17β-Estradiol Inhibits Oxidative Stress-Induced Apoptosis in Keratinocytes by Promoting Bcl-2 Expression

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We examined *in vitro* effects of 17β-estradiol on H_2O_2-induced apoptosis in human keratinocytes. 17β-estradiol prevented the H_2O_2-induced apoptosis. H_2O_2 decreased, whereas 17β-estradiol increased Bcl-2 protein and mRNA levels in keratinocytes, and H_2O_2 plus 17β-estradiol led to basal levels. Overexpression of Bcl-2 protected keratinocytes against H_2O_2-induced apoptosis, indicating the anti-apoptotic effect of Bcl-2. H_2O_2 suppressed, whereas 17β-estradiol enhanced *bcl-2* promoter activity, and H_2O_2 plus 17β-estradiol led to basal activity. Cyclic adenosine monophosphate (cAMP) response element on *bcl-2* promoter was responsible for the effects of 17β-estradiol and H_2O_2. Bcl-2 expression was enhanced by membrane-impermeable bovine serum albumin-conjugated 17β-estradiol, indicating the effects via membrane 17β-estradiol-binding sites. H_2O_2 decreased, whereas 17β-estradiol increased the amount of phosphorylated cAMP response element-binding protein and cAMP response element-dependent transcriptional activity, and H_2O_2 plus 17β-estradiol led to basal levels. H-89, an inhibitor of cAMP-dependent protein kinase A, suppressed basal and 17β-estradiol-induced cAMP response element-binding protein phosphorylation, cAMP response element-dependent transcriptional activity, Bcl-2 expression, and apoptosis resistance. The cAMP analog, dibutyryl cAMP, enhanced cAMP response element-binding protein phosphorylation, cAMP response element-dependent transcriptional activity, Bcl-2 expression, and apoptosis resistance. 17β-estradiol increased intracellular cAMP level and protein kinase A activity, whereas these were not altered by H_2O_2. Keratinocytes expressed mRNA for estrogen receptor β and guanine nucleotide-binding protein-coupled receptor, GPR30. GPR30 anti-sense oligonucleotide did, but anti-sense estrogen receptor β did not suppress 17β-estradiol-induced cAMP signal, cAMP response element-binding protein phosphorylation, Bcl-2 expression, and apoptosis resistance. These results suggest that 17β-estradiol may enhance Bcl-2 expression and prevent H_2O_2-induced apoptosis by phosphorylating cAMP response element-binding protein via cAMP/protein kinase A pathway in keratinocytes. These effects of 17β-estradiol may be mediated via membrane GPR30. ***Key words: cyclic adenosine monophosphate response element-binding protein/GPR30/phosphorylation/protein kinase A. J Invest Dermatol 121:1500–1509, 2003***

It has been reported that estrogen manifests protective effects on the skin, and may aid in maintaining skin homeostasis (Shah and Maibach, 2001). Estrogen is known to sustain skin thickness and water-holding capacity (Shah and Maibach, 2001), and also accelerates cutaneous wound healing (Ashcroft *et al*, 1997). These effects of estrogen are mostly exerted by its direct action on dermal fibroblasts; estrogen induces fibroblasts to produce transforming growth factor β1, which promotes the maturation of fibroblasts and synthesis of the extracellular matrix (Ashcroft *et al*, 1997). On the other hand, little is known about the direct effects of estrogen on epidermal keratinocytes. We recently found that 17β-estradiol (E2) acted on human keratinocytes and inhibited their production of chemokines, IP-10, MCP-1, and RANTES, which recruit activated T helper-1 cells or macrophages (Kanda and Watanabe, 2003a,b,c). These results indicate that E2 may suppress the development of inflammatory skin diseases, such as psoriasis or lichen planus. Urano *et al* (1995) also reported that E2 stimulated proliferation of human keratinocytes; however, it has not been examined if E2 may promote the survival of keratinocytes. Keratinocytes apt to be exposed to a variety of environmental or endogenous stresses, such as oxidative stress, inflammatory cytokines such as tumor necrosis factor-α, ultraviolet (UV) light, or ionizing irradiation (Qin *et al*, 2001; Chang *et al*, 2002). To prevent the stress-induced apoptosis may thus maintain the survival of keratinocytes and contribute to skin homeostasis. Previous studies reported that E2 inhibited staurosporine-induced apoptosis in rat cortical neurons (Honda *et al*, 2001) or tumor necrosis factor-α-induced apoptosis in human breast cancer MCF-7 cells (Burow *et al*, 1999). On the other hand, E2 enhanced tamoxifen-induced apoptosis in hypothalamic neuronal cells (Hashimoto *et al*, 1997). Though E2 inhibited vitamin E succinate-induced apoptosis, it promoted spontaneous apoptosis in MCF-7 cells (Tesarik *et al*, 1999). Thus E2 may either suppress or promote apoptosis dependently on cell types or

