Cell-penetrating heme oxygenase-1 in the therapy of atopic dermatitis in mice

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Abstract. Atopic dermatitis (AD), also referred to as atopic eczema, is a long-term inflammatory condition that is characterized by itchy, red, swollen and cracked skin. Accumulating evidence suggests that AD is caused by genetic factors, environmental exposure and immune system dysfunction; however, its underlying molecular mechanism remains unclear. Current treatment strategies aim to decrease the severity and frequency of flares. Heme oxygenase-1 (HO-1) is a nuclear factor erythroid 2-related factor 2 (Nrf2)-regulated gene that plays crucial roles against stress, inflammation and oxidation, and exerts cytoprotective effects. Previous studies have reported that treatment of AD induces high expression levels of HO-1 and Nrf2, indicating that HO-1 may play an important role in the treatment of AD. The present study constructed the recombinant protein, cell-penetrating peptide-HO-1 (CPP-HO-1), which was expressed in *Escherichia coli* and isolated with a 6xHis-tag using HiTrap His column (1 ml). AD was established using 4-dinitrochlorobenzene (DNCB) in mice. It was observed that the CPP-HO-1 fusion protein decreased the severity of AD, inhibited scratching in mice and decreased skin inflammation. Taken together, the results of the present study suggested that the CPP-HO-1 fusion protein may play a protective role against DNCB-induced AD in mice.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin condition with an increasing prevalence (1). AD affects individuals of all ages, occurring in 15-20% children and 1-3% adults (2). AD is clinically characterized by skin dryness and itchy papules (occasionally vesicles in infants) that become excoriated and lichenified, usually with crusting (3). Accumulating evidence suggests that AD is elicited by skin barrier dysfunction, which is followed by immune system activation, which in turn negatively regulates skin barrier homeostasis, referred to as an 'outside-inside-outside' model of AD pathogenesis (4). Thus, current therapies focus on maintaining skin barrier function and ameliorating inflammation. For example, skin care and moisturizing products are recommended as first-line treatment for mild AD (5). Treatments for moderate-to-severe AD include dupilumab, cyclosporine, phototherapy and systemic glucocorticoids, albeit with limited success due to considerable side effects and inability to affect the recurrence rate (6). Thus, it is crucial to identify novel promising therapies, with fewer side effects, for the effective treatment of AD.

Heme oxygenase-1 (HO-1) catalyzes the first and rate-limiting step in the oxidative degradation of free heme, and may affect several biological processes of aneurysmal diseases, articular diseases and hepatic gluconeogenesis, among others, via its enzymatic by-products (7). Previous studies on AD mouse models have demonstrated that certain agents, including Soshiho-tang, sulforaphane, dihydroaustrasulfone alcohol (WA-25) and *Platycodon grandiflorus* root-derived saponins, can alleviate AD-like skin lesions and skin inflammation by increasing the expression of HO-1 and nuclear factor erythroid 2-related factor 2 (Nrf2) (8-11). Another study also concluded that enhancement of HO-1 expression attenuated the development of skin lesions in mice (12). Based on these findings, HO-1 appears to hold promise for the treatment of AD; however, whether the topical use of HO-1 can alleviate AD remains unclear.

Cell-penetrating peptides (CPPs) are short peptides (<30 amino acids) that are capable of crossing cell membranes and transport small molecules into cells, such as drugs, peptides, proteins, nucleic acids, nanoparticles and imaging agents (13). Previous studies have demonstrated that the CPP-HO-1 fusion protein exerted a protective effect against renal and intestinal ischemia/reperfusion (I/R) injury (14,15). In the present study, a CPP was attached to the HO-1 protein to transportHO-1 into the skin. Given that the efficacy of the topical use of the CPP-HO-1 fusion protein in AD treatment remains unclear, the present study aimed to investigate the efficacy of CPP-HO-1 in a mouse model of AD.

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Abbreviations: AD, atopic dermatitis; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; WA-25, dihydroaustrasulfone alcohol

Key words: HO-1, AD, cell-penetrating peptides, recombinant protein, dinitrochlorobenzene

Materials and methods

Chemicals. The H&E staining kit, SDS-PAGE 12% gel preparation kit and acetone were obtained from Sangon Biotech Co., Ltd. 2,4-Dinitrochlorobenzene (DNCB) was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. Acetone and olive oil were mixed at a ratio of 4:1. DNCB was dissolved in the acetone/olive oil mixture at a concentration of 1%. PBS, xylene and PBST (10% Tween-20) were prepared by the authors' laboratory.

