Biochemical and Biophysical Research Communications 398 (2010) 140–145

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Celastrol suppresses IFN-gamma-induced ICAM-1 expression and subsequent monocyte adhesiveness via the induction of heme oxygenase-1 in the HaCaT cells

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ARTICLE INFO

Article history:
Received 8 June 2010
Available online 17 June 2010

Keywords:
Celastrol
ICAM-1
HO-1
Inflammation
Keratinocyte
IFN-γ

ABSTRACT

Celastrol, a quinone methide triterpenoid derived from the medicinal plant *Tripterygium wilfordii,* possesses various biological activities such as anti-oxidant, anti-tumor, and anti-inflammatory activities. In this study, we examined the suppressive effect of celastrol on IFN-γ-induced expression of ICAM-1 and the molecular mechanism responsible for these activities. We found that celastrol induced mRNA and protein expression of heme oxygenase-1 (HO-1) in the human keratinocyte cell line HaCaT. Treatment of HaCaT cells with tin protoporphyrin IX (SnPP), a specific inhibitor of HO-1, reversed the suppressive effect of celastrol on IFN-γ-induced protein and mRNA expression of ICAM-1. HO-1 knockdown using small interfering RNA (siRNA) led to reverse inhibition of IFN-γ-induced up-regulation of ICAM-1 by celastrol. In addition, SnPP reversed suppression of IFN-γ-induced promoter activity of ICAM-1 by celastrol. Furthermore, blockage of HO-1 activity by SnPP and HO-1 siRNA reversed the inhibitory effect of celastrol on IFN-γ-induced adhesion of monocytes to keratinocytes. These results suggest that celastrol may exert anti-inflammatory responses by suppressing IFN-γ-induced expression of ICAM-1 and subsequent monocyte adhesion via expression of HO-1 in the keratinocytes.

1. Introduction

The epidermal keratinocyte, one of major cell type in the skin, can express adhesion molecules in response to cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α) [1,2]. Up-regulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on the surface of keratinocytes is believed to increase infiltration of monocytes/T cells into the site of inflammation in the skin [3].

Heme oxygenase-1 (HO-1) catalyzes the degradation of heme to generate ferrous iron, carbon monoxide (CO) and biliverdin. The by-products resulting from HO-1 activity seem to contribute to the beneficial effects of HO-1 expression. Induction of HO-1 expression in a variety of cell types has been reported to exert cytoprotective, anti-oxidant, anti-inflammatory, and anti-apoptotic effects in a number of pathological conditions including inflammation [4]. Recently, HO-1 expression has shown to have a regulatory function in skin inflammation such as atopic dermatitis [5–7].

Celastrol, a quinone methide triterpenoid, is present in *Tripterygium wilfordii Hook* that is known as the "Thunder of God Vine" [8]. Celastrol possesses various biological activities such as anti-oxidant, anti-tumor, and anti-inflammatory activities [9–11], even though the exact mechanism has not been fully understood. Previous studies demonstrated that celastrol inhibited production of IL-6, TNF-α, and IL-1β by inhibition of NF-kB activation in LPS-stimulated human monocytes [12], mouse macrophages [13] and microglial cells [14]. Celastrol inhibited FcepsilonRI signaling to exert an anti-allergic effect by binding to ERK in antigen-stimulated mast cells [15]. Therefore, celastrol may exert its biological activity by multiple distinct mechanisms depending on the type of stimulus and cells (for review, see [16]).

In this study, we examined the effect of celastrol on IFN-γ-induced ICAM-1 expression and subsequent monocyte adhesion in a human keratinocyte cell line HaCaT. We observed for the first time that celastrol induced expression of HO-1 at the mRNA and protein levels in the HaCaT cells. Blockage of HO-1 activity by SnPP,

Abbreviations: HO-1, heme oxygenase-1; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ.

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doi:10.1016/j.bbrc.2010.06.053

an HO-1 inhibitor, or HO-1 knockdown using small interfering RNAs (siRNA) reversed the suppressive effect of celastrol on IFN-γ-induced expression of ICAM-1 and subsequent adhesion of monocytes to HaCaT cells. These results suggest that celastrol suppresses IFN-γ-induced expression of ICAM-1 and subsequent monocyte adhesion by a mechanism involving expression of HO-1 in the keratinocytes.