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Abbreviations: AC, adenylate cyclase; CRE, cAMP response element; CREB, CRE-binding protein; E2, 17β-estradiol; E2-BSA, 17β-estradiol 6-(O-carboxymethyl)oxime:bovine serum albumin; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-protein, guanine nucleotide-binding protein; KBM, Keratinocyte basal median; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein; MEK, MAPK or ERK kinase; PI-3K, phosphatidylinositol 3-OH kinase; PKA, protein kinase A.

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experimental conditions. It is reported that hydrogen peroxide, tumor necrosis factor-α, or cell-permeable ceramides suppressed the expression of anti-apoptotic protein Bcl-2 and thus induced apoptosis in leukemia HL-60 or U937 cells or hippocampal neurons (Chen *et al*, 1995; Pugazhenthi *et al*, 2003), whereas overexpression of Bcl-2 counteracted apoptosis in B-lineage cells (Vaux *et al*, 1988; Alam *et al*, 1997). Bcl-2 prevents the activation of caspases, inhibits membrane lipid peroxidation, and blocks the actions of pro-apoptotic bcl-2 family members such as Bax or Bad (Merry and Korsmeyer, 1997). Thus E2 may either suppress or promote apoptosis by regulating the expression of these pro-apoptotic or anti-apoptotic bcl-2 family proteins.

It is known that the effects of E2 are mediated by genomic or nongenomic mechanisms (Beato, 1989; Kelly and Levin, 2001). E2 may thus regulate apoptosis by either or both mechanisms. The genomic mechanism is that E2-bound nuclear estrogen receptor (ER) α or β stimulates or inhibits gene expression by binding to estrogen response element of the target genes or by interacting with other transcription factors (Beato, 1989; Kanda and Watanabe, 2003c). On the other hand, the nongenomic mechanism is that E2 interacts with cell surface E2-binding sites and rapidly induces a variety of intracellular signals (Kelly and Levin, 2001), such as activation of adenylate cyclase (AC) (Aronica *et al*, 1994), phospholipase C (Razandi *et al*, 1999), or extracellular signal-regulated kinase (ERK) (Migliaccio *et al*, 1996). Membrane E2-binding sites are not a unique molecule; some are post-translationally modified forms of nuclear ERα or β, whereas others are structurally different from classical nuclear ER (Papas *et al*, 1995; Razandi *et al*, 1999; Benten *et al*, 2001). It is reported that E2 interacts with several guanine nucleotide-binding protein (G-protein)-coupled receptors such as steroid hormone-binding globulin receptor (Fissore *et al*, 1994; Nakla *et al*, 1994), or an orphan receptor GPR30 (Filardo *et al*, 2002).

In this study, we examined the *in vitro* effects of E2 on H_2O_2-induced apoptosis in human keratinocytes. We used H_2O_2 as an apoptotic model as reactive oxygen species, including H_2O_2 are produced and act as apoptotic regulators in keratinocytes exposed to UV, tumor necrosis factor-α, bacteria, or viruses (Kohen and Gati, 2000). We found that E2 prevented H_2O_2-induced apoptosis in keratinocytes. We further studied the mechanism for the effects, focusing on the regulation of Bcl-2 expression by E2.

