10, 50 and 100 μg/mL) significantly inhibited these 5α-reductase actibities (Fig. 5) in a concentration-dependent manner in LPS-stimulated RAW 264.7 cells ($P < 0.05$ at 10 μg/mL, $P < 0.01$ at 50 μg/mL and $P < 0.001$ at 100 μg/mL, respectively). The presence of dihydrotestosterone, which is converted from testosterone by the role of 5a-reductase,

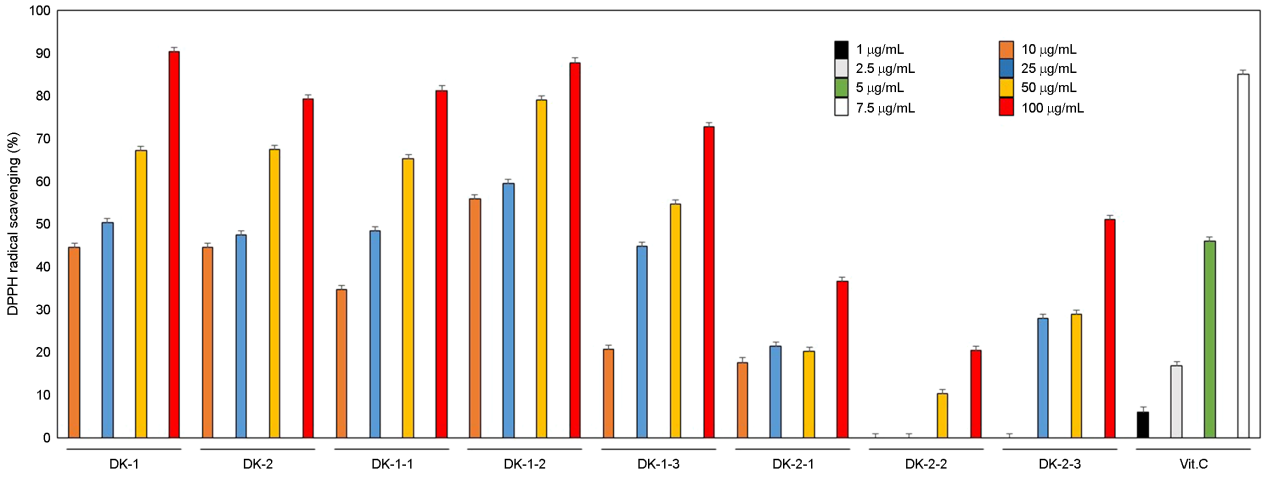

Fig. 2. Effects of DK-peptide on DPPH radical scavenging activity.