2. Materials and methods

2.1. Cell culture and reagents

The immortalized human keratinocyte cell line, HaCaT, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin) at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. Human THP-1 monocytic cells were maintained in RPMI 1640 medium supplemented with 2 mm L-glutamine and 10% fetal bovine serum. Tin protoporphyrin IX (SnPP) were purchased from Calbiochem (La Jolla, CA). Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probe (Eugene, OR, USA). HO-1 specific siRNA, primary antibodies against ICAM-1, HO-1 and actin (Santa Cruz, CA, USA) were obtained commercially. Celastrol, HRP-conjugated anti-rabbit or goat antibodies were supplied by Sigma (St. Louis, MO, USA).

2.2. Immunoblot analysis

Cell lysates were prepared by incubating cells in lysis buffer (125 mM Tris–HCl, pH 6.8, 2% SDS, 10% v/v glycerol.) at 4 °C for 30 min. Proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting. Immunoreaction was performed with the indicated antibodies, and the immunoreactive bands were detected by enhanced chemiluminescence (ECL; Amersham) as recommended by the manufacturer.

2.3. RT-PCR analysis

Total RNA was isolated from HaCaT cells using a Trizol reagent kit (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions [17]. The RNA (2 μg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 μg/μL oligo-$(dT)_{15}$ primer (Promega, Madison, WI, USA), followed by PCR amplification using specific primers, as described previously [18]. The following forward and reverse primers (5′→3′) were used: ICAM-1 forward, GGT GAC GCT GAA TGG GGT TCC; ICAM-1 reverse, GTC CTC ATG GTG GGG CTA TGA CTC; HO-1 forward, GCG CAG CAT GCC CCA GGA TTT G; and HO-1 reverse, AGC TGG ATG TTG AGC AGG A; beta-actin forward, GCG GGA AAT CGT GCG TGA CAT T; and beta-actin reverse, GAT GGA GTT GAA GGT AGT TTC GTG. PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide.

2.4. HO-1 activity assay

HO-1 activity in the HaCaT cells was assessed by bilirubin generation from hemin as described previously with some minor modifications [19]. In brief, the harvested cell was dissolved in 100 mM potassium phosphate buffer (pH 7.4) with 2 mM $MgCl_2$. The cells were frozen (−80 °C), and thawed three times and sonicated on ice. The supernatant was collected after centrifugation at 12,000g for 10 min at 4 °C. The supernatant was added to the reaction mixture (400 μL) containing 3 mg rat liver cytosol, 20 μM hemin, 2 mM glucose-6-phophate, 0.2 U glucose-6-phosphate dehydrogenase, and 0.8 mM NADPH and incubated at 37 °C for 1 h in the dark. One milliliter of chloroform was added to extract the bilirubin, and the difference in absorbance between 464 nm and 530 nm was determined (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO-1 activity was expressed as picomoles of bilirubin formed per mg of protein per hour.

2.5. Transfection

The ICAM-1 luciferase plasmid (pICAM-1-luc) containing regions spanning −485 to +45 of the human ICAM-1 promoter was used [20]. Transfection of HaCaT cells with pICAM-1-luc or pCMV-β-galactosidase construct was performed using the AMAXA nucleofector according to the manufacturer's instructions (Amaxa biosystems, Germany) [18]. After 24 h, the cells were harvested, and luciferase and beta-galactosidase activities were measured. The luciferase activity of each sample was normalized to the beta-galactosidase activity to calculate the relative luciferase activity, and the results were expressed as fold transactivation. To perform the knockdown experiments of HO-1 by siRNA, HaCaT cells were transfected with control siRNA or HO-1 siRNA Mix using Lipofecamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.6. Cell adhesion assay

Adherence of THP-1 cells to HaCaT cells was assayed using a cell–cell adhesion assay as described previously [21]. Briefly, the calcein-AM labeled THP-1 (7.0×10^5) was co-cultured with HaCaT cells for 1 h. Co-cultured cells were washed three times with PBS. For the adhesion quantification, the calcein-AM fluorescent intensity was measured at 485 nm excitation and 538 nm emission by a Fluoroskan ELISA plate reader (Labsystems Oy). The fluorescence images were obtained at 485 nm excitation and 538 nm emission using a SPOT II digital camera-attached fluorescence microscope with Spot II data acquisition software (Diagnostic Instrument).