MATERIALS AND METHODS

Reagents E2-bovine serum albumin (E2-BSA) and 17α-estradiol were purchased from Sigma (St Louis, Missouri). SQ22536, LY294002, SB202190, PD98059, H-89, and W-7 were obtained from Calbiochem (La Jolla, California). Monoclonal antibodies against cyclic adenosine monophosphate (cAMP) response element (CRE) binding protein (CREB) and Ser^{133}-phosphorylated CREB were purchased from New England Biolabs (Beverly, Massachusetts). Anti-Bcl-2 monoclonal antibody was from Transduction Laboratories (Lexington, Kentucky). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was from American Research Products (Belmont, Massachusetts).

Culture of keratinocytes Human neonatal foreskin keratinocytes were cultured in serum-free keratinocyte growth medium (Clonetics, Walkersville, Maryland) 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 third passage were used.

Assays for apoptosis Apoptosis was measured by quantification of cytoplasmic histone-associated DNA fragments using the cell death detection kit ELISA Plus (Roche, Indianapolis, Indiana) according to the manufacturer's instructions. Keratinocytes (5×10^3 per well) were seeded in triplicate into 96-well plates in 200 μL of keratinocyte growth medium, adhered overnight, then the medium was changed to phenol red-free, basal KBM depleted of growth supplements, and incubated for 24 h. The medium was removed and the cells were incubated with 30 μM H_2O_2 in the presence or absence of indicated hormones in phenol red-free KBM for 5 h. The cell lysates were centrifuged, and the amount of apoptotic nucleosomes in the supernatants was measured. The extent of apoptosis was presented as enrichment factor defined as absorbance at 405 nm minus absorbance at 492 nm divided by the same absorbance difference in controls with medium alone. Preliminary dose–response (10–200 μM) and kinetic (1–10 h) experiments led to the determination of the optimal conditions, i.e., 30 μM H_2O_2 for 5 h. Mitochondrial membrane potential was measured by rhodamine 123 fluorescence as described (Sitailo *et al*, 2002). H_2O_2-treated keratinocytes were trypsinized and incubated for 20 min in KBM containing 5 μM rhodamine 123 (Sigma) at room temperature. The cells were washed and analyzed by flow cytometry for reduced rhodamine 123 fluorescence, indicating loss of mitochondrial membrane potential.

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) We performed semiquantitative reverse transcription–PCR to determine relative changes in mRNA levels by densitometric analysis. Keratinocytes were incubated as above, and total cellular RNA was isolated using TRIzol reagent (Invitrogen, Rockville, Maryland). We reverse-transcribed 0.5 μg of total RNA to produce cDNA as described (Tjandrawinata *et al*, 1997), and 1/30 of synthesized cDNA was thermocycled for PCR amplification with 1 μM each primer and 1.5 U of *Taq* polymerase (Invitrogen). The sequences of PCR primers for ERα, ERβ, GPR30, bcl-2, bax, bcl-x_L, bad, bak, and GAPDH were previously described (Carmeci *et al*, 1997; Dong *et al*, 1999; Dubal *et al*, 1999; Vegeto *et al*, 2001; Chernavsky *et al*, 2002; Kanda and Watanabe, 2003c). PCR was performed by one denaturing cycle of 95°C for 3 min, 30 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. In preliminary experiments using control cDNA from human spleen, 30 cycles lay in the linear range of PCR amplification for each gene examined. The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide, viewed by UV light. Densitometric analysis of the bands was performed by NIH Image Software. The mRNA levels of bcl-2, bax, bcl-x_L, bad, and bak were normalized for that of GAPDH. The mRNA level for bcl-2 was further measured by real-time quantitative reverse transcription–PCR using a TaqMan PCR Reagent Kit (Perkin-Elmer Applied Biosystems, Foster City, California) according to the manufacturer's protocol. The sequences of forward and reverse primers and TaqMan fluorogenic probe for bcl-2 and GAPDH were previously described (Ikeguchi *et al*, 2002). The mRNA level of bcl-2 was normalized for that of GAPDH.

