Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 351 (2006) 266–272
BBRC
www.elsevier.com/locate/ybbrc

Mitogen-activated protein kinase kinase 1/extracellular signal-regulated kinase (MEK-1/ERK) inhibitors sensitize reduced glucocorticoid response mediated by TNFα in human epidermal keratinocytes (HaCaT)

Kenji Onda [a,*], Masahiro Nagashima [a], Yo Kawakubo [b], Shota Inoue [a], Toshihiko Hirano [a], Kitaro Oka [a]

[a] Department of Clinical Pharmacology, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[b] Department of Dermatology, Teikyo University School of Medicine, Ichihara Hospital, Chiba, Japan

Received 27 September 2006
Available online 19 October 2006

Abstract

Glucocorticoids (GCs) are essential drugs administered topically or systematically for the treatment of autoimmune skin diseases such as pemphigus. However, a certain proportion of patients does not respond well to GCs. Although studies on the relationship between cytokines and GC insensitivity in local tissues have attracted attention recently, little is known about the underlying mechanism(s) for GC insensitivity in epidermal keratinocytes. Here, we report that tumor necrosis factor (TNF) α reduces GC-induced transactivation of endogenous genes as well as a reporter plasmid which contains GC responsive element (GRE) in human epidermal keratinocyte cells (HaCaT). The GC insensitivity by TNFα was not accompanied by changes in mRNA expressions of GR isoforms (α or β). However, we observed that mitogen-activated protein kinase kinase-1/extracellular signal-regulated kinase (MEK-1/ERK) inhibitors (PD98059 and U0126) significantly sensitized the GC-induced transactivation of anti-inflammatory genes (glucocorticoid-induced leucine zipper (GILZ) and mitogen-activated protein kinase phosphatase (MKP)-1) and FK506 binding protein (FKBP) 51 gene in the presence of TNFα. Additionally, we observed that TNFα reduced prednisolone (PSL)-dependent nuclear translocation of GR, which was restored by pre-treatment of MEK-1 inhibitors. This is the first study demonstrating a role of the MEK-1/ERK cascade in TNFα-mediated GC insensitivity. Our data suggest that overexpression of TNFα leads to topical GC insensitivity by reducing GR nuclear translocation in keratinocytes, and our findings also suggest that inhibiting the MEK-1/ERK cascade may offer a therapeutic potential for increasing GC efficacy in epidermis where sufficient inflammatory suppression is required.

Keywords: TNFα; MEK-1/ERK; HaCaT; Human epidermal keratinocytes; Glucocorticoid receptor; Nuclear translocation

Glucocorticoids (GCs) have been the cornerstone in the treatment of several inflammatory and autoimmune diseases including pemphigus. GCs have contributed to a significant reduction in the mortality rate of this serious skin disease with their strong anti-inflammatory and immunosuppressive potencies [1]. However, current GC therapy still possesses unsolved problems such as GC insensitivity/resistance or serious adverse reactions.

Although their mode of action is not completely understood, the pharmacological effects of GCs in autoimmune skin diseases such as pemphigus are considered to be exerted systemically and locally. First, GCs act on infiltrating and circulating lymphocytes leading to a suppression of autoantibody production or immune/inflammatory responses. Second, GCs directly affect keratinocytes with modification of their inflammatory and pathophysiological

* Corresponding author. Fax: +81 426 76 5798.
E-mail address: knjond@ps.toyaku.ac.jp (K. Onda).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.10.032

gene expressions [2]. In target cells, the molecular effects of GCs are mainly mediated by their binding to GC receptor (GR)α, a classically well-investigated receptor isoform. GRα is, in the absence of its ligand, localized in cytoplasm binding with other chaperon molecules such as heat shock proteins. After binding with GC, GRα translocates into the nucleus and homodimerizes and binds to GC responsive element (GRE) located in the promoter region of genes encoding anti-inflammatory mediators, and consequently transactivates such gene expression in harmony with several co-factors (transactivation; TA) [3]. Meanwhile, GC–GRα complex interacts with other transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), and inhibits the gene expression of inflammatory mediators (transrepression; TR) [3].

There have been studies showing that cytokines alter GC efficacy. It has been reported that a combination of IL-2 and -4, or IL-13 alone, reduces GR affinity with its ligands in T cells [4,5]. Several studies have reported that GRβ, a dominant negative isoform of GRα, is induced by cytokines such as IL-2 and -4 in combination, and TNFα [6,7], although the physiological significance of GRβ is controversial [8,9]. In addition, cytokines have been shown to activate pathways that lead to the stimulation of several inflammatory transcription factors, which also contribute to the attenuation of GC effects [10]. However, although it is known that inflammatory cytokines are crucial for developing a topical pathophysiology of the disease [11], little is known about the effect of cytokines on the GC effects in skin regions such as epidermal keratinocytes.