2.7. Statistical analysis

The results were expressed as the means ± SEM from at least three independent experiments. The values were evaluated via one-way ANOVA, followed by Duncan's multiple range tests using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Celastrol induces the expression of HO-1 in the HaCaT cells

Consistent with previous reports [1,22], HaCaT cells were markedly induced to express ICAM-1 when stimulated with IFN-γ. Treatment with celastrol suppressed IFN-γ-induced mRNA and protein expression of ICAM-1 in the HaCaT cells (Fig. 1A). Celastrol had no significant cytotoxic effect on the HaCaT cells at the concentrations tested (data not shown). To elucidate the possible mechanism involved in the inhibitory effect of celastrol on IFN-γ-induced expression of ICAM-1, we examined whether celastrol can induce expression of HO-1. We observed for the first time that pretreatment with celastrol significantly induced mRNA and protein expression of HO-1 in dose- and time-dependent manners (Fig. 1B and C) in the HaCaT cells. As expected, celastrol increased the enzyme activity of HO-1 in a dose-dependent manner (Fig. 1D). These data suggest that celastrol is a potential inducer of HO-1 in the HaCaT cells.

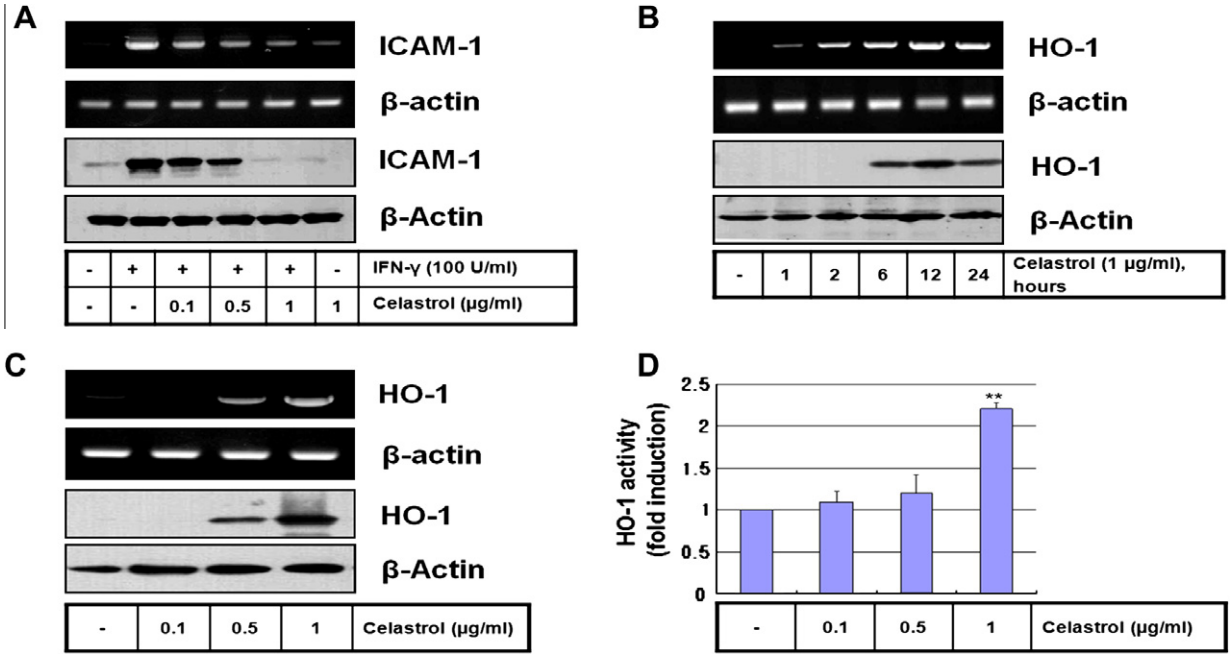

Fig. 1. Celastrol induces HO-1 expression in HaCaT cells. (A) HaCaT cells were incubated with the indicated concentrations of celastrol for 1 h, and then stimulated with IFN-γ (100 U/ml) for 4 h (for RNA) or 12 h (for protein). Total RNA and protein were analyzed by RT-PCR (upper panel) and Immunoblotting (bottom panel), respectively. HaCaT cells were incubated with 1 μg/ml celastrol for the indicated time (B) and various concentrations of celastrol for 12 h (C). Total RNA and protein were analyzed by RT-PCR (upper panel) and immunoblotting (bottom panel), respectively. (D) HO-1 enzyme activity was measured at 12 h after exposure to the indicated concentrations of celastrol.