Animals. A total of 18 ICR mice (male, aged 4-5 weeks; weight, 21±2 g) were purchased from Shanghai Slacker Laboratory Animal Co., Ltd. [(approval no. SCXK(hu)2017-005; Shanghai, China]. All animals were housed under specific pathogen-free conditions at a controlled temperature of 20-25˚C and 50-60% humidity with a 12-h light/dark cycle. The animals were provided access to sterile food and water ad libitum.

The present study was approved by the Ethics Committee of Huzhou University (Huzhou, China) and all animal care and experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (published by the National Institutes of Health and revised in 1996; no. 85-23).

Induction of AD-like lesions and drug treatment. After 2 weeks of acclimation, the mice were divided into three groups (n=6/group) as follows: AD, CPP-enhanced green fluorescent protein (EGFP) (DNCB with 0.5 μg/μl CPP-EGFP) and CPP-HO-1 (DNCB with 0.5 μg/μl CPP-HO-1) groups.

The method for inducing AD was modified from a previous study (10). The dorsal skin hair was clipped and an area ~1x2 cm^2 was depilated with a hair removal cream. A total of 50 μl 1% DNCB was added to the dorsal skin three times per week for a total of 2 weeks. Treatment with 50 μl PBS, 50 μl CPP-EGFP (0.5 μg/μl) or 50 μl CPP-HO-1 (0.5 μg/μl) was applied 3 times for the first week 1 h following DNCB and 7 times for the second week. In order to increase cell penetrating efficiency, CPP-HO-1, CPP-EGFP or PBS solution was kept on the skin for at least 3 h. The animals were sacrificed on day 15, and dorsal dermal tissues were collected for further analysis. The experimental schedule is summarized in Fig. 1.

Preparation of CPP-HO-1. The preparation method of the CPP-HO-1 was as follows: The CPP-HO-1 and CPP-EGFP genes were synthesized by Sangon Biotech Co., Ltd. and inserted into the pET28b vector by the *Nde*I and *Eco*RI restriction enzymes and T4 DNA ligase (Sangon Biotech Co., Ltd.), and subsequently transferred into the Novagen's Rosetta™ 2 (pLysS) host strains from Hangzhou Biogroup Technology Co., Ltd. CPP-HO-1 was induced at 0.7 mm IPTG for 18 h at 37˚C, and was subsequently centrifuged at 13.8 x g for 15 min at 4˚C. The resultant pellet was stored at -80˚C for at least 24 h. For protein extraction, the *E. coli* were released with PBS buffer (pH 7.4) containing 20 mM imidazole and sonicated with an ultrasonic homogenizer (Ningbo Xinzhi Biological Technology Co., Ltd.) at 60% amplitude for 5 min on ice, for 15 cycles of 5 sec on and 15 sec off. The supernatant was subsequently centrifuged at 14.95 x g for 20 min at 4˚C and bound to a HiTrap His column (1 ml, GE Healthcare) at a rate of 0.5 ml/min. Elution was performed using 500 mM imidazole. Subsequently, 2.5 ml eluent was added to the pre-balanced G25 desalination column (GE Healthcare) for desalination and was finally stored in 10% glycerol (Sangon Biotech Co., Ltd.) at -80˚C.

Table I. Evaluation criteria of atopic dermatitis (8).

Items	Response intensity	Score
Erythema	None	0
	Mild	1
	Moderate	2
	Severe	3
Erosion	None	0
	Mild	1
	Moderate	2
	Severe	3
Scarring	None	0
	Mild	1
	Moderate	2
	Severe	3
Edema	None	0
	Mild	1
	Moderate	2
	Severe	3

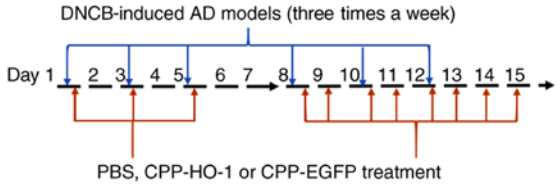

Figure 1. Protocol for induction of AD in specific-pathogen-free mice. DNCB, 4-dinitrochlorobenzene; AD, atopic dermatitis; CPP, cell-penetrating peptide; HO-1, heme oxygenase-1; EGFP, enhanced green fluorescent protein.

Evaluation of AD severity. AD was observed in all mice and the score was recorded on the last day of the experiment, according to the criteria described in Table I. The severity of AD was determined based on four symptoms: i) Erythema; ii) erosion; iii) scar; and iv) edema. The score of each clinical symptom ranged from 0 to 3 (none, 0; mild, 1; moderate, 2; and severe, 3). The total AD score (maximum score, 12) was the sum of individual scores.