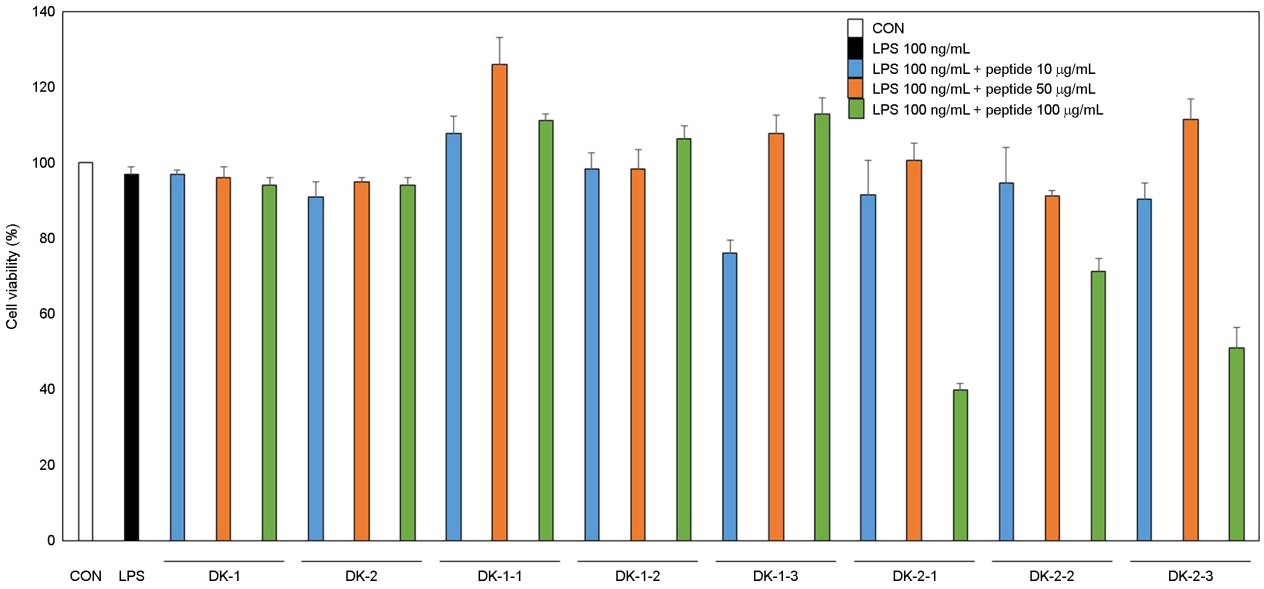

Fig. 3. Effects of DK-peptide on the viability of RAW 264.7 microglial cells. Cell viability in combination extract of DK-peptide treated cells was determined using MTT assay. The results are displayed in percentage of control samples.

is related to aberrant of hair follicle cycling, miniaturization of hair follicles, and finally hair loss. Therefore, the compounds that could inhibit 5α-reductase would be useful for anti-hair loss.

DISCUSSION

To produce new hairs, existing follicles perpetually undergoes through three stages: growth (anagen), involution (catagen), and rest (telogen). Determining the molecular signals that orchestrate the follicle's transit between these stages is one of the key challenges of hair research. Numerous growth factors and growth factor receptors are critical for normal hair follicle development and cycling, but no single growth factor appears to exert ultimate control over these processes (Paus and Cotsarelis, 1999). Alopecia is

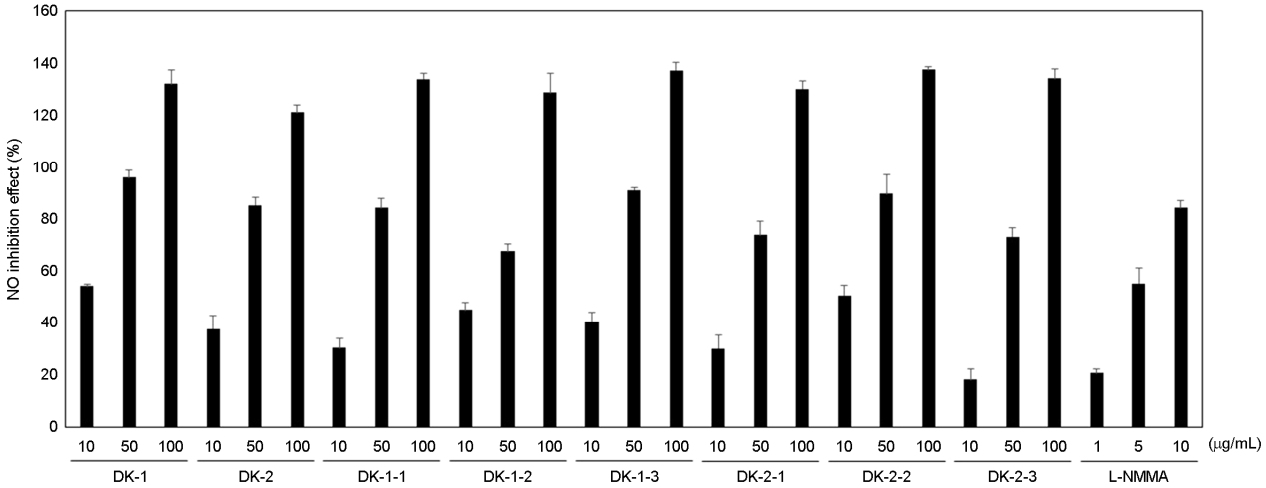

Fig. 4. Effects of DK-peptide on NO Production in LPS-stimulated RAW 264.7 microglial cells. RAW 264.7 cells were treated with DK-peptide with or without LPS (100 ng/mL) for 24 h. The nitrite in the culture supernatant was evaluated using Griess reagent. L-NMMA; N-monomethyl-L-arginine.