In the current study, we report TNFα modulates GC sensitivity by suppressing GR nuclear translocation and GC-induced transactivation in human epidermal keratinocyte cells (HaCaT). We also demonstrate that mitogen-activated protein kinase kinase-1/extracellular signal-regulated kinase (MEK-1/ERK) inhibitors restore GR translocation and sensitize the reduced GC response mediated by TNFα.

Materials and methods

Reagents. Human recombinant TNFα was purchased from R&D systems (Tokyo, Japan). Prednisolone (PSL) was obtained from Sigma–Aldrich, (Tokyo, Japan). PD98059 and U0126 (MEK-1/ERK inhibitors), SC-514 (IKKβ inhibitor), SB203580 (p38 MAPK inhibitor), wortmannin (PI3 kinase inhibitor), and SP600125 (JNK inhibitor) were purchased from Calbiochem (Tokyo, Japan).

Cells and culture conditions. HaCaT cells were kindly provided by Dr. Norbert E. Fusenig (German Cancer Research Centre, Heidelberg, Germany). Cells were grown to confluence in Dulbecco's modified essential medium (DMEM) (Sigma–Aldrich) containing 10% FBS at 37 °C and 5% CO_2. We have preliminarily confirmed that none of the concentrations of cytokines or enzyme inhibitors presently examined showed significant changes in cell viability of HaCaT cells assessed by trypan blue staining and Celltiter Glo (Promega, Tokyo, Japan).

RNA extraction and quantitative real-time RT-PCR. Total RNA from HaCaT was extracted using a GenElute Total RNA Extraction Kit (Sigma–Aldrich, Tokyo, Japan) according to the manufacturer's instructions. The amount of total RNA was quantified by spectrophotometry. To exclude genomic DNA contamination, DNase I treatment was performed on all samples. Reverse transcription was performed with a stepwise reaction at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. For the subsequent PCR procedure, 2.5 μl cDNA mixture was used. PCR was performed using real-time SYBR green technology and analyzed by an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, USA). Primers were either originally designed by Primer Express software (Applied Biosystems) for GAPDH (sense: GAAGGTGAAGGTCG GAGTC, antisense: GAAGATGGTGATGGGATTTC) and FK506 binding protein (FKBP51) (sense: CCAAAGCTGTTGAATGCTGTGA, antisense: CAAACTCGTTCATGAGCAGCTG), or purchased from a manufacturer (Quantitect Primer Assay, Qiagen, Tokyo, Japan) for glucocorticoid inducible leucine zipper (GILZ, also known as TSC22D3) and map kinase phosphatase (MKP)-1 (DUSP1). All PCR products were analyzed by dissociating curve analysis to ensure that amplification was appropriately performed. To express the relative amounts of gene expressions, a $\Delta\Delta C_t$ method was adopted for the analysis of mRNA for GILZ, FKBP51, and MKP-1. To analyze the effects of kinase inhibitors on TNFα-mediated GC insensitivity, percent recovery rates were calculated as follows; recovery rates $(\%) = (A - B)/(C - B) \times 100$, where A indicates amounts of gene expressions in the presence of each inhibitor, TNFα, and PSL, B indicates data in the case of TNFα and PSL treatment, and C indicates data in the case of PSL treatment alone.

GRα and GRβ mRNA expressions were determined by RT-PCR using specific Taqman probes with an ABI Prism 7000 sequence detector (Applied Biosystems). The raw fluorescence data of the Taqman assay were exported and graphically analyzed by GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, USA). Primers and probes for GRα (sense: GAGGAAGTTATCCTCTGCCTC, antisense: TGTAAGCAC CACCTTCCTGTCT, probe: 6FAM-TTCCAACAGTGAGTCTGTCA GCGCA-TAMRA) and GRβ (sense: GGCAGCGGTTTTATCAACT GA, antisense: GTGTGAGATGTGCTTTCTGGTTTT, probe: 6FAM-A AAACTCTTGGATTCTATGCATGAAAATGTTATGTGG-TAMRA) were used.

Reporter gene assay. HaCaT cells were transiently transfected by using LipofectAMINE PLUS (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Briefly, cells were placed in 24-well plates two days prior to transfection and grown to 95% confluence. Plasmid DNA was prepared in 100 μl of serum-free DMEM and incubated with 3 μl of PLUS reagent at room temperature for 15 min, followed by 2.5 μl of LipofectAMINE in an additional 100 μl of DMEM. The mixture was incubated for another 15 min and then added to the wells containing 500 μl of serum- or antibiotic-free DMEM. Transfections were allowed to proceed for 6 h. One microgram of pGRE-luc (Clontech, Tokyo, Japan) was co-transfected along with 10 ng *Renilla* luciferase reporter (pRL-TK) (Promega, Tokyo, Japan) to control for transfection efficiency. The cells were then washed twice with serum-free DMEM and incubated for an additional 24 h in DMEM with TNFα followed by PSL treatment for 4 h. After treatments, cells were washed twice with PBS and lysed in a passive lysis buffer (dual-luciferase assay, Promega). These treatments were done in triplicate for each experiment. Luciferase activity was measured by using a dual-luciferase reporter assay system (Promega) and a MicroLumat Plus LB96V luminometer (Berthold, Tokyo, Japan). Luciferase activity was calculated as the ratio of Firefly/*Renilla* and expressed as the relative value to its control experiment.