Measurement of scratching behavior. All mice were acclimated in acrylic cages for 15 min. Following acclimation, the mice were videotaped for 30 min and the number of scratches was counted by the same observer.

Histological analysis. The mice were anesthetized with an i.p. injection of a mixed solution containing xylazine (20 mg/kg; Bayer) and ketamine (150 mg/kg; Bayer). All mice were decapitated and the dorsal dermal skin tissues were collected and fixed in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc.) for 24 h at -80˚C. Tissue samples were cut into 50-μm

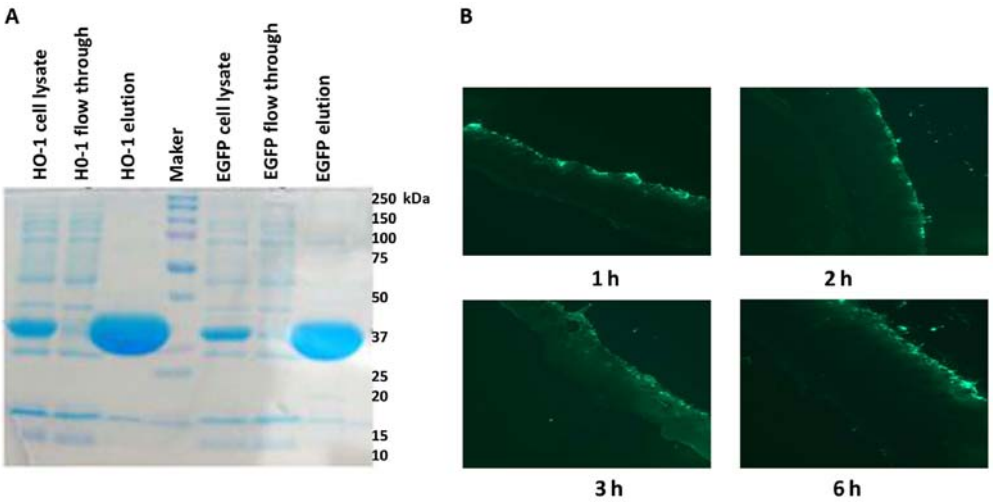

Figure 2. CPP-HO-1 purification. (A) Purified proteins were analyzed via 12% SDS-PAGE (stained with Coomassie blue). (B) Fluorescence microscopy examination at 1, 2, 3 and 6 h after application of EGFP. Scale bar, 100 μm. CPP, cell-penetrating peptide; HO-1, heme oxygenase-1; EGFP enhanced green fluorescent protein.

