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2,3-Dimethoxy-2′-hydroxychalcone ameliorates TNF-α-induced ICAM-1 expression and subsequent monocyte adhesiveness via NF-kappaB inhibition and HO-1 induction in HaCaT cells

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Up-regulation of adhesion molecules plays an important role in the infiltration of leukocytes into the skin during the development of various inflammatory skin diseases, such as atopic dermatitis. In this study, we investigated the modulatory effects of 2,3-dimethoxy-2′-hydroxychalcone (DMHC) on tumor necrosis factor (TNF)-α-induced intercellular adhesion molecule-1 (ICAM-1) expression and monocyte adhesiveness, as well as the molecular mechanisms underlying its action in the HaCaT human keratinocyte cell line. Pre-treating HaCaT cells with DMHC significantly suppressed TNF-α-induced ICAM-1 expression and subsequent monocyte adhesiveness. DMHC inhibited TNF-α-induced activation of NF-κB. In addition, DMHC induced HO-1 expression as well as NRF2 activation. Furthermore, HO-1 knockdown using siRNA reversed the inhibitory effect of DMHC on TNF-α-induced ICAM-1 expression and adhesion of monocytes to keratinocytes. These results suggest that DMHC may inhibit TNF-α-induced ICAM-1 expression and adhesion of monocytes to keratinocytes by suppressing the signaling cascades leading to NF-κB activation and inducing HO-1 expression in keratinocytes. [BMB Reports 2016; 49(1): 57-62]

INTRODUCTION

Infiltration of leukocytes into the skin is a characteristic feature of the inflammatory immune response involved in the development of various skin diseases such as atopic dermatitis (AD) (1). Upregulation of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), may accelerate infiltration of leukocytes into the inflamed skin area (2, 3). Epidermal keratinocytes, a major skin cell type, express ICAM-1 in response to inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon-gamma (4). Increased levels of ICAM-1 expression are observed in keratinocytes of inflamed lesions in patients with AD and psoriasis (5, 6), suggesting that upregulation of ICAM-1 reflects the progression of inflammatory skin diseases (7). As ICAM-1 is critical for interactions between keratinocytes and leukocytes during skin inflammation, modulating ICAM-1 expression provides a rationale for developing therapeutic agents against various inflammatory skin diseases.

Nuclear factor-kappaB (NF-κB) is a major transcriptional factor mediating ICAM-1 expression (8). Stimulating keratinocytes with TNF-α activates the IκB-kinase (IKK) complex, consisting of two kinase subunits (IKKα and IKKβ) and a regulatory subunit IKKγ/NEMO. The activated IKK complex phosphorylates IκBα, resulting in its ubiquitination and subsequent proteasomal degradation. NF-κB moves from the cytosol to the nucleus, where it induces transcription of the ICAM-1 gene (9).

A growing body of evidences suggests that many pharmacological compounds exert their anti-inflammatory activities by inducing heme oxygenase-1 (HO-1) expression in inflammatory disease models (10). HO-1 catalyzes the degradation of heme, leading to the generation of ferrous iron, carbon monoxide, and biliverdin. These by-products mediate the beneficial effects of HO-1 expression in a number of pathological conditions (11). Previous studies have shown that HO-1 expression exerts immune-modulatory effects against inflammatory skin diseases, such as AD (12-14).

2,3-Dimethoxy-2′-hydroxychalcone (DMHC) is a derivative of 2′-hydroxychalcone in the flavonoid family (15). 2′-Hydroxychalcone derivatives exert potent anti-inflammatory activity in in vitro and *in vivo* models. 2′-Hydroxychalcone derivatives inhibit polymixin B-induced hind-paw edema in mice (16), and 2′-hydroxychalcone suppresses TNF-α- and lipopolysaccharide (LPS)-induced ICAM-1, VCAM-1, and E-selectin expression by blocking activation of NF-κB in human umbilical vein endothelial cells (17). 2′-Hydroxychalcone derivatives inhibit nitric oxide (NO) and TNF-α production in LPS-stimulated RAW 264.7 macrophages by inhibiting NF-κB and AP-1 activation (18). In contrast, 2′-hydroxychalcone inhibits LPS-induced NO and TNF-α production by inducing HO-1, without affect-

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ing activation of NF-κB in RAW 264.7 macrophages (19), suggesting that 2′-hydroxychalcone exerts anti-inflammatory effects via multiple mechanisms. However, very little is known about the protective effects of 2′-hydroxychalcone and its mechanism of action in keratinocytes.