Western blotting. Cells were cultured in 100 mm diameter dishes and nuclear proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Protein concentration was quantified by a bicinchoninic acid (BCA) assay method (Pierce). Equivalent amounts of nuclear protein (5 μg) were separated by SDS–polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Tokyo, Japan). Proteins were detected by immunoblotting followed by ECL chemiluminescence detection (Amersham Biosciences, Buckinghamshire, England). GR (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA) and histone H1 (AE-4) (Santa Cruz Biotechnology) antibodies were used as first antibodies. Secondary antibodies used were horseradish peroxidase conjugates of either anti-mouse

or anti-rabbit immunoglobulin G (Amersham Biosciences). Signals were detected by a luminoimage analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Data analysis. A Williams test was performed by Excel (Microsoft, Tokyo, Japan) originally programmed to calculate dose dependency. A Dunnet's multiple comparison test was applied to compare data among the control and treated samples. A Bonferroni's multiple comparison test was applied to compare data in each group. p Values less than 0.05 were regarded as statistically significant. These analyses were performed using GraphPad PRISM 4.0 (GraphPad Software Inc.).

Results

TNFα reduces PSL-dependent transactivation of pGRE-luc and an endogenous gene in HaCaT cells

We investigated the effect of TNFα on GC-induced transactivation in HaCaT cells. PSL (100 ng/ml) treatment for 4 h induced relative luciferase activity in HaCaT cells transiently transfected with pGRE-luc and pRL-TK plasmids (Fig. 1A). This transactivation was significantly reduced by a pre-treatment of cells with TNFα (0.1–10 ng/ml) in a dose-dependent manner. Statistically significant differences were observed when TNFα concentrations were higher than 1 ng/ml ($p < 0.05$ and $p < 0.01$ at TNFα concentrations of 1 and 10 ng/ml, respectively). PSL induction of an endogenous gene (glucocorticoid-induced leucine zipper; GILZ) was also significantly suppressed by TNFα pre-treatment in a similar manner (Fig. 1B). These results demonstrate TNFα reduces PSL sensitivity by decreasing its transactivational effect in HaCaT cells.

GRα and GRβ mRNA expressions were not changed by TNFα treatment in HaCaT cells

As shown in Fig. 1, TNFα-induced PSL insensitivity by suppressing GC responsive gene expressions in HaCaT cells. We next sought to determine the molecular mechanism(s) of PSL insensitivity. Taqman RT-PCR was performed to investigate changes in amounts of GRα or β mRNA expressions in the cultured conditions. However, results showed mRNA expressions of GR isoforms (α and β) were not changed by a 24 h TNFα treatment (10 ng/ml) in HaCaT cells (Fig. 2).

Effects of signaling cascade inhibitors on TNFα-induced GC insensitivity in HaCaT cells

Next, we investigated the effects of several enzyme inhibitors related to TNFα signaling. Cells were pre-treated with SC-514 (20 μM), SB203580 (10 μM), SP600125 (20 μM), wortmannin (1 μM), PD98059 (50 μM), or U0126

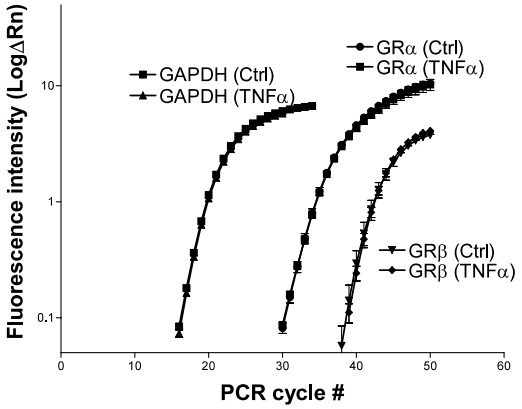

Fig. 2. Amplification plots of Taqman RT-PCR for GR isoforms (α and β) and effect of TNFα treatment on the expressions of GR isoforms. HaCaT cells were treated with TNFα (10 ng/ml) for 24 h. No significant change was observed in the mRNA expressions of both GR isoforms. GAPDH mRNA expressions, as a housekeeping control, were also determined. The data performed in triplicate are representative of three independent experiments.

