17β-estradiol Inhibits the Production of RANTES in Human Keratinocytes

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A chemokine, regulated upon activation, normal T cell expressed and secreted (RANTES) attracts T helper-1 cells and macrophages. The production of RANTES is enhanced in keratinocytes of psoriatic skin lesions, which may contribute to the inflammatory infiltrate. It is known that estrogen regulates the natural course of psoriasis. We examined the *in vitro* effects of 17β-estradiol on RANTES production by human keratinocytes. 17β-estradiol inhibited tumor necrosis factor-α or interleukin-1β-induced RANTES secretion, mRNA expression, and promoter activity in keratinocytes, and these effects of 17β-estradiol were counteracted by estrogen receptor antagonist ICI 182 780. Two nuclear factor κB elements on RANTES promoter were required for tumor necrosis factor-α or interleukin-1β-induced transcription and involved in the inhibition by 17β-estradiol. 17β-estradiol inhibited nuclear factor κB transcriptional activity, whereas it did not inhibit DNA binding of nuclear factor κB or phosphorylation or degradation of the inhibitor of nuclear factor κB α in tumor necrosis factor-α or interleukin-1β-stimulated keratinocytes. 17β-estradiol-induced inhibition of nuclear factor κB transcriptional activity and RANTES promoter activity was rescued by overexpression of a coactivator cyclic AMP response element-binding protein (CREB) or nuclear factor κB p65 but not by steroid receptor coactivator-1 or nuclear factor κB p50. The overexpression of CREB-binding protein rescued 17β-estradiol-induced inhibition of transcription mediated by a chimeric protein, GAL4-p65$^{286–551}$, which contained GAL4 DNA binding domain fused to C-terminal transactivating domain of p65 (amino acids 286–551). The transfection of estrogen receptor α or estrogen receptor β into estrogen receptor-negative SKBR3 cells resulted in 17β-estradiol-mediated inhibition of transcription via GAL4-p65$^{286–551}$. These results suggest that 17β-estradiol-bound estrogen receptor may inhibit nuclear factor κB-dependent transcription of RANTES gene by competing with p65 for limiting amounts of CREB-binding protein. ***Key words: CREB binding protein/nuclear factor κB/psoriasis. J Invest Dermatol 120:420–427, 2003***

Psoriasis is characterized by epidermal hyperplasia associated with vascular proliferation and inflammatory infiltrates composed of T cells, macrophages, or neutrophils (Bos and de Rie, 1999). Such infiltrates may be attributable to the increased expression of various chemokines by lesional keratinocytes (Giustizieri *et al*, 2001). The lymphocytes infiltrating into psoriatic lesions are predominantly activated T helper-1 cells (Uyemura *et al*, 1993; Bos and de Rie, 1999). CXC-chemokines, interferon-induced protein of 10 kDa, and monokines induced by interferon-γ, are thought to be primary chemokines attracting T helper-1 cells into psoriatic lesions (Giustizieri *et al*, 2001). A CC-chemokine, regulated upon activation, normal T cell expressed and secreted (RANTES), also preferentially attracts T helper-1 cells rather than T helper-2 cells (Siveke and Hamann, 1998). It is reported that RANTES production by keratinocytes is increased in psoriatic skin lesions (Fukuoka *et al*, 1998; Raychaudhuri *et al*, 1999). RANTES may thus contribute to the T helper-1-dominant infiltration in psoriatic lesions as well as CXC-chemokines. RANTES also recruits macrophages (Ying *et al*, 1995). T helper-1 cells or macrophages infiltrating into psoriatic lesions in turn may produce RANTES (Schrum *et al*, 1996) and proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, or interferon-γ (Uyemura *et al*, 1993), which promote RANTES production by keratinocytes (Fukuoka *et al*, 1998). Thus RANTES production in psoriatic lesions may be regulated in an autocrine/paracrine manner, which may amplify the inflammation in the lesions. The blockage of RANTES production by keratinocytes is a key therapeutic target for psoriasis.

It has been suggested that estrogen may regulate the natural course of psoriasis (Farber and Nall, 1974; Dunna and Finlay, 1989; Stevens *et al*, 1993; Boyd *et al*, 1996; Boyd and King, 1999); however, the reports are somewhat conflicting; psoriasis improves during pregnancy, whereas it worsens after delivery (Farber and Nall, 1974; Dunna and Finlay, 1989; Boyd *et al*, 1996) or at menopause (Mowad *et al*, 1998), indicating the protective effects of estrogen. In contrast, psoriasis flares premenstrually, and anti-estrogen tamoxifen improves the symptoms (Stevens *et al*, 1993; Boyd and King, 1999), indicating the disease-accelerating effect

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Abbreviations: CAT, chloramphenicol acetyl transferase; CBP, CREB binding protein; E2, 17β-estradiol; ER, estrogen receptor; IκB, inhibitor of nuclear factor κB; NF-κB, nuclear factor-κB; RANTES, regulated upon activation, normal T cell expressed and secreted; SRC-1, steroid receptor coactivator-1.

of estrogen. Such discrepancy may be caused by the diversity in the immunomodulatory effects of estrogen. Estrogen has both anti-inflammatory and pro-inflammatory functions; 17β-estradiol (E2) enhances the production of proinflammatory interferon-γ and anti-inflammatory IL-10 in T cells (Correale *et al*, 1998). The effects of E2 also differ with its concentration; antigen-stimulated TNF-α production in $CD4^+$ T cells was enhanced by 20–40 nM of E2 but was inhibited by 200–400 nM of E2 (Gilmore *et al*, 1997). The balance of such diverse functions of estrogen may determine its total effect on the course of psoriasis. Estrogen exerts its effects via intracellular estrogen receptor (ER); estrogen-bound ER upregulates or downregulates the transcription of various genes by binding to the estrogen response element of the genes or by interacting with other transcription factors (Beato, 1989; Paech *et al*, 1997). ER exists as two subtypes, ERα and ERβ, encoded by separate genes (Mendelsohn and Karas, 1999).

It has recently been reported that estrogen treatment *in vivo* inhibited RANTES production in murine liver, spinal cord, and spleen (Matejuk *et al*, 2001, 2002; Evans *et al*, 2001). This suggests that E2 may prevent the infiltration of T helper-1 cells or macrophages and thus suppress the inflammation mediated by these cells in the respective tissues; however, it has been not precisely examined how E2 inhibits RANTES production in these tissues. Besides it is unknown if such an inhibitory effect of E2 may occur in the skin. In this study, we examined if E2 may *in vitro* inhibit RANTES production by human keratinocytes in order to test the preventive effect of E2 on the development of psoriatic skin lesions. We found that E2 inhibited TNF-α or IL-1β-induced RANTES production by suppressing the activity of nuclear factor (NF)-κB.