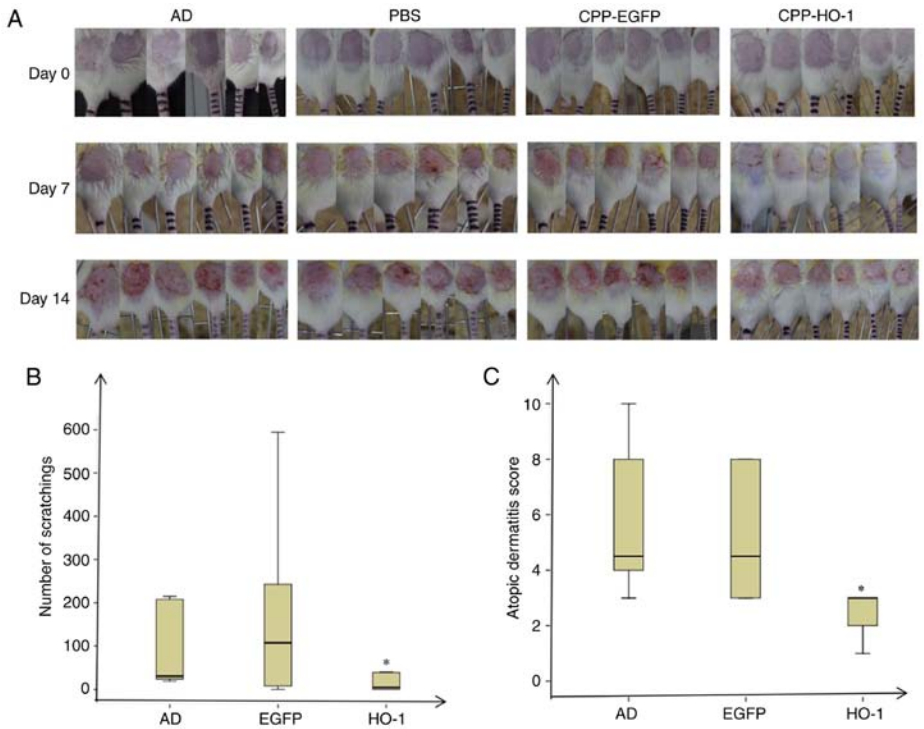

Figure 3. AD severity and scratching behavior. (A) Images of the AD, EGFP and HO-1 groups were capturedon days 0, 7 and 14. (B) Scratching behavior was assessed after 30 min. (C) AD scores of the AD, EGFP and HO-1 groups. *P<0.05 vs. AD and HO-1 groups. AD, atopic dermatitis; CPP, cell-penetrating peptide; HO-1, heme oxygenase-1; EGFP, enhanced green fluorescent protein.

sections. H&E staining was performed at 37˚C to determine epidermal thickness and inflammatory cell infiltration in each group. The tissue sections were observed under an optical light microscope (magnification, x1,000), and the degrees of keratinization, dermaledema and lymphocyte infiltration were analyzed.

Statistical analysis. Statistical analysis was performed using SPSS 20.0 (IBM Corp.). All the experiments were performed in triplicate (n=6 mice per group) and data are presented as median and interquartile range. Kruskal-Wallis test followed by Dunn's post hoc test was used to compare differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

CPP-HO-1 purification. The SDS-PAGE (coloration) results demonstrated high purity of CPP-HO-1 and CPP-EGFP (Fig. 2A). CPP-EGFP was applied to the dorsal skin of the mice for 1, 2, 3 and 6 h, and examination under a fluorescence microscope revealed that CPP-EGFP infiltrated through the mouse skin barrier in a time-dependent manner (Fig. 2B).

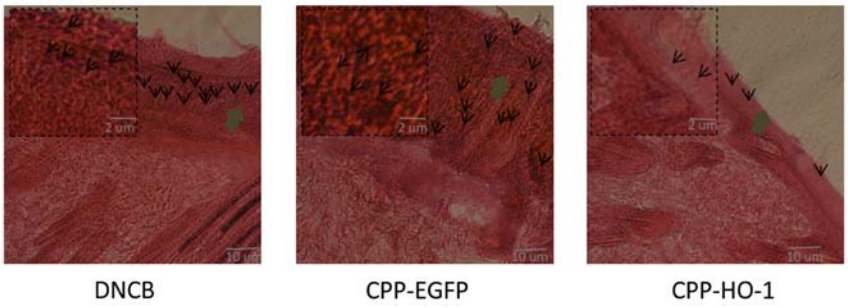

Figure 4. Hematoxylin and eosin staining of skin lesion tissue from the AD, EGFP and HO-1 groups (magnification, x1,000; scale bar, 10 μm). DNCB, 4-dinitrochlorobenzene; AD, atopic dermatitis; HO-1, heme oxygenase-1; CPP, cell-penetrating peptide; EGFP, enhanced green fluorescent protein.

Dermatitis severity and scratching behavior. The AD model was induced by DNCB, and 50 μl CPP-EGFP (0.5 μg/μl) or 50 μl CPP-HO-1 (0.5 μg/μl) was added to study the therapeutic effect (Fig. 1). The AD group exhibited severe dermatitis with erythema, scarring, edema and erosion on day 14 (Fig. 3). The skin condition was significantly improved in CPP-HO-1-treated mice compared with those in the AD group (Fig. 3A). All mice were acclimated in acrylic cages for 15 min and subsequently videotaped for 30 min on day 15, and the number of scratches was counted by the same observer. The results demonstrated that the number of scratching events was lower in the CPP-HO-1 group compared with the AD group (Fig. 3B; $P<0.05$), while there was no significant difference between the AD and CPP-EGFP groups ($P>0.05$). The score was recorded on the last day of the experiment, according to the criteria described in Table I (8). The results demonstrated that CPP-HO-1 decreased the dermatitis score of DNCB-induced skin lesions on day 15 compared with the AD group (Fig. 3C; $P<0.05$), whereas the AD score was not significantly improved in the CPP-EGFP group ($P>0.05$).

H&E staining. Examination of H&E stained sections of the skin lesions revealed decreased epidermal thickness and inflammatory cells in the CPP-HO-1 group. However, the epidermal thickness and inflammatory cell infiltration exhibited no significant differences between the CPP-EGFP and AD groups (Fig. 4). Taken together, these results indicated that topical application of CPP-HO-1 can improve the histological signs of AD.