In this study, we examined the inhibitory effect of DMHC on TNF-α-induced ICAM-1 expression and the molecular mechanism responsible for these activities in the HaCaT human keratinocyte cell line. Our results suggest that DMHC may exert anti-inflammatory effects by inhibiting NF-κB activation and inducing HO-1 expression in keratinocytes.

RESULTS

Effect of DMHC on TNF-α-induced ICAM-1 expression and subsequent monocyte adhesion in HaCaT cells

Cell viability was examined with the MTT assay to exclude the possibility that DMHC cytotoxicity (Fig. 1A) might contribute to its anti-inflammatory effects. As shown in Fig. 1B, DMHC had no significant cytotoxic effect on HaCaT cells in the absence or presence of TNF-α up to a concentration of 20 μM. We next examined the effect of DMHC on TNF-α-induced ICAM-1 expression in HaCaT cells. DMHC significantly inhibited TNF-α-induced ICAM-1 expression at the mRNA and protein levels in a dose-dependent manner (Fig. 1C). We further investigated the effect of DMHC on TNF-α-induced monocyte adhesion to HaCaT cells. As shown in Fig. 1D, DMHC significantly suppressed TNF-α-induced monocyte adhesion to HaCaT cells in a dose-dependent manner (Fig. 1D).

Effect of DMHC on TNF-α-induced NF-κB activation in HaCaT cells

NF-κB signaling pathway is activated upon stimulation with TNF-α to induce ICAM-1 expression (9). We investigated the effect of DMHC on signaling cascades leading to NF-κB activation in TNF-α-stimulated HaCaT cells. We examined the effect of DMHC on TNF-α-induced IKKα/β activation using antibodies against total and phosphorylated IKKα/β. DMHC significantly inhibited TNF-α-induced IKKα/β phosphorylation (Fig. 2A). DMHC also suppressed TNF-α-induced degradation of IκBα and phosphorylation of NF-κB p65 in a dose-dependent manner (Fig. 2B). We next examined the effect of DMHC on TNF-α-induced NF-κB p65 DNA binding activity using an electrophoretic mobility shift assay (EMSA). DMHC significantly reduced TNF-α-induced p65 DNA binding activity in a dose-dependent manner in HaCaT cells (Fig. 2C). We further evaluated the effect of DMHC on NF-κB promoter activity in TNF-α-stimulated HaCaT cells. As shown in Fig. 2D, DMHC suppressed NF-κB promoter activity in a dose-dependent manner. These results suggest that DMHC exerts its anti-inflammatory activity by inhibiting the signaling cascades leading to NF-κB activation.

DMHC induces HO-1 expression in HaCaT cells

As many chalcone derivatives exert their biological activities by inducing HO-1 expression (15), we next examined whether DMHC induced HO-1 expression in HaCaT cells. Cells were treated with various doses of DMHC (5-15 μM) for the indicated times, and HO-1 expression was analyzed by RT-PCR and Western blotting. As shown in Fig. 3A and B, DMHC significantly induced HO-1 mRNA and protein expression in