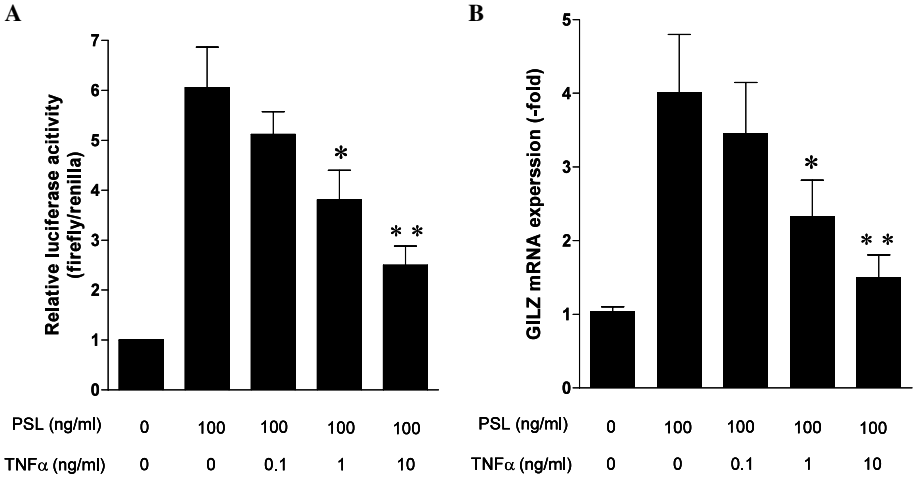

Fig. 1. (A) Effects of TNFα on the transcriptional activity of pGRE-luc determined by dual-luciferase assay. HaCaT cells were preincubated in the presence or absence of TNFα (0.1, 1, and 10 ng/ml) for 24 h and treated with or without PSL (100 ng/ml) for 4 h. Pre-treatment of TNFα inhibited PSL-induced luciferase activity in a dose-dependent manner ($n = 4$, the error bars are indicated as means ± SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ by Williams test). (B) TNFα also decreased PSL-induced endogenous GILZ mRNA expression dose-dependently ($n = 4$, the error bars are indicated as means ± SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ by Williams test).

(20 μM) as well as DMSO (final conc. 0.4%) as a vehicle for 1 h. Subsequently, the cells were treated with TNFα (10 ng/ml) for 24 h followed by PSL (100 ng/ml) treatment for 4 h. Then the amounts of GILZ and FKBP51 mRNA expressions were determined by real-time RT-PCR (Fig. 3A and B). The pre-treatments of PD98059 and U0126 significantly sensitized the PSL responses of GILZ and FKBP51 mRNA expressions by as much as 300% ($p < 0.01$). Although SP600125 tended to restore both PSL-dependent gene expressions, the difference was not statistically significant. Other signaling cascade inhibitors did not also show statistically significant recoveries of PSL-dependent gene expressions.