MATERIALS AND METHODS

Reagents E2 was purchased from Sigma (St Louis, MO). ICI 182 780 was from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human TNF-α and IL-1β were purchased from R&D (Minneapolis, MN). Antibodies used in the electrophoretic mobility shift assay, immunoprecipitation, and immunoblotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), except for rabbit IgG antibody against phosphorylated inhibitor of NF-κB (IκB) α (Calbiochem, La Jolla, CA).

Culture of normal human epidermal keratinocytes Human neonatal foreskin keratinocytes were cultured in serum-free keratinocyte growth medium (Clonetics, Walkersville, MD) consisting of basal medium MCDB153 supplemented with 0.5 μg hydrocortisone per ml, 5 ng epidermal growth factor per ml, 5 μg insulin per ml, and 0.5% bovine pituitary extract. The cells in the third passage were used for the experiments.

Measurement of RANTES secretion Keratinocytes (2×10^4 per well) were seeded in triplicate into 24-well plates in 1 ml keratinocyte growth medium, adhered overnight, then the medium was changed to basal keratinocyte basal medium depleted of phenol red, hormones, growth factors, and bovine pituitary extract, and incubated for 24 h. The medium was removed and the cells were preincubated with E2 in phenol red-free keratinocyte basal medium for 10 min, then incubated with TNF-α or IL-1β in the presence of E2 for another 48 h. The supernatants were assayed for RANTES by enzyme-linked immunosorbent assay (Biosource, Camarillo, CA). The sensitivity of the assay was 3 pg per ml.

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) Keratinocytes were incubated as above for 6 h, and RNA was extracted using mRNA purification kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. cDNA was made from RNA samples as described (Tjandrawinata *et al*, 1997). Primers for amplification and the sizes of respective PCR products were as follows: RANTES, 5′-ACCACACCCTGCTGCTTTGCCTACATTGCC-3′ (sense) and 5′-CTCCCGAACCCATTTCTTCTCTGGGTTGGC-3′ (anti-sense) for 162 bp; and glyceraldehyde-3-phosphate dehydrogenase, 5′-CCACCCATGG-CAAATTCCATGGCA-3′ (sense) and 5′-TCTACAC-GGCAGGTC-AGGTCCACC-3′ (anti-sense) for 600 bp (Hu *et al*, 1999). PCR was performed by one denaturing cycle of 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 3 min. The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide, viewed by ultraviolet light, and photographed. Densitometric analysis was performed by scanning the bands into Photoshop and performing densitometry with NIH Image Software.

Plasmids A firefly luciferase reporter plasmid driven by human RANTES promoter (−397/+5 bp relative to the transcriptional start site), denoted as pRANTES-luc, was constructed by PCR and insertion into pGL3 basic vector (Promega, Madison, WI) as described (Lin *et al*, 1999; Genin *et al*, 2000). Site-specific mutations were created by multiple rounds of PCR using appropriate primers with altered bases as described (Moriuchi *et al*, 1997). A chloramphenicol acetyl transferase (CAT) reporter plasmid driven by human IL-8 promoter (−98/+44 bp relative to the transcriptional start site), denoted as pIL-8-CAT, was constructed as described (Kanda and Watanabe, 2001). p3xNF-κB1-SV-luc and p3xNF-κB2-SV-luc were constructed by inserting three copies of distal NF-κB (NF-κB1) (5′-ATTTTGGAAACTCCCCTTAGG-3′, with consensus sequences underlined), proximal NF-κB (NF-κB2) (5′-TTGAGGGGATGCCC-CTAAGG-3′) from human RANTES promoter in front of heterologous minimal SV40 promoter upstream of firefly luciferase reporter as described (Ohmori *et al*, 1994). pFR-luc (Stratagene, La Jolla, CA) contains five copies of the sequences for GAL4 DNA binding sites in front of TATA box upstream of firefly luciferase reporter. pGAL4-$p65^{286–551}$ expresses a chimeric protein containing GAL4 DNA binding domain and C-terminal region (amino acids 286–551) of p65 and was constructed as described (Schmitz and Baeuerle, 1991). pCMV-CBP and pCMV-SRC-1 express full-length CREB-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1), respectively and were constructed as described (Kamei *et al*, 1996; Na *et al*, 1998). pCMV-p65 and pCMV-p50 express full-length NF-κB p65 and p50, respectively, and were constructed as described (Stein *et al*, 1993). pCMV-ERα and pCMV-ERβ expressing full-length human ERα (595 amino acids) and ERβ (530 amino acids), respectively, were constructed as described (Bhat *et al*, 1998).

Transfections Transient transfections were performed with Effectene (Qiagen, Tokyo, Japan) as described (Zellmer *et al*, 2001). Keratinocytes were plated in 10 cm dishes and grown to about 80% confluence. Twenty-four hours before the transfection, the medium was changed to phenol red-free KBM. Keratinocytes in KBM were incubated for 6 h with 5 μg of pRANTES-luc, pIL-8-CAT, or p3xNF-κB-SV-luc and 1 μg of Rous sarcoma virus β-galactosidase vector (pRSV-βgal) with or without 0.5, 1, or 2.5 μg pCMV-CBP or pCMV-SRC-1 or 1 μg pCMV-p65 or pCMV-p50, premixed with enhancer, transfection buffer, and Effectene. The total amount of plasmid DNA in all samples was made up to 10 μg with empty CMV vector. The transfected cells were washed and incubated in KBM for 18 h, then treated with E2 for 10 min prior to the addition of TNF-α or IL-1β. After 24 h, cell extracts were prepared and luciferase activities were quantified using luciferase assay system (Promega). The same cell extracts were assayed for β-galactosidase activity using chemiluminescent Galacto-Light kit (Tropix, Bedford, MA). All readings were taken using a Lumat 9501 luminometer (Berthold, Wildbach, Germany). The results obtained in each transfection were normalized for β-galactosidase activity and expressed as relative luciferase activity. CAT expression was analyzed by CAT-ELISA (Roche Diagnostics, Tokyo, Japan) for the lysates of the cells transfected with pIL-8-CAT in place of luciferase reporter vectors, and the results were also normalized for β-galactosidase activity. In the chimeric protein-mediated transcription experiments, keratinocytes were transfected with 2.5 μg pFR-luc and 0.5 μg pRSV-βgal, 2.5 μg pGAL4-$p65^{286–551}$, with or without 0.5, 1, or 2.5 μg pCMV-CBP. The total amount of plasmid DNA was made up to 10 μg with empty vector DNA. Human breast carcinoma SKBR3 cells (ERα and ERβ negative) (Dainippon) were maintained with 1:1 mixture of Dulbecco minimal Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum. SKBR3 cells were transfected with 2.5 μg pFR-luc and 0.5 μg pRSV-βgal, 2.5 μg pGAL4-$p65^{286–551}$, with or without 1 μg pCMV-ERα or pCMV-ERβ, 2.5 μg pCMV-CBP using Effectene in serum, and phenol red-free Dulbecco minimal Eagle's medium/F-12 (1:1). The total amount of plasmid DNA was made up to 10 μg with empty vector.