3.2. HO-1 mediates the inhibitory effect of celastrol on IFN-γ-induced expression of ICAM-1 in the HaCaT cells

To examine the involvement of HO-1 expression in the inhibitory effect of celastrol on IFN-γ-induced expression of ICAM-1, we exploited pharmacological HO-1 inhibitor and siRNA knockdown strategy against HO-1. As shown in Fig. 2A and B, pretreatment with a HO-1 inhibitor, SnPP, reversed the inhibitory effect of celastrol on IFN-γ-induced mRNA and protein expression of ICAM-1. Fig. 2C and D shows the effect of HO-1 siRNA on inhibition of IFN-γ-induced expression of ICAM-1 by celastrol. HO-1 knockdown significantly reversed the inhibitory effect of celastrol on

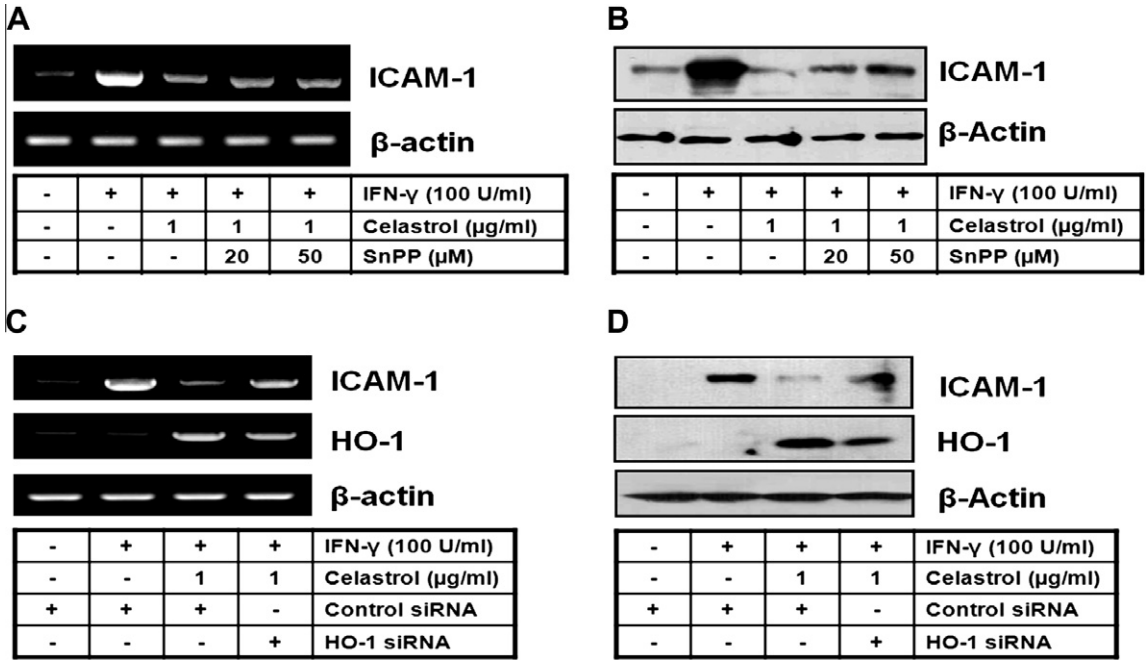

Fig. 2. HO-1 induction by celastrol suppresses IFN-γ-induced expression of ICAM-1 mRNA and protein in HaCaT cells. HaCaT cells were incubated with 1 μg/ml celastrol for 1 h in the absence or presence of SnPP, and then exposed to IFN-γ for 4 h (for RNA) or 12 h (for protein). The total RNA and protein were analyzed by RT-PCR (A) and immunoblotting (B), respectively. HaCaT cells were transiently transfected with control or HO-1 siRNA. After 24 h, the transfected HaCaT cells were incubated with 1 μg/ml celastrol for 1 h, and then stimulated with IFN-γ for 4 h (for RNA) or 12 h (for protein). The total RNA and protein were analyzed by RT-PCR (C) and immunoblotting (D), respectively.