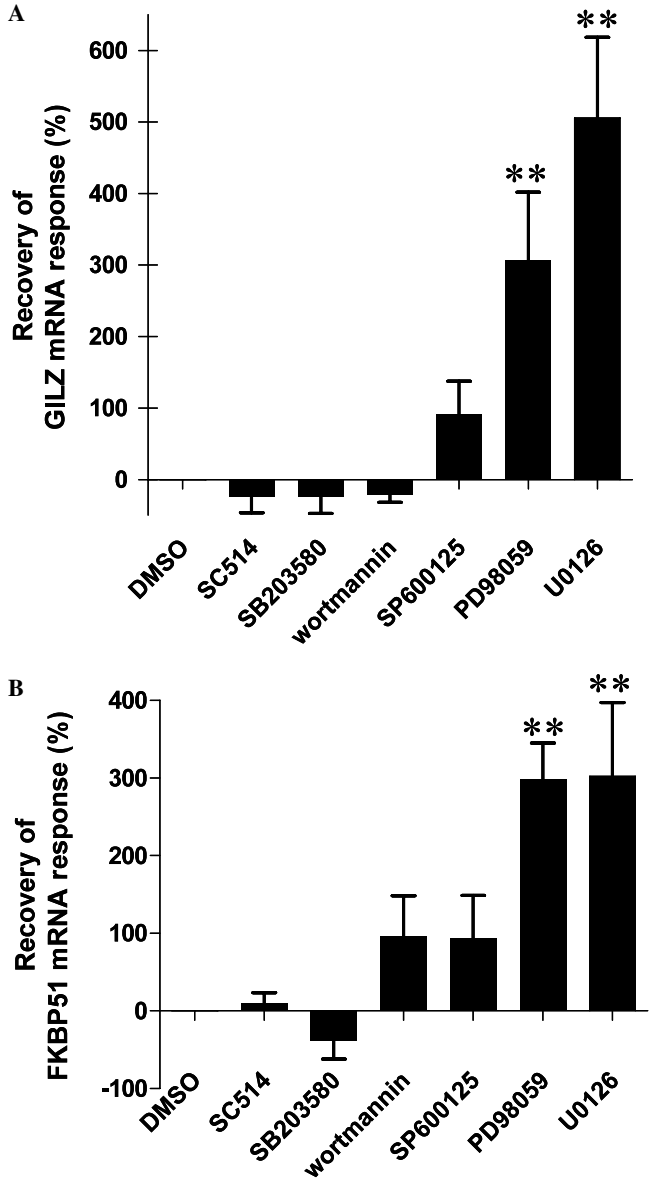

Fig. 3. Effects of inhibitors of signaling cascades on (A) GILZ mRNA and (B) FKBP51 mRNA expression in keratinocytes. HaCaT cells were pre-treated for 1 h with either an IKKβ inhibitor, SC-514 (20 μM), a p38 MAPK inhibitor, SB203580 (10 μM), a JNK inhibitor, SP600125 (20 μM), a PI3K inhibitor, wortmannin (1 μM), MEK-1/ERK inhibitors, PD98059 (50 μM), or U0126 (20 μM) as well as DMSO (final conc. 0.4%). The cells were then treated with TNFα (10 ng/ml) for 24 h followed by PSL (100 ng/ml) treatment for 4 h. GILZ and FKBP51 mRNA expressions were determined using RT-PCR. Data are shown as percent recovery rate by each specific inhibitor as described in Materials and methods. PD98059 and U0126 treatment significantly sensitized PSL-dependent GILZ and FKBP51 mRNA expressions ($n = 5$, Dunnet's test; $^{**}p < 0.01$ as compared to the vehicle data (DMSO)).

Effects of MEK-1/ERK inhibitors on MKP-1 gene expression

MKP-1 is an important GC-induced anti-inflammatory gene that contains GRE sequences in the 5′ promoter region. We also investigated the effects of TNFα and PSL on MKP-1 gene expressions in the presence of MEK-1/ERK inhibitors. We did not observe a significant increase in MKP-1 mRNA expression by PSL (100 ng/ml) treatment after 4 h in HaCaT cells, whereas TNFα (10 ng/ml) slightly increased MKP-1 mRNA expression. MKP-1 mRNA expression showed an additive increase by a combination of TNFα and PSL treatments. However, pre-treatment of PD98059 (50 μM) and U0126 (20 μM) strongly increased MKP-1 mRNA expression in the presence of TNFα and PSL treatments (Fig. 4).

Effects of TNFα and MEK-1/ERK inhibitors on PSL-dependent GR nuclear translocation

To further investigate TNFα-mediated GC insensitivity and its recovery by MEK-1/ERK inhibitors, we

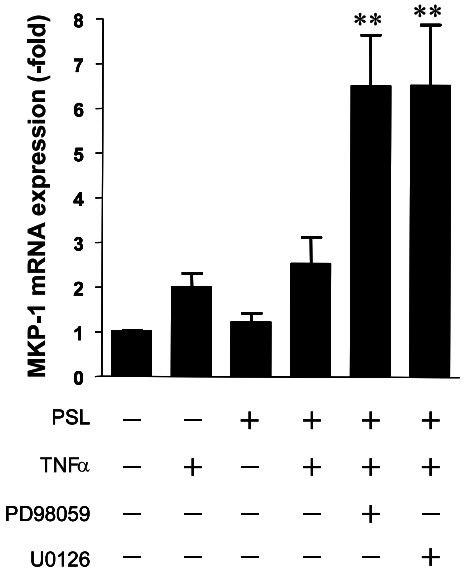

Fig. 4. Effect of MEK-1/ERK inhibitors on MKP-1 mRNA expression in the presence of TNFα and PSL in HaCaT cells. Cells were pre-treated with either PD98059 (50 μM), or U0126 (20 μM) for 1 h followed by TNFα treatment (10 ng/ml) for 24 h and then PSL treatment (100 ng/ml) for 4 h. MEK-1/ERK inhibitors strongly upregulated MKP-1 mRNA expression in the presence of TNFα and PSL ($n = 4$, Bonferroni's test; $^{**}p < 0.01$ as compared to PSL + TNFα treated group).

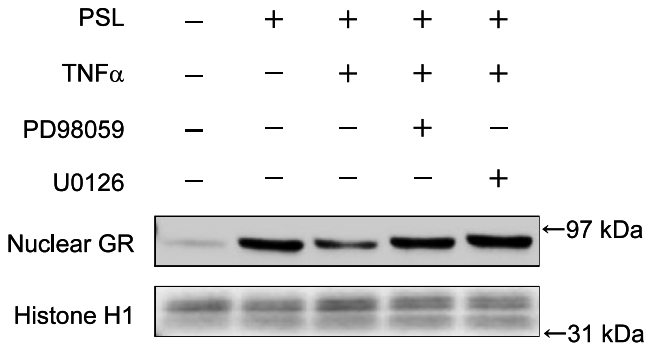

Fig. 5. Western blot analysis for PSL-dependent GR (94 kDa) nuclear translocation. Pre-treatment of TNFα (10 ng/ml) for 24 h reduced PSL- (100 ng/ml) dependent nuclear translocation of GR in HaCaT cells. However, this reduced GR translocation was restored by pre-treatment of PD98059 (50 μM), or U0126 (20 μM) for 1 h. Cells were harvested 2 h after PSL treatment. Histone H1 (32–33 kDa) was also detected as a nuclear loading control. The results were highly reproducible and are representative of four independent experiments.