EMSA The probes used were annealed double-stranded DNA containing NF-κB2 or NF-κB1 sequences from the human RANTES promoter. The probes were labeled by incorporation of [^{32}P]deoxycytidine triphosphate with Klenow DNA polymerase. For gel shift assays, 2–5 μg of nuclear protein extracts were incubated at room temperature for 5 min with a mixture containing 6 mM HEPES (pH 7.9), 0.4 mM ethylenediamine tetraacetic acid, 125 mM KCl, 10% glycerol, 0.05 μg per

μl poly(dI-dC), 1 mM dithiothreitol, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 50 μg aprotinin per ml, and 50 μg leupeptin per ml. Approximately, 1 ng labeled probe was added and the reactions were incubated at room temperature for another 20 min. In antibody supershift experiments, the nuclear extracts were preincubated with anti-NF-κB p65 (sc-8008), p50 (sc-8414), or c-Rel (sc-6955) antibody on ice for 30 min before the addition of probe. Reactions were then fractionated on a nondenaturing 5% polyacrylamide gel. The gels were dried and visualized with PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western blotting Keratinocytes were lyzed in lysis buffer (20 mM Tris, pH 8.0, 0.1 M NaCl, 0.5% Nonidet P-40, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 μg leupeptin per ml, 1 μg pepstatin per ml, 1 mM dithiothreitol). The lysates were centrifuged ($8000 \times g$, 5 min) and the protein concentration of the supernatants was determined by Bio-Rad protein assay reagents (Bio-Rad, Hercules, CA). Twenty micrograms of total cellular protein was electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with 1 μg per ml of mouse anti-inhibitor of NF-κB (IκB) α (sc-1643) or rabbit anti-phospho IκBα antibody (Calbiochem, La Jolla, CA), then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005) or horseradish peroxidase-conjugated goat anti-rabbit IgG (sc-2301), respectively, and developed by enhanced chemiluminescence (Amersham). Immunoblots for ER were similarly performed using rabbit anti-ERα (sc-7207) or anti-ERβ (sc-8974) antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG.

Immunoprecipitation and immunoblotting Whole cell extracts were prepared from keratinocytes in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM Na_3VO_4, 20 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μg leupeptin per ml, 2 μg aprotinin per ml, 1 μg pepstatin per ml, 1% Nonidet P-40, 0.25% deoxycholate, and 0.1% sodium dodecyl sulfate). Extracts were immunoprecipitated with 10 μg per ml of anti-NF-κB p65 or p50 antibodies. The immune complexes were captured on protein G-Sepharose beads (Pharmacia, Piscataway, NJ) for 1 h at 4°C. Precipitated proteins were washed by lysis buffer and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with 1 μg per ml of anti-ERβ antibody, then incubated with horseradish peroxidase-conjugated secondary antibody, and developed. Following the analysis of ERβ, the blots were stripped and reprobed with anti-p65 or anti-p50 antibodies as described above. The same cell extracts were also immunoprecipitated with anti-ERβ antibody, then immunoblotted with anti-p65 or p50 antibodies as described above.

RESULTS

Keratinocytes express ERβ, but not ERα The presence of ER subtypes was analyzed by western blot (**Fig 1**). A 55 kDa band for ERβ was detected (lane 4) but 66 kDa band for ERα was not detected (lane 2) in human keratinocytes. These results in western blot were consistent with those in immunohistochemical studies,[1] and indicate that E2 may act by binding to ERβ in human keratinocytes.

E2 inhibits RANTES secretion induced by TNF-α or IL-1β The constitutive RANTES secretion with medium alone was minimal in keratinocytes (mean ± SEM 15 ± 3 pg per ml, n = 5), and TNF-α 50 ng per ml or IL-1β 50 U per ml enhanced the RANTES secretion up to 310 ± 32 pg per ml or 248 ± 21 pg per ml (n = 5), respectively. Though E2 did not alter the constitutive RANTES secretion, it suppressed TNF-α or IL-1β-induced RANTES secretion (**Fig 2*a***); the significant effect of E2 occurred at 10^{-10} M, and increased in a concentration-dependent manner and maximized at 10^{-8} M, which inhibited the TNF-α or IL-1 β-induced RANTES secretion by 75% or 72%, respectively. Though ER antagonist ICI 182 780 alone did not alter TNF-α or IL-1β-induced RANTES secretion in the absence of E2, it counteracted the E2-mediated inhibition on TNF-α or IL-1β-induced RANTES secretion (**Fig 2*b***), indicating that the effects of E2 may be mediated via ER. We then examined if E2 may inhibit RANTES mRNA expression induced by TNF-α or IL-1β.

[1]Thornton MJ, Taylor AH, Mulligan K, Al-Azzawi F: Estrogen receptor beta is present in the hair follicle and sebaceous gland in male human skin. *J Invest Dermatol* 114:351, 2000 (Abstr.)

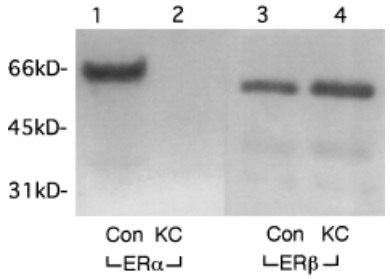

Figure 1. Western blot analysis for ER subtypes in keratinocytes. The whole cell lysates from keratinocytes (KC) (*lanes 2 and 4*) were analyzed for the expression of ERα (*lanes 1 and 2*) and ERβ (*lanes 3 and 4*). The positive controls (Con) (*lanes 1 and 3*) were the lysates from breast cancer MCF-7 cells. The results shown in the figure are representative of five separate experiments.