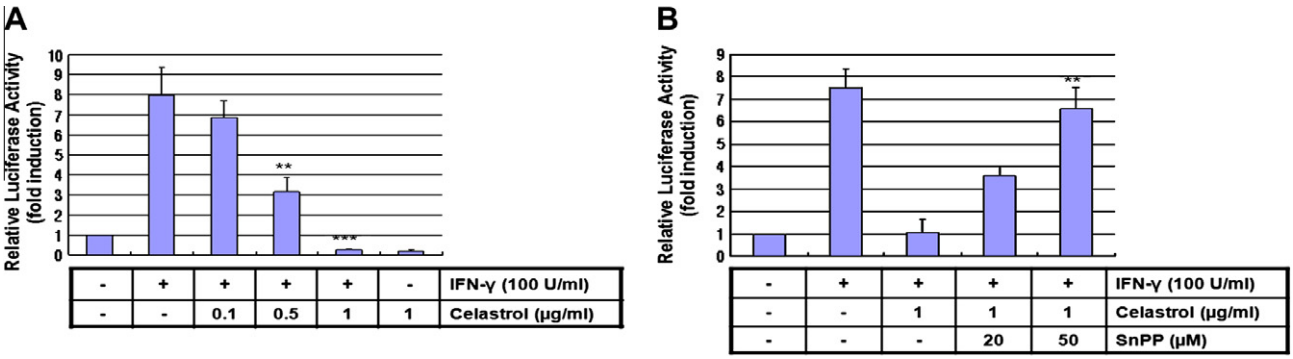

Fig. 3. Induction of HO-1 by celastrol suppresses IFN-γ-induced luciferase activity of ICAM-1 promoter in HaCaT cells. HaCaT cells were transiently transfected with an ICAM-1 promoter-luciferase construct and a β-galactosidase construct (pCMV-lacZ) for 24 h. (A) The transfected cells were incubated with the indicated concentration of celastrol for 1 h, and then stimulated with IFN-γ (100 U/ml) for 24 h. (B) The transected cells were incubated with 1 μg/ml celastrol for 1 h in the absence or presence of SnPP to block the HO-1 expression and then stimulated with IFN-γ (100 U/ml) for 24 h. The luciferase activity was measured as described under Section 2. Luciferase activities are normalized to the β-galactosidase activities and expressed as fold induction over the control. RLA, relative luciferase activity. Result are means ± SD. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with IFN-γ alone.

IFN-γ-induced expression of ICAM-1 mRNA and protein. The results confirm that HO-1 expression produced by celastrol mediates the inhibitory effects of celastrol on IFN-γ-induced expression of ICAM-1.

3.3. HO-1 reverses the inhibitory effect of celastrol on IFN-γ-induced promoter activity of ICAM-1 in the HaCaT cells

We further evaluated the effect of HO-1 expression on the inhibitory effect of celastrol on IFN-γ-induced promoter activity of ICAM-1. As expected, celastrol suppressed IFN-γ-induced luciferase activity in a dose-dependent manner in HaCaT cells transfected with an ICAM-1 promoter-luciferase construct (Fig. 3A). Pretreatment of SnPP reversed the inhibitory effect of celastrol on IFN-γ-induced luciferase activity in a dose-dependent manner (Fig. 3B). These results confirm that the inhibitory effect of celastrol on IFN-γ-induced ICAM-1 expression is mediated by HO-1 expression.