Western blot analysis Keratinocytes incubated under the indicated conditions were lyzed and centrifuged as described (Kanda and Watanabe, 2002). Protein concentration in the supernatant was determined by Bio-Rad DC reagent (Bio-Rad Laboratories, Hercules, California). For western analysis, 20 μg of proteins were resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel. The proteins were transferred to a PVDF membrane. The membrane was blocked, and exposed to primary antibodies, followed by peroxidase-conjugated secondary antibodies (Bio-Rad). The blots were developed with an enhanced chemiluminescence kit (Amersham, Arlington Heights, Illinois). Densitometric analysis of the bands was performed by NIH Image Software.

Plasmids and transfections The firefly luciferase reporter plasmids driven by human *bcl-2* P1 promoter (–1640/–1287 bp relative to the transcriptional start site) were constructed by PCR and insertion into pGL3 basic vector (Promega, Madison, Wisconsin) as described (Wilson *et al*, 1996), and was denoted as pbcl-2 luc. Site-specific mutation of the promoter was created by multiple rounds of PCR using primers with altered bases as described (Dong *et al*, 1999). p4xCRE-TATA-luc was constructed by inserting four copies of CRE-like element (5′-ACGTGTGACGTTACGCAC-3′) from *bcl-2* promoter in front of TATA box upstream of firefly luciferase reporter as described (Kanda and Watanabe, 2003c). Transient transfections were performed with Effectene (Qiagen, Tokyo, Japan) as described (Zellmer *et al*, 2001). The efficiency of transfection into keratinocytes by this method was mean ± SEM 28.5 ± 3.1% (n = 9) as determined by flow cytometry using β-galactosidase vector. Keratinocytes were plated in 10 cm dishes and grown to about 60% confluence. Twenty-four hours before the transfection, the medium was changed to phenol red-free KBM. Keratinocytes were incubated for 6 h with 5 μg of pbcl-2 luc or p4xCRE-TATA-luc and 1 μg of SV40 promoter-linked β-galactosidase reporter vector, pCH110 (Amersham), premixed with enhancer, transfection buffer, and Effectene. The transfected cells were washed and incubated in fresh phenol red-free KBM for 18 h, then incubated with 30 μM H_2O_2 in the presence or absence of indicated hormones. After 5 h, luciferase activities of the cell extracts were quantified by luciferase assay system (Promega). The

same cell extracts were assayed for β-galactosidase activity using chemiluminescent Galacto-Light kit (Tropix, Bedford, Massachusetts). 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. In our preliminary experiments, H_2O_2 did not alter the control β-galactosidase activity (data not shown). Human *bcl-2* expression vector, pSV2-neo-bcl-2 was constructed by inserting human *bcl-2* cDNA (Tsujimoto and Croce, 1986) into pSV2-neo vector as described (Fernandez *et al*, 1994, 1995). Ten micrograms of the expression vector or empty vector were transfected into 10^7 keratinocytes using Effectene as described above. After 2 d of growth, individual *bcl-2*-overexpressing or control cell clones were isolated following G418 selection (400 μg per mL for 2 wk) as described (Fernandez *et al*, 1994, 1995).

Treatment with anti-sense oligodeoxynucleotides Anti-sense oligonucleotides were synthesized as described (O'Dowd *et al*, 1998; Lau *et al*, 2000). The oligonucleotides were ERβ, 5′-CATCACAGCAGGGC-TATA-3′; GPR30, 5′-TTGGGAAGTCACATCCAT-3′; random control, 5′-GATCTCAGCACGGCAAAT-3′. For anti-sense experiments, keratinocytes were washed twice in phosphate-buffered saline, then transfected, finally, with 0.2 μM of the indicated oligonucleotides premixed with Superfect Reagent (Qiagen) in phenol red-free KBM for 4 h. The medium was aspirated and fresh medium containing E2 was added. The efficiency of transfection was 89.3 ± 9.5% (n = 9) as examined microscopically using fluorescein isothiocyanate-labeled oligonucleotides.