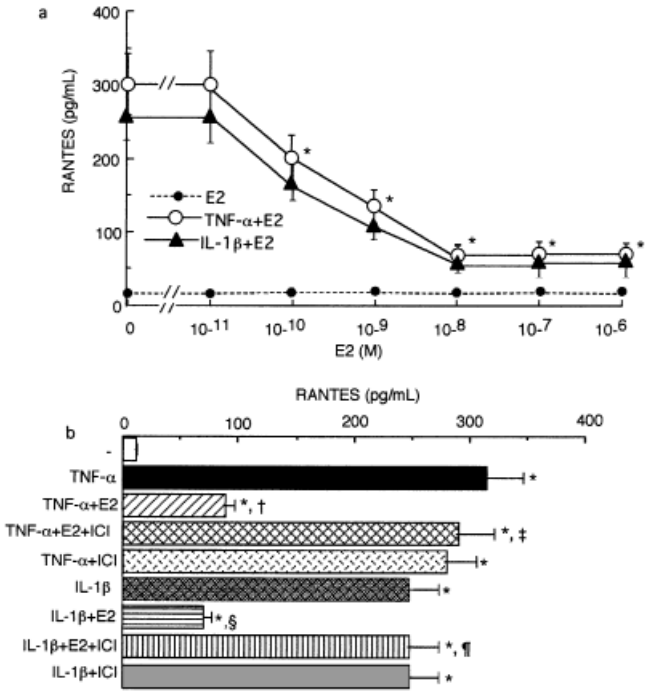

Figure 2. Dose dependency for the effects of E2 on TNF-α- or IL-1β-induced RANTES secretion in human keratinocytes (*a*) and the reversal by ER antagonist on the effects of E2 (*b*). (*a*) Human keratinocytes were preincubated with indicated concentrations of E2 for 10 min, then incubated with 50 ng TNF-α per ml or 50 U per ml IL-1β in the presence of E2. After 48 h, the culture supernatants were assayed for RANTES secretion. Values are mean ± SD of triplicate cultures. *$p < 0.05$ *vs* control cultures without hormones, by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. The data shown in the figure are representative of five separate experiments. (*b*) Keratinocytes were preincubated for 10 min with 10^{-8} M E2, 10^{-6} M ICI 182 780 (ICI), alone or in combination, then incubated with 50 ng TNF-α per ml or 50 U per ml IL-1β for another 48 h. Values are mean ± SEM (n = 5). *$p < 0.05$ *vs* control values with medium alone, †$p < 0.05$ *vs* values with TNF-α alone, ‡$p < 0.05$ *vs* values with TNF-α plus E2, §, $p < 0.05$ *vs* values with IL-1β alone, ¶$p < 0.05$ *vs* values with IL-1β plus E2, by one-way ANOVA with Scheffe's multiple comparison test.

E2 inhibits RANTES mRNA expression induced by TNF-α or IL-1β Though E2 alone did not alter RANTES mRNA level in unstimulated keratinocytes, E2 suppressed TNF-α or IL-1β-induced RANTES mRNA expression by 67% or 65% of control, respectively (**Fig 3**). ICI 182 780 counteracted the E2-mediated inhibition on TNF-α or IL-1β-induced RANTES mRNA expression. Thus E2 inhibited TNF-α- or IL-1β-induced RANTES production at pretranslational level. We then examined if E2 may suppress RANTES promoter activity induced by TNF-α or IL-1β.

E2 inhibits RANTES promoter activity induced by TNF-α or IL-1β We transiently transfected human RANTES promoter-luciferase reporter constructs into keratinocytes, and the promoter activity was assessed by the relative luciferase activities of the cell lysates. TNF-α increased wild-type RANTES promoter activity 9.3-fold of controls (**Fig 4*b***, uppermost three columns), and E2 suppressed the response to TNF-α by 71% (**Fig 4*b***), and the effect of E2 was counteracted by ICI 182 780 (data not shown). IL-1β also increased wild-type RANTES promoter activity 8.5-fold of controls, which was suppressed by E2 by 65%.

RANTES promoter contains a number of putative *cis*-acting elements that may contribute to the promoter activity (Moriuchi *et al*, 1997) (**Fig 4*a***). We thus examined which of these elements may be responsible for the inhibition by E2 on the TNF-α or IL-1β-induced RANTES transcription, using mutated promoters. The mutation of NF-κB2 (proximal NF-κB) or NF-κB1 (distal NF-κB) reduced the basal promoter activity and fold induction by TNF-α (**Fig 4*b***, the second and third three columns), and the mutation of both NF-κB elements completely abrogated the induction by TNF-α of RANTES promoter and the suppression by E2 on the response to TNF-α

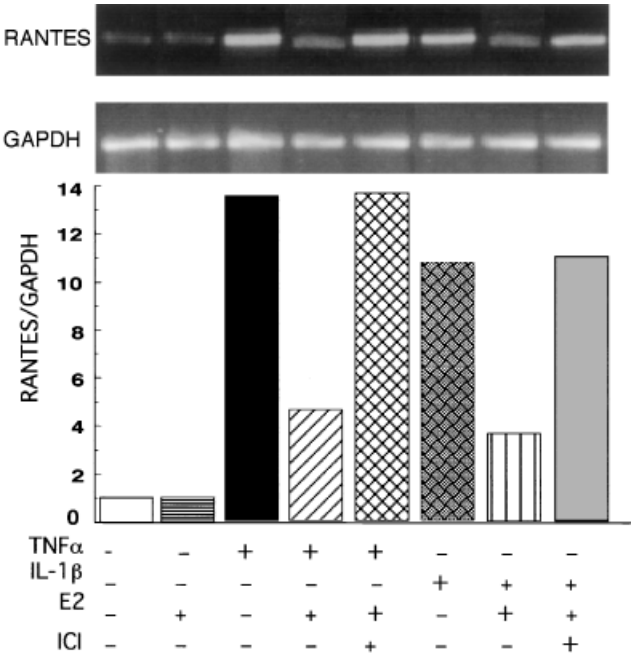

Figure 3. The effects of E2 on TNF-α- or IL-1β-induced RANTES mRNA expression in human keratinocytes. Keratinocytes were preincubated with 10^{-8} M E2, 10^{-6} M ICI 182 780 (ICI), alone or in combination for 10 min, then incubated with 50 ng TNF-α per ml or IL-1β 50 U per ml in the presence of hormones for another 6 h. RNA was isolated, and reverse transcription–PCR was performed. The intensity of the band for RANTES was corrected to that for glyceraldehydes-3-phosphoate dehydrogenase (GAPDH). The lower graph shows the corrected intensities relative to that in control cells cultured with medium alone (set as 1.0). The results shown in the figure are representative of five separate experiments.