3.4. HO-1 expression induced by celastrol suppresses IFN-γ-induced monocyte adhesion

We next examined the effect of a HO-1 inhibitor and siRNA knockdown on the inhibitory effect of celastrol on IFN-γ-induced monocyte adhesion to HaCaT cells. Treatment with celastrol suppressed IFN-γ-induced monocyte adhesion to HaCaT cells in a

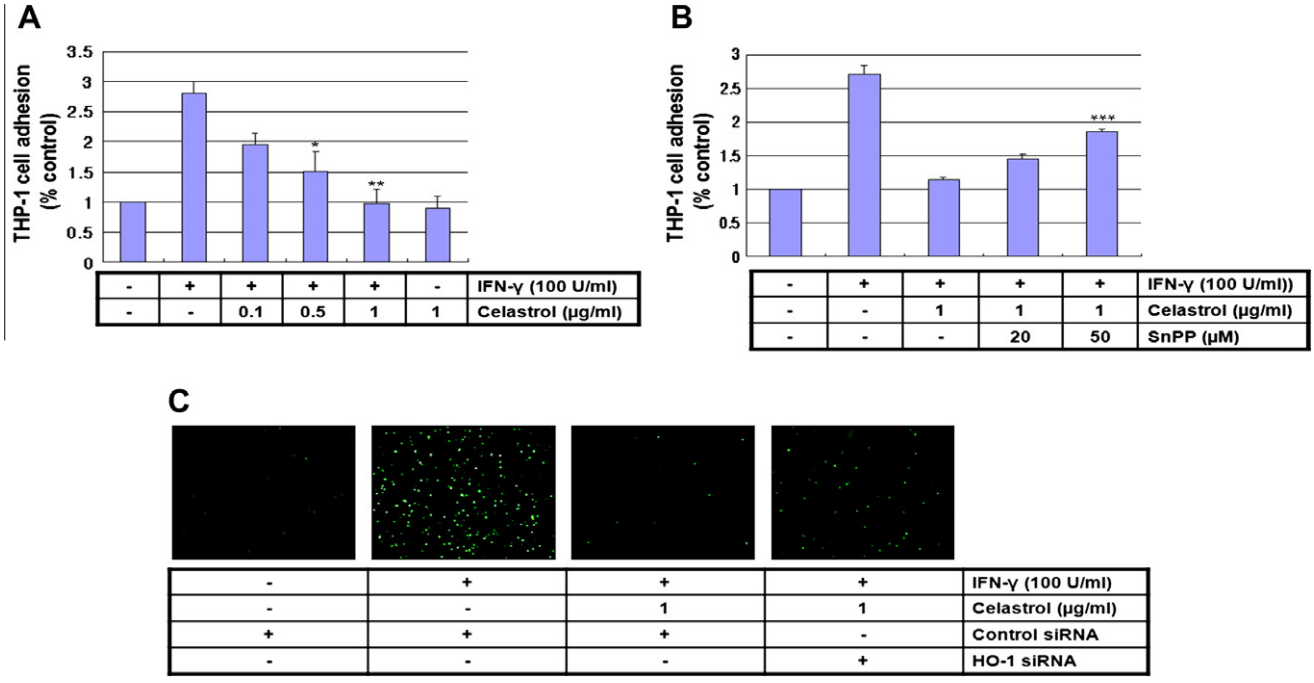

Fig. 4. HO-1 induction mediates the suppressive effect of celastrol on IFN-γ-induced monocyte adhesion in HaCaT cells. (A) HaCaT cells were incubated with the indicated concentrations of celatrol for 1 h, and then simulated with IFN-γ (100 U/ml) for 8 h. HaCaT cells were co-cultured with calcein-AM-labeled THP-1 monocytes for 1 h. The calcein-AM fluorescent intensity was measured by an ELISA plate reader. (B) HaCaT cells were incubated with 1 μg/ml celastrol for 1 h in the absence or presence of SnPP, and then exposed to IFN-γ (100 U/ml) for 8 h. HaCaT cells were co-cultured with calcein-AM-labeled THP-1 monocytes for 1 h. (C) HaCaT cells transfected with control or HO-1 siRNA were incubated with 1 μg/ml celastrol for 1 h, and stimulated with IFN-γ (100 U/ml) for 8 h. HaCaT cells were co-cultured with calcein-AM-labeled THP-1 monocytes for 1 h. Microphotographs were obtained using fluorescence microscopy (original magnification, 40×). Results are means ± SD. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with IFN-γ alone.

dose-dependent manner (Fig. 4A). SnPP significantly reversed the inhibitory effect of celastrol on IFN-γ-induced monocyte adhesion (Fig. 4B). As shown in Fig. 4C, HO-1 knockdown using siRNA significantly reversed the inhibitory effect of celastrol on IFN-γ-induced monocyte adhesion. These results confirm the relevance between HO-1 expression and the inhibitory effect of celastrol on IFN-γ-induced monocyte adhesion to HaCaT cells.