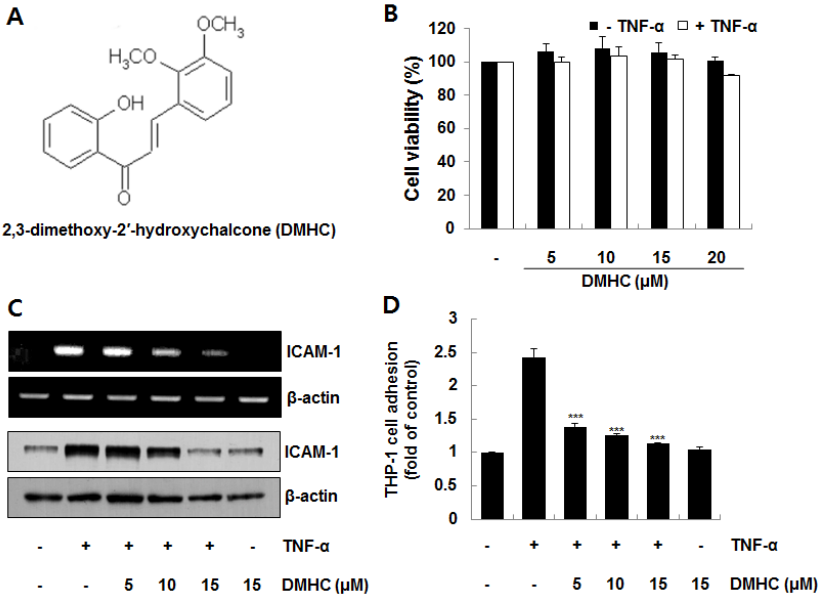

Fig. 1. DMHC inhibits TNF-α-induced ICAM-1 expression and subsequent monocyte adhesion in HaCaT cells. (A) Chemical structure of DMHC. (B) Cytotoxic effects of DMHC in HaCaT cells. The cells were incubated with the indicated doses of DMHC in the absence or presence of TNF-α (10 ng/ml) for 24 h and cell viability was determined with the MTT assay. (C) Effects of DMHC on TNF-α-induced expression of the ICAM-1 gene in HaCaT cells. Cells were pretreated with the indicated doses of DMHC for 3 h, and then exposed to TNF-α (10 ng/ml) for 1 h (for RNA) or 12 h (for protein). Total RNA and protein were analyzed by RT-PCR (upper panel) and Western blot (lower panel), respectively. (D) Effects of DMHC on monocyte adhesiveness in TNF-α-stimulated HaCaT cells. Cells were pretreated with various doses of DMHC for 3 h, and then exposed to TNF-α (10 ng/ml) for 12 h. Calcein-AM-labeled THP-1 monocytes were added to the HaCaT cells and incubated for 1 h. Calcein-AM fluorescence intensity was quantitated using an ELISA plate reader. Data are presented as the means ± SD of three experiments. ***P < 0.001 compared to TNF-α alone.

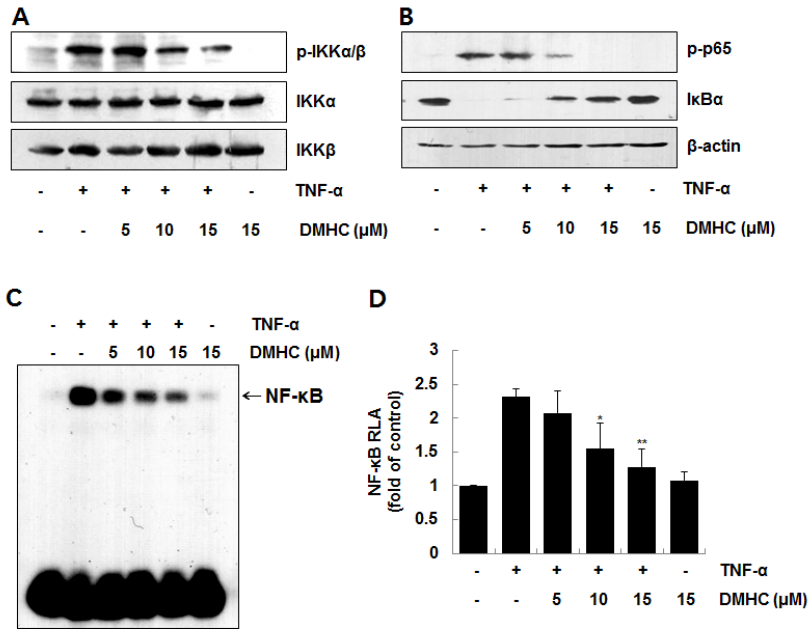

Fig. 2. DMHC suppresses the TNF-α-induced NF-κB signaling cascade in HaCaT cells. Cells were pretreated with various doses of DMHC for 3 h and then exposed to TNF-α (10 ng/ml) for 15 min. (A) Whole cell lysates were analyzed for IKKα/β activation by Western blotting using phospho-specific antibodies. (B) Cell lysates were analyzed for the levels of total IκBα and phosphorylated p65 by Western blot analysis. (C) Cells pretreated with DMHC for 3 h were exposed to TNF-α (10 ng/ml) for 15 min. Nuclear extracts were prepared and analyzed for p65 DNA-binding activity by EMSA. (D) DMHC decreased NF-κB promoter activity. HaCaT cells were transiently transfected with an NF-κB promoter-luciferase construct and a β-galactosidase construct (pCMV-lacZ). The cells were pretreated with DMHC for 3 h at 48 h after transfection and then exposed to TNF-α for 24 h. Cell extracts were prepared and analyzed for the luciferase activity assay using a luminometer. Luciferase activities are normalized to β-galactosidase activities and expressed as fold increases over the control. RLA, relative luciferase activity. Data are presented as means ± SD of three experiments. *P < 0.05 and **P < 0.01 compared to TNF-α alone.