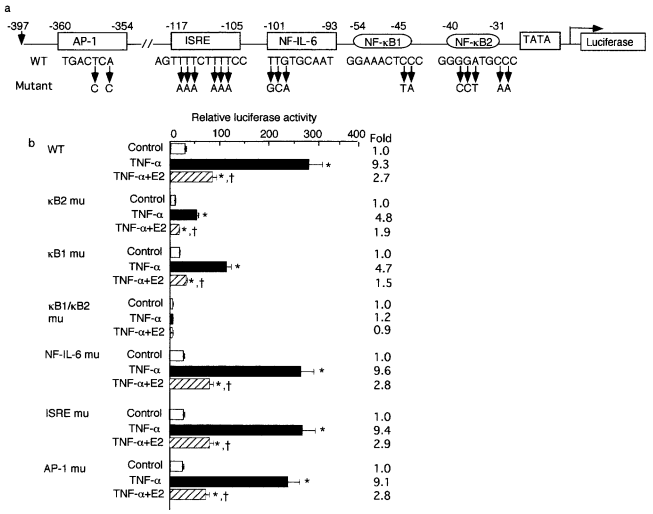

Figure 4. The effects of E2 on TNF-α-induced activities of wild-type or mutated RANTES promoters in human keratinocytes. (*a*) Schematic representation of human RANTES promoter. The locations of *cis*-elements are shown with their sequences, and substituted bases for mutation are indicated. The nucleotide positions are relative to the transcriptional start site. ISRE, interferon-stimulated response element; NF-IL-6, nuclear factor IL-6; AP-1, activator protein-1. (*b*) Keratinocytes were transiently transfected with wild-type (WT) or mutated human RANTES promoter linked to luciferase reporter together with β-galactosidase vector, and preincubated with 10^{-8} M E2 for 10 min, then incubated with 50 ng TNF-α per ml for 24 h. Relative luciferase activities normalized to β-galactosidase activities were shown. The data are mean ± SEM (n = 4). Values at right indicate the fold induction *vs* basal promoter activity. $^{*}p < 0.05$ *vs* control values and †$p < 0.05$ *vs* values with TNF-α alone, by one-way ANOVA with Scheffe's multiple comparison test.

(**Fig 4*b***, the fourth three columns). These results suggest that two NF-κB elements are necessary for TNF-α-induced promoter activation and may be involved in the suppression by E2 on the response to TNF-α. When NF-IL-6, interferon-stimulated response element, or activator protein-1 element was mutated, the basal promoter activity, the response to TNF-α, and the suppression by E2 on the response to TNF-α were not altered (**Fig 4*b***, fifth, sixth, and seventh three columns), indicating that these elements may not be required for TNF-α-induced RANTES transcription and may not be involved in the suppression by E2 on the response to TNF-α. The results were similar in IL-1β-stimulated keratinocytes (data not shown). We then analyzed if E2 may suppress NF-κB transcriptional activity induced by TNF-α or IL-1β.

E2 inhibits NF-κB transcriptional activity induced by TNF-α or IL-1β Keratinocytes were transiently transfected with plasmid containing three repeats of NF-κB2 or NF-κB1 sequences linked to the heterologous minimal SV40 promoter and luciferase reporter, and were incubated with E2 together with TNF-α. TNF-α increased the NF-κB2 or NF-κB1-dependent transcriptional activity, and E2 suppressed the response to TNF-α by 74% or by 72% of controls, respectively (**Fig 5**, first and second four columns). ICI 182 780 counteracted the E2-mediated inhibition on NF-κB2 or NF-κB1-dependent transcriptional activity induced by TNF-α. IL-1β also enhanced NF-κB2 or NF-κB1-dependent transcriptional activity by 10.2-fold or 10.1-fold of controls, respectively, and these were also reduced by E2 by 68% or 66%, respectively. We then examined if E2 may suppress the TNF-α or IL-1β-induced DNA binding of NF-κB.

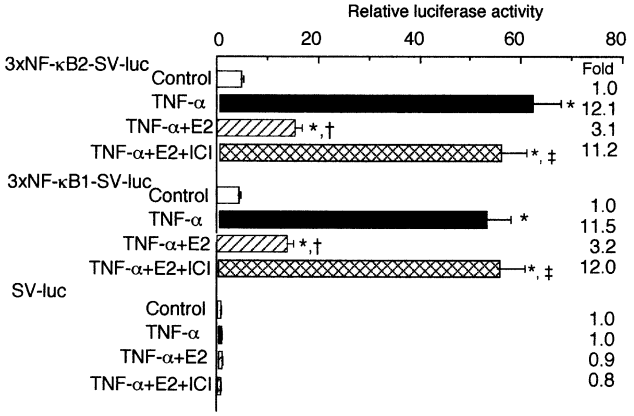

Figure 5. The effect of E2 on TNF-α-induced NF-κB transactivation. Keratinocytes were transiently transfected with luciferase reporter plasmids driven by NF-κB2 or NF-κB1 linked to heterologous SV-40 minimal promoter together with β-galactosidase vector. The cells were preincubated with 10^{-8} M E2, 10^{-6} M ICI 182 780 (ICI), alone or in combination, then incubated with 50 ng TNF-α per ml for 24 h. The results are shown as relative luciferase activities normalized for β-galactosidase activities, and represent mean ± SEM (n = 4). Values at right indicate the fold induction *vs* basal activity. *$p < 0.05$ *vs* control values with medium alone, †$p < 0.05$ *vs* values with TNF-α alone, and ‡$p < 0.05$ *vs* values with TNF-α plus E2, by one-way ANOVA with Scheffe's multiple comparison test.

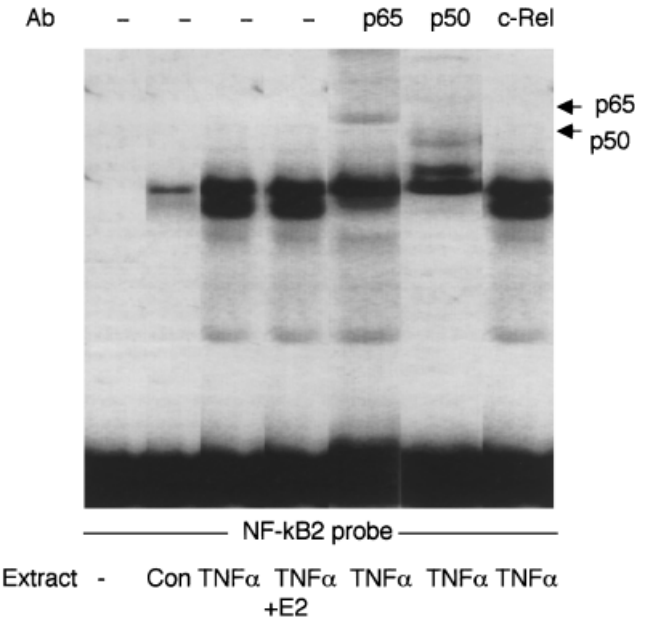

Figure 6. The effect of E2 on TNF-α-induced NF-κB binding to DNA. Keratinocytes were preincubated with 10^{-8} M E2 for 10 min, then incubated with 50 ng TNF-α per ml for 30 min, and nuclear extracts were prepared. The nuclear extracts were incubated with ^{32}P-labeled oligonucleotides containing NF-κB2 from human RANTES promoter. In supershift assays, antibodies against transcription factors were incubated for 30 min before the addition of the probe. *Arrows* indicate the supershifted complexes. The results shown in the figure are representative of four separate experiments.