investigated PSL-dependent nuclear translocation of GR by Western blotting for nuclear proteins. Results showed that TNFα (10 ng/ml) treatment reduced PSL- (100 ng/ml) induced nuclear translocation of GR in HaCaT cells (Fig. 5). This inhibitory effect of TNFα was restored by pre-treatment of cells with PD98059 (50 μM) or U0126 (20 μM) for 1 h.

Discussion

In the current study, we investigated the effect of TNFα on GC sensitivity of HaCaT, a human epidermal keratinocyte cell line. Data showed that pre-treatment of cells with TNFα suppressed PSL-induced transactivation of pGRE-luc and GILZ mRNA expressions in dose-dependent manners. This GC insensitivity was not accompanied by an increase in GRβ or a decrease in GRα mRNA expression, but was significantly recovered by pre-treatments of cells with an MEK-1/ERK specific inhibitor (PD98059 or U0126). We also observed that GR nuclear translocation by PSL treatment was reduced by TNFα treatment and that MEK-1/ERK inhibitors also restored this phenomenon.

We have focused on three endogenous GC responsive genes (GILZ, FKBP51, and MKP-1), all of which have been shown to contain at least one GRE sequence in either the 5′ promoter or intronic sequence. GILZ is known to be produced by B and T lymphocytes, and its production is strongly increased by GCs [12]. Promoter analysis showed that the 5′ regulatory sequence of GILZ gene contains at least four GREs [13]. GILZ has been shown to have inhibitory effects on AP-1 DNA binding and NF-κB activity by interfering with the p65 subunit [14,15], suggesting that GILZ plays an important role in suppressing the inflammatory response of cells. It has recently been reported that GILZ inhibits phosphorylation of Raf-1 and consequently inhibits Raf-MEK/ERK activation in T cells [16]. Our results demonstrate, for the first time, constitutive GILZ gene expression in HaCaT keratinocytes and strong induction of its mRNA by GC treatment, suggesting that GILZ mediates the effects of GC, at least partly, in suppressing the inflammatory responses in keratinocytes. Our data also suggest that TNFα-mediated GC insensitivity in keratinocytes leads to decreased GILZ expression in response to GC treatment. This suggests GC fails to repress sustained inflammatory processes mediated by the Raf-MEK/ERK pathway. Our data also imply that inhibiting MEK-1/ERK by specific inhibitors latently cancels this vicious circle of GC insensitive inflammatory states.

FKBP51 is one of the GC inducible genes, which has been proposed as a general indicator of GC sensitivity [17]. Recently, two different groups independently reported the existence of functional GREs of FKBP51 in intron 1 [18] or intron 5 (E) [19]. One of the physiological functions of FKBP51 is to form a complex with GRα in the absence of its ligand together with other chaperon proteins, which may contribute to a negative feedback loop for partial desensitization of GC effects [20].

MKP-1 is also an important GC responsive anti-inflammatory gene because this molecule, known as a dual specific phosphatase, is capable of suppressing phospholylated p38 and JNK pathways [21,22]. This gene also contains GREs sequence in the 5′ promoter region [23]. MKP-1 is also inducible under some inflammatory conditions and this molecule plays a significant role in downregulating the inflammatory process [21].

One of the striking results observed in our study is that pre-treatment of MEK-1/ERK inhibitors strongly sensitized PSL-dependent gene expressions in the presence of TNFα. The amounts of these gene expressions in the treatment condition (MEK-1/ERK inhibitor + TNFα + PSL) were higher than those in the PSL treatment alone. Although it is reasonable to consider that the restoration of GR nuclear translocation by MEK-1/ERK inhibitors substantially accounts for this increase in GC-induced gene expressions, we cannot exclude the possibility that other mechanisms such as, for instance, modulation of co-factors that associate with GR may also exist.

Our data demonstrated that GRα and GRβ mRNA expressions were not affected by TNFα treatment. For Western blotting, we used anti-GR antibody which recognizes a common epitope in both GRα (94 kDa) and GRβ (90 kDa). However, our results clearly showed a single band which can be regarded as GRα, since our RT-PCR experiment showed GRα predominantly expressed over GRβ in HaCaT cells. Several studies have reported on the relationships between cytokines with GRβ overexpressions. Webster et al. have shown that pro-inflammatory cytokines such as TNFα and IL-1β are able to accumulate GRβ expression and lead to GC insensitivity [7]. In a recent study, Tliba et al. reported that combinations of TNFα and IFNβ or IFNγ treatment reduced GC-dependent CD38 suppression and GRE-mediated transactivation in airway smooth muscle (ASM) cells. They reported that GRβ mRNA and protein expression were significantly increased

by TNFα and/or IFN treatment in ASM cells [24]. Although the reason for the different observations from our experiments is not clear, further research is required to clarify how significant the role is of GRβ expressions in cytokine-mediated GC insensitivity in several tissues where GCs are applied.