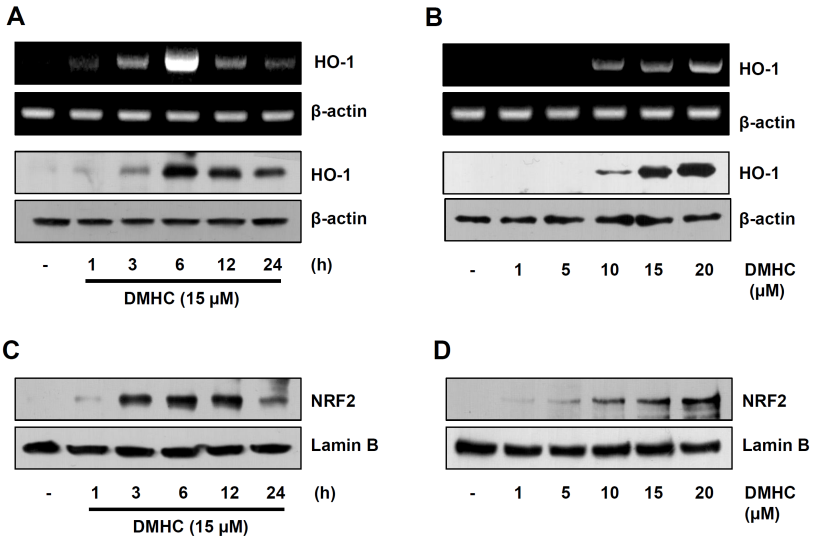

Fig. 3. DMHC induces HO-1 expression and activates NRF2 in HaCaT cells. (A) Cells were treated with DMHC for the indicated periods. Total RNA and protein were analyzed by RT-PCR (upper panel) and Western blotting (bottom panel), respectively. (B) Cells were incubated with DMHC for 3 h (for RNA) or 12 h (for protein). Total RNA and protein were analyzed by RT-PCR (upper panel) and Western blotting (bottom panel), respectively. (C) Cells were treated with DMHC for the indicated periods. Cell nuclear extracts were analyzed for NRF2 level by Western blotting. (D) After incubating with various DMHC concentrations for 3 h, nuclear extracts were prepared from the cells and were analyzed for NRF2 level by Western blotting.

time- and dose-dependent manners. As nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a major transcriptional factor in the regulation of HO-1 expression (11), we examined NRF2 activation in HaCaT cells. Cells were treated with DMHC, and the cell nuclear fraction was prepared and analyzed for translocation of NRF2 into the nucleus by Western blotting. As shown in Fig. 3C and D, DMHC strongly induced nuclear accumulation of NRF2 in time- and dose-dependent manners in HaCaT cells. These results suggest that DMHC is a potential inducer of HO-1 by activating NRF2 in HaCaT cells.

HO-1 mediates the inhibitory effect of DMHC on TNF-α-induced ICAM-1 expression and monocyte adhesion in HaCaT cells

We used a siRNA knockdown strategy against HO-1 to examine the functional relevance of HO-1 expression in the inhibitory effect of DMHC on TNF-α-induced ICAM-1 expression. As shown in Fig. 4A and B, knockdown of HO-1 using siRNA significantly reversed the inhibitory effect of DMHC on TNF-α-induced ICAM-1 expression at the mRNA and pro-