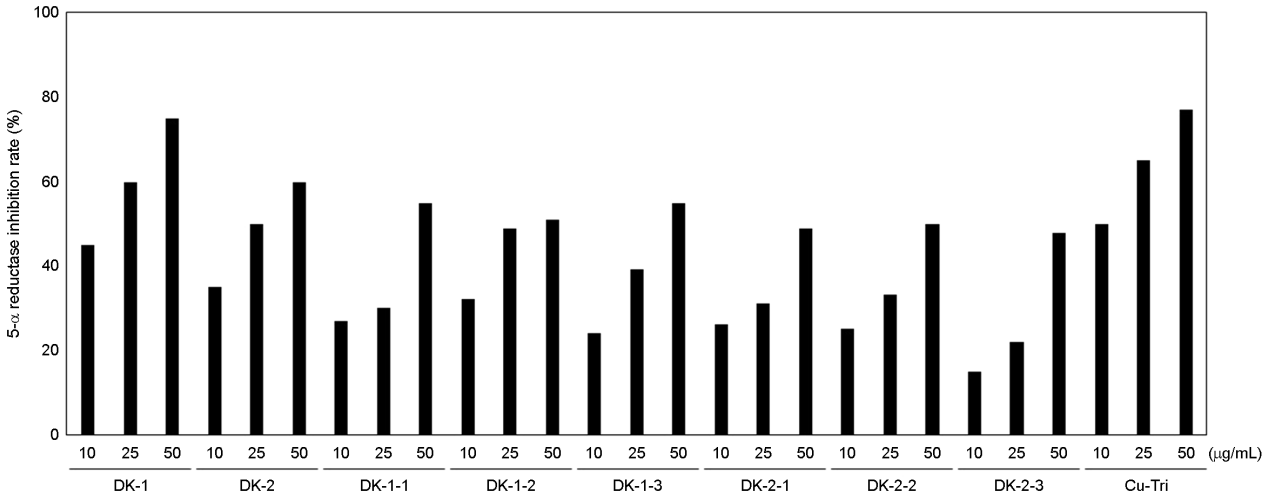

Fig. 5. Effects of DK-peptide on 5α-reductase inhibition activity.

defined as abnormal hair loss and is mainly caused by genetic factors, immune disorders and aging. In the United States, a research study among patients with diagnosed alopecia shows that above 70% of the patients have inflammatory diseases like atopy and contact dermatitis and mental health problems are seen in above 25% (Huang et al., 2013). Also, Alopecia is strongly related to an autoimmune disease. Alopecia' agonist effect can decreased fibroblast follicle cell growth and renewal, but antagonist effects can increased dihydrotestosterone (Dmytriw et al., 2015). 5α-reductase converts testosterone to dihydrotestosterone, which has a higher affinity for androgen receptors than testosterone and induces the expression of genes related to minimizing follicles, thus stimulating hair loss (Yim et al., 2014). A treatment for alopecia should prevent hair loss and promote hair growth. 5α-reductase inhibitors and hair growth agents has been shown to improve the androgenetic alopecia (Amory et al., 2007). Alopecia may be treated by hair transplantation, but,

hair transplantation is expensive, oral medicines have side effects such as hepatotoxicity, and oriental medicines are not clearly effective in improving hair growth (Traish et al., 2014). Identifying alternative treatments especially peptides that exhibit relatively low toxicity compared with synthetic drugs is therefore a dynamic area of research and development. All the peptides were designed to be perfectly amphipathic when folded into α-helical structures, by converging the hydrophobic leucines into one side and the cationic lysines into the other side of the helical axis. The triptophan residues were always positioned at the critical amphipathic interface between the hydrophilic ending side and the hydrophobic starting side, in the helical wheel projection. In addition, the 5α-reductase inhibitory activity of DK peptides was firstly described in the present study. The results noted that, among eight DK peptides, DK-1 and DK 1-3 possessed the significantly highest 5α-reductase inhibition. Beside the role of 5α-reductase and DHT, several cytokines are also related to the hair loss. IL-6 is one of the cytokines which has been more upregulated in balding dermal papilla cells (Kwak et al., 2012). In addition, IL-6 has been reported to inhibit the hair shaft elongation and suppressed proliferation of matrix human hair follicles cells and finally lead to the hair loss (Kwak et al., 2012). DK peptides that perhaps inhibit the IL-6 secretion might have several health benefits other than anti-hair loss.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication this article.

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