It is generally known that TNFα and other inflammatory cytokines are able to induce NF-κB signaling cascades. The GC–GR complex can directly or indirectly interact with other transcription factors including NF-κB, AP-1, and STAT families that are activated by cytokine stimulation [25]. Bantel et al. reported that in GC-resistant patients with Crohn's disease, strong expressions of NF-κB and TNFα in epithelial cells have been observed, whereas these activations are restricted to macrophages in lamina propria in GC-sensitive patients [26]. They also reported that in human colorectal CaCo2 cells, overexpression of RelA, JNK1, or p38, which leads to the activation of NF-κB and TNFα, suppressed GR-induced transactivation [26]. In contrast to this report, our data showed that SC-514, IKKβ specific inhibitor did not affect reduced GC sensitivity mediated by TNFα, suggesting that there was little role of NF-κB in interfering with GR function in our experimental conditions.

Recent studies have demonstrated an association of the MEK-1 pathway with cellular GC resistance. Li et al. demonstrated that in GC resistance induced by microbacterial superantigens, MEK-1 activation is involved in inhibiting GR nuclear translocation in T cells [27]. They also showed that GR is phospholylated by *in vitro* co-incubation of ERK 1 or 2 with GR. Another study suggests that TCR signaling by co-stimulatory signal (CD28 or IL-2) induces MEK-ERK activation which leads to GC resistance in T cells [28]. These studies indicate the MEK-1 pathway may be an attractive target for overcoming GC resistance in T cells.

In the current study, we have clarified a novel link of TNFα, MEK-1/ERK, and reduced GR nuclear translocation in human epidermal keratinocytes. Since clinical studies report that higher concentrations of TNFα in skin blister fluids are sometimes observed and are associated with disease severities [29–31], our data imply that patients who have high TNFα expression at the skin inflammatory region acquire topical GC insensitivity with reduced GR nuclear translocation. Inhibiting the MEK-1/ERK cascade may offer a therapeutic potential for increasing GC efficacy in epidermis where sufficient immune/inflammatory suppression is required.

Acknowledgments

We thank Dr. Norbert E. Fusenig for generously providing the HaCaT cell line. We also thank Mr. Kamogawa, Uchida, Nakamura, and Taniguchi for their technical assistance. This study was supported by a grant from the Japan Research Foundation for Clinical Pharmacology.

References

[1] J.D. Fine, Management of acquired bullous skin diseases, N. Engl. J. Med. 333 (1995) 1475–1484.
[2] V.T. Nguyen, J. Arredondo, A.I. Chernyavsky, Y. Kitajima, M. Pittelkow, S.A. Grando, Pemphigus vulgaris IgG and methylprednisolone exhibit reciprocal effects on keratinocytes, J. Biol. Chem. 279 (2004) 2135–2146.
[3] P.J. Barnes, Anti-inflammatory actions of glucocorticoids: molecular mechanisms, Clin. Sci. (Colch) 94 (1998) 557–572.
[4] J.C. Kam, S.J. Szefler, W. Surs, E.R. Sher, D.Y. Leung, Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids, J. Immunol. 151 (1993) 3460–3466.
[5] J.D. Spahn, S.J. Szefler, W. Surs, D.E. Doherty, S.R. Nimmagadda, D.Y. Leung, A novel action of IL-13: induction of diminished monocyte glucocorticoid receptor-binding affinity, J. Immunol. 157 (1996) 2654–2659.
[6] D.Y. Leung, Q. Hamid, A. Vottero, S.J. Szefler, W. Surs, E. Minshall, G.P. Chrousos, D.J. Klemm, Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta, J. Exp. Med. 186 (1997) 1567–1574.
[7] J.C. Webster, R.H. Oakley, C.M. Jewell, J.A. Cidlowski, Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance, Proc. Natl. Acad. Sci. USA 98 (2001) 6865–6870.
[8] C.M. Bamberger, A.M. Bamberger, M. de Castro, G.P. Chrousos, Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans, J. Clin. Invest. 95 (1995) 2435–2441.
[9] P. de Lange, J.W. Koper, A.O. Brinkmann, F.H. de Jong, S.W. Lamberts, Natural variants of the beta isoform of the human glucocorticoid receptor do not alter sensitivity to glucocorticoids, Mol. Cell. Endocrinol. 153 (1999) 163–168.
[10] S.J. Lane, I.M. Adcock, D. Richards, C. Hawrylowicz, P.J. Barnes, T.H. Lee, Corticosteroid-resistant bronchial asthma is associated with increased c-fos expression in monocytes and T lymphocytes, J. Clin. Invest. 102 (1998) 2156–2164.
[11] C. Feliciani, P. Toto, P. Amerio, S.M. Pour, G. Coscione, G. Shivji, B. Wang, D.N. Sauder, In vitro and in vivo expression of interleukin-1alpha and tumor necrosis factor-alpha mRNA in pemphigus vulgaris: interleukin-1alpha and tumor necrosis factor-alpha are involved in acantholysis, J. Invest. Dermatol. 114 (2000) 71–77.
[12] F. D'Adamio, O. Zollo, R. Moraca, E. Ayroldi, S. Bruscoli, A. Bartoli, L. Cannarile, G. Migliorati, C. Riccardi, A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death, Immunity 7 (1997) 803–812.
[13] J.C. Wang, M.K. Derynck, D.F. Nonaka, D.B. Khodabakhsh, C. Haqq, K.R. Yamamoto, Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes, Proc. Natl. Acad. Sci. USA 101 (2004) 15603–15608.
[14] P.R. Mittelstadt, J.D. Ashwell, Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ, J. Biol. Chem. 276 (2001) 29603–29610.
[15] E. Ayroldi, G. Migliorati, S. Bruscoli, C. Marchetti, O. Zollo, L. Cannarile, F. D'Adamio, C. Riccardi, Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB, Blood 98 (2001) 743–753.
[16] E. Ayroldi, O. Zollo, A. Macchiarulo, B. Di Marco, C. Marchetti, C. Riccardi, Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1, Mol. Cell. Biol. 22 (2002) 7929–7941.
[17] H. Vermeer, B.I. Hendriks-Stegeman, B. van der Burg, S.C. van Buul-Offers, M. Jansen, Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability, J. Clin. Endocrinol. Metab. 88 (2003) 277–284.