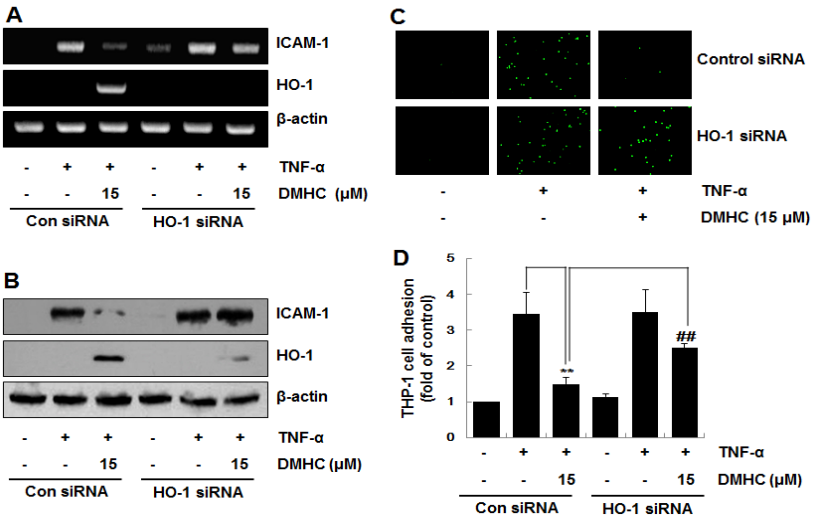

Fig. 4. HO-1 expression mediates the inhibitory effect of DMHC on TNF-α-induced ICAM-1expression and monocyte adhesion in HaCaT cells. (A, B) The cells were transiently transfected with control or HO-1 siRNA to knockdown the HO-1 gene. After 48 h of transfection, the cells were pretreated with DMHC for 3 h, and then stimulated with TNF-α for 1 h (for RNA) or 12 h (for protein). Total RNA and protein were analyzed by RT-PCR (A) and Western blotting (B), respectively. (C, D) Cells transfected with control or HO-1 siRNA were incubated with DMHC for 3 h, and then stimulated with TNF-α for 12 h. HaCaT cells were co-cultured with calcein-AM-labeled THP-1 monocytes for 1 h. (C) Microscopic images were obtained using a fluorescence microscope (original magnification, ×40). (D) Calcein-AM fluorescence intensity was quantified using a fluorescence plate reader. Data are presented as means ± SD of three independent experiments. $^{**}P < 0.01$ compared to TNF-α alone, $^{\#\#}P < 0.01$ compared to TNF-α and DMHC.

tein levels. In addition, HO-1 knockdown significantly reversed the inhibitory effect of DMHC on TNF-α-induced monocyte adhesion (Fig. 4C and D). These results support the notion that HO-1 expression is involved in mediating the suppressive effects of DMHC on TNF-α-induced ICAM-1 expression and monocyte adhesion.

DISCUSSION

Infiltration of leukocytes into inflamed areas is a critical step in the development of inflammatory skin diseases. Various pro-inflammatory stimuli, such as TNF-α, participate in upregulating adhesion molecules, such as ICAM-1, in keratinocytes, which play an important role in leukocyte infiltration into the skin (4). Therefore, modulating the expression of adhesion molecules is a good strategy to treat inflammatory skin diseases. In this study, we evaluated the anti-inflammatory effects of DMHC on keratinocytes. We showed that DMHC suppressed TNF-α-induced ICAM-1 expression by inhibiting NF-κB activation and upregulating HO-1 expression in keratinocytes.

Upon stimulation with TNF-α, keratinocytes express adhesion molecules, such as ICAM-1, that facilitates infiltration of leukocytes into the inflamed skin area. Several studies have reported that increased ICAM-1 expression levels in keratinocytes are observed in inflamed lesions of patients with AD and psoriasis (5, 6). We performed experiments to examine the effect of DMHC on TNF-α-induced ICAM-1 expression in HaCaT cells. Pretreatment with DMHC significantly inhibited ICAM-1 expression at the mRNA and protein levels in TNF-α-stimulated HaCaT cells. In addition, DMHC significantly suppressed TNF-α-induced monocyte adhesion to HaCaT cells. These results suggest that DMHC modulates against inflammatory responses.

NF-κB is the principle transcription factor mediating expression of various pro-inflammatory genes including ICAM-1 (8). NF-κB is associated with IκBα in unstimulated cells and resides in the cytosol. Stimulating cells with TNF-α activates the IKK complex, consisting of two kinase subunits (IKKα and IKKβ) and a regulatory subunit IKKγ/NEMO. The activated IKK complex phosphorylates IκBα, which is, in turn, degraded by proteasomes. NF-κB moves to the nucleus, where it induces the transcription of pro-inflammatory genes. We performed experiments to evaluate the modulatory activity of DMHC on the signaling pathways leading to NF-κB activation in TNF-α-stimulated HaCaT cells. DMHC significantly inhibited IKK phosphorylation, IκBα degradation, and p65 phosphorylation in TNF-α-stimulated HaCaT cells. In addition, DMHC decreased TNF-α-induced p65 DNA-binding and NF-κB promoter activities (Fig. 2C and 2D). These results indicate that DMHC modulates the signaling cascades leading to NF-κB activation.