Discussion

It was previously indicated that induction of HO-1 plays a protective role in several inflammation-related diseases (7), including AD, and HO-1 has been reported to exhibit therapeutic efficiency in AD (8-11). Chen and Zhong (16) revealed that HO-1 combined with microRNAs may affect certain skin diseases, such as ischemia, hypoxia, rheumatoid arthritis and AD, by regulating the functions of T cells, dendritic cells and mast cells, and the release of chemokines and cytokines. Another study reported that the HO-1 inducer, cobaltic protoporphyrin, inhibited T-cell-dependent skin inflammation by suppressing antigen-presenting cells (17). In 2016, Kim *et al* (18) reported that 2,3-dimethoxy-2'-hydroxychalcone, a derivative of 2'-hydroxychalcone in the flavonoid family, can alleviate skin inflammation by inhibiting TNF-α-induced intercellular adhesion molecule-1 expression and adhesion of monocytes to keratinocytes, by suppressing NF-κB activation and inducing HO-1 expression in keratinocytes. Hung *et al* (10) demonstrated that WA-25 can protect against AD by increasing the expression levels of HO-1 and Nrf2. Gene therapy of AD via targeting the HO-1 gene has been reported in several studies; however, AD therapy using the HO-1 protein is limited by its inability to enter cells. In previous studies, CPPs have been conjugated with HO-1 to form the CPP-HO-1 fusion protein, which can transfer the HO-1 protein into cells and decrease the extent of I/R injury (14,15); however, to the best of our knowledge, this method has not been reported in AD to date.

In the present study, the CPP was conjugated to HO-1 or EGFP (control protein with similar molecular weight) (19) to form the fusion proteins CPP-HO-1 and CPP-EGFP, and CPP-HO-1 was studied in the therapy of AD in mice. The CPP-EGFP could effetely enter the skin time-dependently, as shown in Fig. 2A, similar to CPP-EGFP penetration reported in heart tissues (20,21). In the present study, in order to increase cell penetrating efficiency, the CPP-HO-1, CPP-EGFP or PBS solution were kept on the skin for at least 3 h. The AD model was induced using DNCB, the immune phenotype of AD, such as upregulated iNOS protein expression, was not included in the present study, as it was described in a previous study (10). The DNCB-treated skin area exhibited itchy, red, swollen and cracked skin, which indicated that the AD model had been successfully established. In order to determine the optimal time and concentration of CPP-HO-1 treatment, the benefits in mice were compared between different concentrations of CPP-HO-1 (0.1 and 0.5 μg/μl) in a pre-study (data not shown), and the result revealed that 0.5 μg/μl was the most effective. However, the hair in the depilated area grew rapidly, and the frequent use of hair remover would also further damage the skin; therefore, in order to reduce the use of hair remover, the CPP-HO-1, CPP-EGFP or PBS treatment was applied 1 h after DNCB induction. Furthermore, the treatment times were also increased in the second week to increase the therapeutic efficacy, according to a previously published study (10). Compared with the PBS and CPP-EGFP groups, CPP-HO-1 effectively alleviated scratching, lowered skin score, and decreased skin swelling and inflammatory cell infiltration, as shown in Fig. 3. Similar conclusions were also reported by Kirino *et al* (12) and Hung *et al* (10) via enhancing HO-1 expression. H&E staining of the skin lesions also revealed that CPP-HO1 decreased epidermal thickness and inflammatory cell infiltration compared with the PBS and CPP-EGFP groups (Fig. 4). Taken together, these results suggest that CPP-HO-1 may have a therapeutic effect on AD.

However, there were certain limitations to the present study. A normal control group, which was not set in our research, is required to better demonstrate the effect of HO-1 on AD. The present study used 50-μm OCT sections for HE staining, although 5-μm sections may exhibit a higher resolution and thus will be used in future studies. To strengthen the evidence on the benefits of HO-1 for AD, immunohistochemistry and ELISA must also be performed to investigate additional parameters of AD in mice, such as the serum levels of IL-4, IL-13 and inducible nitric oxide synthase. Furthermore, the detailed mechanism underlying the therapeutic effect of HO-1 on AD must be further elucidated.

In conclusion, the results of the present study suggested that CPP-HO-1 may have a therapeutic effect on AD, and thus may hold promise as a therapeutic strategy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL designed the study and edited manuscript; FT drafted the initial manuscript. WJ, TQ and SQ isolated the proteins; FT, JS, XM and MR performed the animal experiments. WJ and TQ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Huzhou University (Huzhou, China) and all animal care and experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (published by the National Institutes of Health and revised in 1996; no. 85-23).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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