[18] K. Horie-Inoue, K. Takayama, H.U. Bono, Y. Ouchi, Y. Okazaki, S. Inoue, Identification of novel steroid target genes through the combination of bioinformatics and functional analysis of hormone response elements, Biochem. Biophys. Res. Commun. 339 (2006) 99–106.
[19] T.R. Hubler, J.G. Scammell, Intronic hormone response elements mediate regulation of FKBP5 by progestins and glucocorticoids, Cell Stress Chaperones 9 (2004) 243–252.
[20] W.B. Pratt, D.O. Toft, Steroid receptor interactions with heat shock protein and immunophilin chaperones, Endocr. Rev. 18 (1997) 306–360.
[21] A.R. Clark, MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? J. Endocrinol. 178 (2003) 5–12.
[22] D.D. Hirsch, P.J. Stork, Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways in vivo, J. Biol. Chem. 272 (1997) 4568–4575.
[23] O. Kassel, A. Sancono, J. Kratzschmar, B. Kreft, M. Stassen, A.C. Cato, Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1, EMBO J. 20 (2001) 7108–7116.
[24] O. Tliba, J.A. Cidlowski, Y. Amrani, CD38 expression is insensitive to steroid action in cells treated with tumor necrosis factor-alpha and interferon-gamma by a mechanism involving the up-regulation of the glucocorticoid receptor beta isoform, Mol. Pharmacol. 69 (2006) 588–596.
[25] K. De Bosscher, W. Vanden Berghe, G. Haegeman, The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression, Endocr. Rev. 24 (2003) 488–522.
[26] H. Bantel, M.L. Schmitz, A. Raible, M. Gregor, K. Schulze-Osthoff, Critical role of NF-kappaB and stress-activated protein kinases in steroid unresponsiveness, FASEB J. 16 (2002) 1832–1834.
[27] L.B. Li, E. Goleva, C.F. Hall, L.S. Ou, D.Y. Leung, Superantigen-induced corticosteroid resistance of human T cells occurs through activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK-ERK) pathway, J. Allergy Clin. Immunol. 114 (2004) 1059–1069.
[28] D.C. Tsitoura, P.B. Rothman, Enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4+ T cells, J. Clin. Invest. 113 (2004) 619–627.
[29] E. Lopez-Robles, E. Avalos-Diaz, E. Vega-Memije, T. Hojyo-Tomoka, R. Villalobos, S. Fraire, L. Domiguez-Soto, R. Herrera-Esparza, TNFalpha and IL-6 are mediators in the blistering process of pemphigus, Int. J. Dermatol. 40 (2001) 185–188.
[30] L. D'Auria, P. Cordiali Fei, F. Ameglio, Cytokines and bullous pemphigoid, Eur. Cytokine Netw. 10 (1999) 123–134.
[31] F. Ameglio, L. D'Auria, C. Bonifati, C. Ferraro, A. Mastroianni, B. Giacalone, Cytokine pattern in blister fluid and serum of patients with bullous pemphigoid: relationships with disease intensity, Br. J. Dermatol. 138 (1998) 611–614.