E2 did not alter DNA binding of NF-κB The nuclear extracts from unstimulated keratinocytes formed a small amount of DNA–protein complex with the NF-κB2 probe (**Fig 6**, lane 2), and that was enhanced by TNF-α (**Fig 6**, lane 3), and the antibodies against NF-κB p65 and p50 but not anti-c-Rel antibody supershifted the complex (**Fig 6**, lanes 5–7), indicating the presence of p65 and p50, and absence of c-Rel in the complex. The addition of E2 did not reduce the amount of the TNF-α-induced complex with NF-κB2 probe (**Fig 6**, lanes 3 and 4; the band intensity compared with control was 15.1-fold in TNF-α and 14.5-fold in TNF-α plus E2-treated samples, respectively). Similar results were obtained using NF-κB1 probe (data not shown). IL-1β also increased the amounts of DNA–protein complexes with both NF-κB probes, and those were not reduced by E2 (data not shown). These results suggest that E2 may not suppress TNF-α- or IL-1β-induced p65/p50 binding to NF-κB elements.

ERβ and NF-κB are not coimmunoprecipitated It is known that ER can bind to NF-κB p65 or p50 *in vitro*, which may result in the repression of NF-κB transactivation (Stein and Yang, 1995; Ray *et al*, 1997). To examine a possible direct protein–protein interaction between ER and NF-κB in intact cells, we performed coimmunoprecipitation assays on the extracts from keratinocytes treated with TNF-α plus E2 for 30 min. These cell extracts precipitated with anti-ERβ antibody were positive when blotted for anti-ERβ, but were negative when blotted with anti-p65 or p50 antibodies. The cell extracts precipitated with anti-p65 or p50 antibodies, which were positive when blotted with anti-p65 or p50 antibodies, respectively, but were negative when blotted with anti-ERβ antibody. Similar results were obtained when keratinocytes were incubated with IL-1β plus E2 (data not shown). Thus specific association between ERβ and NF-κB p65 or p50 in keratinocytes could not be detected by coimmunoprecipitation.

E2 does not inhibit TNF-α-induced IκBα phosphorylation or degradation In unstimulated cells, NF-κB p50/p65 is associated with inhibitory protein IκBα in the cytoplasm, and certain stimuli induce the serine phosphorylation of IκBα by IκB kinase, leading to the proteolytic degradation of IκBα and the release of p50/p65, which results in the nuclear translocation of p50/p65 and its binding to NF-κB sites on certain genes (Naumann and Scheidereit, 1994). It is reported that E2 suppressed phorbol myristate acetate-induced IκBα degradation in ERα-transfected HeLa cells (Sun *et al*, 1998), or inhibited lipopolysaccharide-induced IκB kinase activity in human endothelial cells (Simoncini *et al*, 2000). We thus examined the involvement of IκBα in the E2-induced inhibition of NF-κB transcriptional activity in TNF-α-stimulated keratinocytes or IL-1β-stimulated keratinocytes. TNF-α-stimulated keratinocytes showed a rapid decrease and disappearance of IκBα protein within 30 min, which was associated with IκBα phosphorylation within 7.5 min. E2 did not inhibit the TNF-α- or IL-1β-induced IκBα degradation or phosphorylation (data not shown). It is thus indicated that IκBα may not be involved in the E2-mediated inhibition of NF-κB activity.

Overexpression of CBP reverses E2-induced inhibition on NF-κB transactivation and RANTES, and IL-8 promoter activities It is known that transcriptional coactivator CBP and SRC-1 play important parts in NF-κB transactivation (Gerritsen *et al*, 1997; Na *et al*, 1998; Sheppard *et al*, 1999). CBP binds to p65, whereas SRC-1 binds to p50, and both cooperatively potentiate NF-κB-mediated transactivation. These coactivators are also involved in the transactivation by E2-bound ER through estrogen response elements on certain genes (Torchia *et al*, 1998; Mckenna *et al*, 1999). It is suggested that E2-bound ER transrepresses NF-κB by competing with NF-κB for coactivators (Harnish *et al*, 2000; Glass and Rosenfeld, 2000; Speir *et al*, 2000; Evans *et al*, 2002). We thus examined if the supplementation of coactivators may reverse the E2-mediated inhibition of NF-κB transactivation and RANTES promoter activity in TNF-α or IL-1β-stimulated keratinocytes. Vectors expressing the coactivators CBP or SRC-1 were cotransfected with NF-κB-luciferase reporter or RANTES promoter-luciferase reporter vector into keratinocytes. Overexpression of

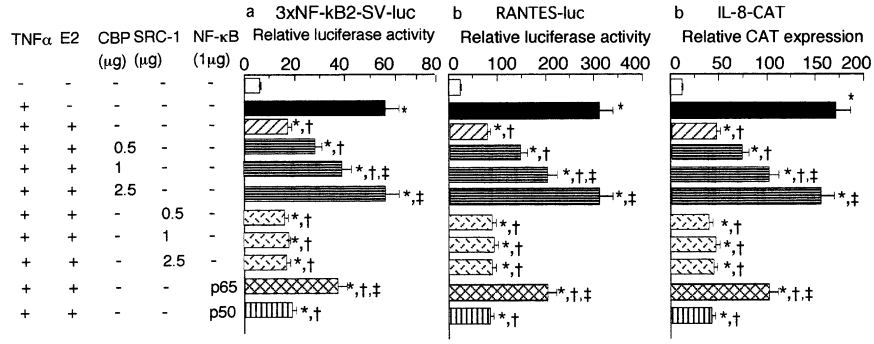

Figure 7. The reversal by CBP on E2-mediated repression of NF-κB activity (*a*) and RANTES (*b*) or IL-8 promoter activities (*c*). Keratinocytes were transfected with 5 μg p3xNF-κB2-SV-luc (*a*), or 5 μg pRANTES-luc (*b*), or 5 μg pIL-8-CAT (*c*), together with 1 μg pRSVβ-gal, and increasing concentrations of pCMV-CBP (0.5, 1, 2.5 μg) or pCMV-SRC-1 (0.5, 1, 2.5 μg) or 1 μ pCMV-p65 or pCMVp50. The total amount of plasmid DNA was made up to 10 μg with empty vector. The cells were preincubated with 10^{-8} M E2 for 10 min, then incubated with 50 ng TNF-α per ml in the presence of E2 for 24 h. Luciferase activities or CAT expression of the cell lysates were analyzed and were normalized for β-galactosidase activities. Values are mean ± SEM of five separate experiments. *$p < 0.05$ *vs* control values, †$p < 0.05$ *vs* values with TNF-α, and ‡$p < 0.05$ *vs* values with TNF-α plus E2, by one-way ANOVA with Scheffe's multiple comparison test.