As various chalcone derivatives exert their anti-inflammatory activities by inducing HO-1 expression in various inflammatory models (15, 19), we first performed an experiment to evaluate the ability of DMHC to induce HO-1 expression. DMHC induced HO-1 expression at the mRNA and protein levels in time- and dose-dependent manners in HaCaT cells, indicating that DMHC is an inducer of HO-1 expression. NRF2 is a major transcriptional factor responsible for expression of the HO-1 gene (11). We observed that DMHC activated NRF2 by promoting translocation of NRF2 to the nucleus in time- and dose-dependent manners in HaCaT cells. HO-1 knockdown significantly reversed the inhibitory effect of DMHC on TNF-α-induced ICAM-1 expression and subsequent monocyte adhesion to HaCaT cells, suggesting the involvement of HO-1 expression in the anti-inflammatory effects of DMHC. A growing body of evidence suggests that HO-1 expression is involved in the protective effects against inflammatory skin diseases (12-14). HO-1 expression exerts a modulatory effect on the keratinocyte in-

flammatory response (20, 21). Ferrous iron, carbon monoxide, and bilirubin, generated from HO-1 activity, may contribute to the beneficial effect of HO-1 inducers (11). Activating NRF2 may affect the expression of a variety of antioxidant and detoxification genes. Because HO-1 expression partly contributed to the anti-inflammatory effects of DMHC in HaCaT cells, we cannot rule out possible involvement of antioxidant and detoxification genes other than HO-1 in this process. Taken together, these results indicate that DMHC-induced HO-1 expression contributes to the inhibitory effects of DMHC on TNF-α-induced ICAM-1 expression and subsequent monocyte adhesion in keratinocytes.

In conclusion, we provide evidence that DMHC exerts its inhibitory effect on TNF-α-induced ICAM-1 expression and subsequent monocyte adhesion to keratinocytes by blocking the signaling cascades leading to NF-κB activation. In addition, the anti-inflammatory activity of DMHC is mediated via HO-1 induction. These results provide molecular evidence that DMHC may have therapeutic potential against inflammatory skin diseases.

MATERIALS AND METHODS

Cell culture and reagents

Human immortalized keratinocyte cell line, HaCaT, and human THP-1 monocytes were maintained as described previously (20). Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). MTT was obtained from Duchefa (Haarlem, the Netherlands). Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probes (Eugene, OR, USA). Primary antibodies against ICAM-1, Nrf2, lamin B, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HO-1 (Enzo Life Sciences, Basel, Switzerland), IκBα, and phospho-p65 (Cell Signaling Technology, Beverly, MA, USA) were obtained commercially. DMHC was purchased from Extrasynthese (Genay, France). A 25 mM DMHC stock was prepared in ethanol, stored at $-20^{\circ}C$, and diluted with DMEM for experiments.

MTT assay

Cell viability was evaluated using a MTT colorimetric assay as described previously (22).

Western blot analysis

Total proteins in cell extracts or nuclear extracts were measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). The protein samples (30-50 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and were transferred to a nitrocellulose membrane that was blocked with 10% dry milk in TBST (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% Tween 20) and incubated with the indicated antibodies. Immuno-reactive bands were visualized by an enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA) (21).

RT-PCR analysis

Total RNA from HaCaT cells was analyzed as described previously (20).

Cell adhesion assay

Monocyte adhesiveness to HaCaT cells was determined using a cell-cell adhesion assay described previously (22).

EMSA

HaCaT cells were treated with DMHC for 3 h and then exposed to TNF-α (10 ng/ml) for 15 min. Cell nuclear extracts were prepared and analyzed for NF-κB binding activity using an EMSA as described previously (23, 24).

Transfection

Co-transfection of HaCaT cells with NF-κB-luc reporter plasmid (Stratagene, La Jolla, CA, USA) and a control (pCMV-β-galactosidase) plasmid was performed for the reporter assay as described previously (23). To perform the HO-1 siRNA knockdown experiments, HaCaT cells were transfected with control siRNA or HO-1 siRNA using Lipofectamine 3000. After a 48 h transfection, the cells were treated with DMHC for 3 h and then exposed to TNF-α (10 ng/ml) for analysis of ICAM-1 expression and monocyte adhesion.

Statistical analysis

Results are expressed as the means ± standard errors from at least three independent experiments. The values were evaluated via one-way analysis of variance, followed by Duncan's multiple range test using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

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