4. Discussion

One of characteristic features of skin inflammation is the infiltration of monocytes/T cells into the skin area. Pro-inflammatory cytokines such as IFN-γ up-regulate expression of ICAM-1 in the keratinocytes, which is an important regulator to initiate interaction between monocytes/T cells and keratinocytes during the processes of infiltration [3]. Because of its critical role in skin inflammation, the down-regulation of ICAM-1 expression is considered an effective target for treatment of inflammatory skin diseases.

Previous studies reported that celastrol exerts anti-inflammatory effects [12–14], even though the relevant anti-inflammatory mechanisms have not been fully understood. In this study, we identified that celastrol is an inducer of HO-1 expression which is responsible for suppression of IFN-γ-induced ICAM-1 expression and monocyte adhesion in the keratinocytes.

Upon stimulation with IFN-γ, HaCaT cells expressed the increased level of ICAM-1 which was effectively decreased by celastrol in a dose-dependent manner (Fig. 1A). In addition, as shown in Fig. 4A, celastrol significantly inhibited adhesion of monocytes to the HaCaT cells stimulated with IFN-γ. Since previous studies have reported that HO-1 expression mediated the biological activities of several natural compounds with therapeutic potential against diseases related to inflammation and oxidative stress [23–25], we examined whether celastrol can induce HO-1 expression. As shown in Fig. 1, treatment with celastrol induced HO-1 expression and the enzyme activity of HO-1 in time- and dose-dependent manners in the HaCaT cells, indicating that celastrol is a potent inducer of HO-1 expression. Pharmacological HO-1 inhibitor and siRNA knockdown against HO-1 significantly reversed the inhibitory effect of celastrol on IFN-γ-induced ICAM-1 expression and subsequent monocyte adhesiveness to the HaCaT cells (Fig. 2 and Fig 4), suggesting the functional consequence of celastrol-induced HO-1 expression. Although the mechanisms by which HO-1 induction by celastrol inhibits IFN-γ-induced ICAM-1 expression remain to be elucidated, the by-products resulting from HO-1 activity, such as carbon monoxide and bilirubin, may contribute to the inhibitory effect of celastrol. Since various transcriptional factors including Nrf2 were responsible for expression of HO-1 gene [4], further studies are required to define the molecular mechanisms underlying celastrol-induced HO-1 expression in the keratinocytes. Taken together, these results demonstrated that HO-1 expression by celastrol regulates IFN-γ-induced ICAM-1 expression and subsequent monocyte adhesiveness in the keratinocytes.

In consistent with our results, several reports have indicated that HO-1 has the inhibitory effect on expression of various adhesion molecules. Overexpression of HO-1 protected against TNF-α-mediated airway inflammation via the down-regulation of adhesion molecules in vitro and in vivo [26]. HO-1 overexpression inhibited up-regulation of ICAM-1, VCAM-1 and E-selectin expression upon TNF-α stimulation in endothelial cells [27,28]. These studies suggest that HO-1 has anti-inflammatory activities by modulating the signaling pathways leading to expression of adhesion molecules during inflammation response. HO-1 has been reported to have a regulatory role in the process of inflammatory skin diseases. Expression of HO-1 inhibits inflammation in the atopic dermatitis-like lesions and the contact hypersensitivity in mice [5–7]. In addition, HO-1 inhibits T cell-dependent skin inflammation [5].

In conclusion, better understanding of the mechanism underlying the anti-inflammatory activity of celastrol might help to develop a therapeutic agent for inflammatory skin diseases. This study provides the evidences that HO-1 induction mediates the suppressive effect of celastrol on IFN-γ-induced ICAM-1 expression and subsequent adhesion of monocytes to keratinocytes. Therefore, the signaling pathway leading to HO-1 induction may represent a mechanism that contributes to the anti-inflammatory activity of celastrol.

Acknowledgments

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MEST) (The Regional Research Universities Program/Medical & Bio-Materials Research Center) and in part by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093812).

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