CBP but not of SRC-1 reversed the E2-mediated repression of TNF-α-induced NF-κB2 activity (**Fig 7*a***) and RANTES promoter activity (**Fig 7*b***) in a concentration-dependent manner. The overexpression of p65 but not of p50 partially rescued the E2-mediated inhibition of TNF-α-induced NF-κB2 activity and RANTES promoter activity. Similar results were obtained in NF-κB1 activity (data not shown). Overexpression of CBP and p65 but not of SRC-1 or p50 rescued the E2-mediated inhibition on IL-1β-induced NF-κB2 or NF-κB1 transcriptional activity and RANTES promoter activity in keratinocytes (data not shown). These results indicate that E2-bound ER may compete with p65 for binding to coactivator CBP, and thus repress NF-κB transcriptional activity and NF-κB-dependent transcription of RANTES in TNF-α-stimulated keratinocytes or IL-1β-stimulated keratinocytes. E2 also inhibited NF-κB-dependent transcription of another chemokine gene, IL-8 by a similar mechanism (**Fig 7*c***). IL-8 promoter (–98/+44 bp) contains NF-κB element (–80/–70), which confers this promoter induction by TNF-α or IL-1β (Mukaida *et al*, 1994). TNF-α (**Fig 7*c***) or IL-1β (data not shown) enhanced IL-8 promoter activity, which was inhibited by E2. The overexpression of CBP and p65 but not of SRC-1 or p50 rescued the inhibition by E2 on TNF-α (**Fig 7*c***) or IL-1β (data not shown)-induced IL-8 promoter activation.

It is known that CBP binds to p65 C-terminal transactivation domain (amino acids 286–551) (Gerritsen *et al*, 1997). To examine the role of C-terminal transactivation domain of p65 in E2-mediated transrepression, further experiments were performed using GAL4-$p65^{286–551}$, a chimeric protein of GAL4 DNA binding domain fused to p65 transactivation domain. GAL4-$p65^{286–551}$-mediated transactivation of a plasmid containing multiple GAL4-binding sites (pFR-luc) was inhibited by E2 (**Fig 8*a***, lanes 2 and 3) in a manner similar to that of NF-κB transrepression. E2-mediated inhibition of GAL4-$p65^{286–551}$ transactivation was counteracted by ER antagonist ICI 182 780 (**Fig 8*a***, lanes 3 and 4). These results suggest that C-terminal region of p65 is involved in NF-κB transrepression by E2-bound ER. Furthermore, cotransfection with CBP reversed the E2-mediated inhibition of transcription by GAL4-$p65^{286–551}$ in a concentration-dependent manner (**Fig 8*a***, lanes 5–7). These results indicate that E2-bound ER may inhibit the transcription mediated by the C-terminal region of p65 by competing for limiting amounts of CBP. To test this hypothesis further, we examined if ER transfection into ER-negative cells may result in E2-mediated inhibition of GAL4–$p65^{286–551}$ transactivation. E2 did not inhibit GAL4–$p65^{286–551}$ transactivation in ER-negative SKBR3 cells (**Fig 8*b***, lanes 1 and 2). Transfection of ERα or ERβ slightly (10–15%) suppressed GAL4–$p65^{286–551}$ transactivation in the absence of E2, indicating the ligand-independent transrepression by ER as reported previously (Valentine *et al*, 2000). E2 significantly suppressed GAL4–$p65^{286–551}$ transactivation in ERα or ERβ-transfected SKBR3 cells (**Fig 8*b***, lanes 4 and 7), and the suppression was reversed by the overexpression of CBP (**Fig 8*b***, lanes 5 and 8), suggesting that E2-bound ERα or ERβ may suppress the transcription mediated by C-terminal region of p65 by competing for limiting amounts of CBP.

DISCUSSION

This study demonstrated that E2 inhibited TNF-α- or IL-1β-induced RANTES production in keratinocytes by inhibiting NF-κB transcriptional activity. E2-bound ER may sequester CBP from binding to NF-κB p65. As human keratinocytes express ERβ and not ERα (**Fig 1**), the effect of E2 may be mediated by ERβ. It is also reported that E2-bound ERβ attenuated IL-1β- or TNF-α-induced NF-κB transcriptional activity as well as ERα (Bhat *et al*, 1998; Quaedackers *et al*, 2001; Evans *et al*, 2002).

CBP is associated with RNA polymerase II, and can interact with TFIIB, a basal transcriptional component (Gerritsen *et al*, 1997). NF-κB p65 C-terminal transactivation domain (amino acids 286–551) can bind to both N-terminal (amino acids 1–446) and C-terminal portions of CBP (amino acids 1892–2441) (Gerritsen *et al*, 1997). The presence of two functional NF-κB elements on the RANTES promoter suggests that both N-terminal and C-terminal binding sites of CBP may simultaneously interact with p65 molecules bound to different NF-κB elements, which may create a highly stable and potent multiprotein transcriptional complex. Thus CBP may play an important part for NF-κB-dependent RANTES transcription. SRC-1 binds to NF-κB p50 and coactivates NF-κB-mediated transcription in synergy with CBP (Na *et al*, 1998; Sheppard *et al*, 1999). CBP and SRC-1 also act as coactivators for E2-bound ER-mediated transactivation through an estrogen response element (Torchia *et al*, 1998; McKenna *et al*, 1999). E2 binding to ER changes the conformation of ER and promotes the recruitment of CBP and SRC-1 (Torchia *et al*, 1998; McKenna *et al*, 1999).

In keratinocytes, E2-bound ER appeared to compete with p65 for binding to CBP, which is likely to occur in the cells whose CBP levels are limiting (Harnish *et al*, 2000; Evans *et al*, 2002). In contrast to CBP, neither SRC-1 nor p50 rescued the E2-mediated transrepression of NF-κB, which is consistent with the study by Cerillo *et al* (1998), indicating that competition between p50 and E2-bound ER for SRC-1 may not occur in keratinocytes. This is possibly because SRC-1 may exist more abundantly

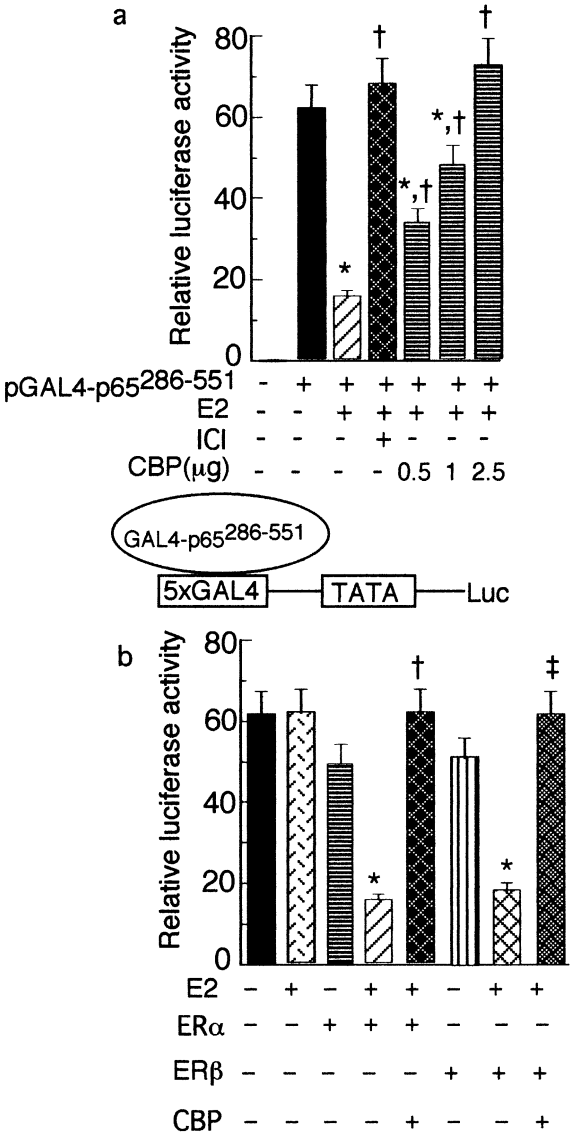

Figure 8. The effects of E2 on GAL4-p65$^{286-551}$-mediated transcription in keratinocytes (*a*) and repression of GAL4-p65$^{286-551}$-mediated transcription by E2 in ERα or ERβ-transfected SKBR3 cells (*b*). (*a*) Keratinocytes were transfected with 2.5 μg pFR-luc, 0.5 μg pRSV-βgal, and 2.5 μg pGAL4-p65$^{286-551}$ with or without increasing concentrations of pCMV-CBP (0.5, 1, 2.5 μg). The total amount of plasmid DNA was made up to 10 μg with empty vector. The cells were incubated with or without 10^{-8} M E2 in the presence or absence of 10^{-6} M ICI 182 780 (ICI) for 24 h. Luciferase activities of the cell lysates were analyzed and were normalized for β-galactosidase activities. Values are mean ± SEM of five separate experiments. *$p < 0.05$ *vs* values of pGAL4-p65$^{286-551}$-transfected cells without E2, †$p < 0.05$ *vs* values of pGAL4-p65$^{286-551}$-transfected cells incubated with E2, by one-way ANOVA with Scheffe's multiple comparison test. (b) ER-negative SKBR3 cells were transfected with 2.5 μg pFR-luc, 0.5 μg pRSV-βgal, and 2.5 μg pGAL4-p65$^{286-551}$ with or without 1 μg pCMV-ERα or pCMV-ERβ, 2.5 μg pCMV-CBP. The cells were incubated with or without 10^{-8} M E2 for 24 h and analyzed for relative luciferase activities as above. Values are mean ± SEM of five separate experiments. *$p < 0.05$ *vs* control values of empty vector-transfected cells without E2 treatment, and †$p < 0.05$ *vs* values of ERα-transfected cells treated with E2, and ‡$p < 0.05$ *vs* values of ERβ-transfected cells treated with E2, by one-way ANOVA with Scheffe's multiple comparison test.

than CBP, or because other SRC family proteins such as SRC-2 or SRC-3 can substitute for SRC-1 as a coactivator for ER (Mckenna *et al*, 1999). As there also exists the other transcriptional coactivators common to NF-κB and ER, such as CBP-associated factor, p/CAF (Sheppard *et al*, 1999), further studies should examine the involvement of these coactivators in E2-mediated NF-κB transrepression.

To date, several different mechanisms are suggested for E2-induced NF-κB transrepression and which mechanism is used may depend on cell types and NF-κB-activating stimuli: (i) E2-bound ER may physically interact with NF-κB and inhibit its DNA binding (Stein and Yang, 1995; Galien and Garcia, 1997; Ray *et al*, 1997); (ii) E2 may inhibit IκBα phosphorylation (Simoncini *et al*, 2000); (iii) E2 may inhibit IκBα degradation (Sun *et al*, 1998) or increase IκBα protein level (McMurray *et al*, 2001); or (iv) E2 induces the expression of NF-κB precursor p105, which inhibits NF-κB nuclear translocation and DNA binding (Hsu *et al*, 2000). These possibilities are rather unlikely in keratinocytes, however, as E2 did not alter IκBα degradation or phosphorylation, or DNA binding of NF-κB in keratinocytes, and coimmunoprecipitation assays failed to demonstrate the stable complexes between ERβ and NF-κB.

E2 *in vitro* inhibited RANTES production in human keratinocytes. On the other hand, E2 did not alter RANTES production in TNF-α plus interferon-γ-stimulated human bronchial epithelial cells (Stellato *et al*, 1995) or human endometrial stromal cells (Hornung *et al*, 1997). Wood *et al* (1997) reported that RANTES production in murine uterus was enhanced during early pregnancy, indicating the stimulatory effect of E2 on RANTES production. Possibly, the regulation of RANTES production by E2 may differ dependently on cell types or stimuli. The amounts or activities of CBP, p65, or ER may determine if E2 may inhibit NF-κB activity. Besides in certain cell types, RANTES promoter activity may be dependent on transcription factors other than NF-κB, such as activator protein-1, whose activity may be stimulated by E2-bound ERα (Paech *et al*, 1997). At least in human keratinocytes, however, TNF-α- or IL-1β-induced RANTES transcription was exclusively dependent on NF-κB, and was inhibited by E2. This indicates that E2 may suppress the infiltration of T helper-1 cells or macrophages into psoriatic skin lesions by inhibiting RANTES production in keratinocytes, implicating the preventive role of E2 in the course of psoriasis.

The serum E2 level is about 10^{-10} M in men, whereas in women that ranges from about 4×10^{-10} M in the follicular phase to 2×10^{-9} M at the time of ovulation, and may rise to nearly 7×10^{-8} M during pregnancy and fall to values below 10^{-10} M after menopause (Mendelson and Karros, 1999). In this study, significant inhibition of RANTES production by E2 appeared at 10^{-10} M, and increased in a concentration-dependent manner, and maximized at 10^{-8} M (**Fig 2**). It is thus suggested that E2 may *in vivo* suppress RANTES production by keratinocytes in men and women, and the effect may be enhanced with ovulation or pregnancy, and reduced after menopause in women, which may be related to the improvement of psoriasis during pregnancy and its exacerbation at menopause. Furthermore, E2 also inhibited TNF-α- or IL-1β-induced IL-8 promoter activation via NF-κB inhibition (**Fig 7**). Psoriatic skin lesions are associated with focal dense infiltration of neutrophils, and the production of the neutrophil-attracting chemokine, IL-8, is enhanced in keratinocytes of those lesions (Gillitzer *et al*, 1996). E2 may thus inhibit neutrophil infiltration into psoriatic lesions by inhibiting IL-8 production in keratinocytes. These results totally suggest that E2 may broadly suppress T helper-1-, macrophage-, and possibly neutrophil-mediated inflammation in psoriatic skin lesions by inhibiting the production of chemokines attracting these cells in keratinocytes. Thus E2 may be therapeutically used for the treatment of psoriatic patients. We are now studying if E2 may also suppress the production of other psoriasis-related chemokines such as monocyte chemoattractant protein-1.

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