Measurement of cAMP amount Keratinocytes were seeded into 96-well plates at 5×10^3 cells per well, adhered, and were incubated with phenol-red free KBM for 24 h, then incubated with hormones in the presence or absence of 30 μM H_2O_2 for 30 min. The medium was discarded, and the cells were lyzed with acetate buffer (pH 5.8) containing 0.25% dodecyltrimethylammonium bromide. The cAMP amount in the lysate was measured by ELISA (Amersham) according to the manufacturer's instructions. The sensitivity of the assay was 12 fmol per assay well. The intracellular cAMP level was presented as pmol per 10^6 cells.

Assays of cAMP-dependent protein kinase A (PKA) Keratinocytes were cultured under the indicated conditions, and were lyzed in the buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid, 1 μg aprotinin per mL, 1 μg pepstatin per mL, 1 μg leupeptin per mL, 15 mM benzamidine, and 3.75 mM β-mercaptoethanol. The cell lysate was assayed for the activity of PKA using an ELISA kit (Medical and Biological Laboratories, Nagoya, Japan) by examining the phosphorylation of plate-bound peptide substrate in the presence or absence of cAMP. The absorbance at 492 nm was read, and the net absorbance was calculated by subtracting the absorbance in the presence of specific PKA inhibitor KT-5720 (Calbiochem) from the total absorbance. The PKA activity was expressed as an activity ratio, which is defined as the net absorbance in the absence of exogenous cAMP divided by the net absorbance in the presence of cAMP.

RESULTS

E2 and E2-BSA prevented oxidative stress-induced apoptosis in keratinocytes The incubation with H_2O_2 at 30 μM for 5 h induced apoptosis in keratinocytes; A_{405}–A_{492} in H_2O_2-incubated cells was mean ± SEM 0.408 ± 0.041 (n = 5), compared with 0.037 ± 0.005 in controls ($p < 0.05$, by paired t test), which corresponds to enrichment factor of 11.0 ± 1.2 in the former group. The H_2O_2-induced apoptosis was suppressed by E2; the anti-apoptotic effect of E2 appeared at 10^{-10} M, increased in a concentration-dependent manner, and was maximized at 10^{-8} M, which reduced the apoptosis rate by 94.5%, and IC_{50} was 0.18 nM (**Fig 1**). E2 stereoisomer 17α-estradiol did not prevent the H_2O_2-induced apoptosis. E2-BSA, membrane-impermeable bovine serum albumin-conjugated E2, suppressed the H_2O_2-induced apoptosis with 0.79 nM of IC_{50}, indicating that the effect of E2 may be mediated via membrane E2-binding sites, and not via intracellular ER. BSA alone did not alter the H_2O_2-induced apoptosis. Thus E2 rescued keratinocytes from H_2O_2-induced apoptosis, and the effect appeared to occur at the membrane. As apoptosis is regulated by anti-apoptotic or pro-apoptotic Bcl-2 family members, we then examined if E2 may alter their expression.

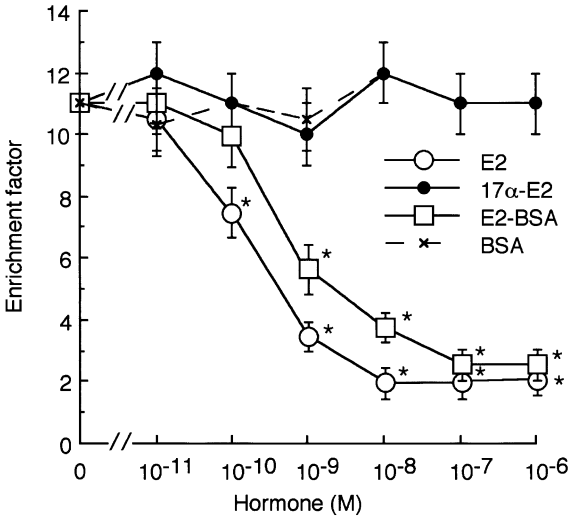

Figure 1. Dose dependency for the effects of E2 or E2-BSA on H_2O_2-induced apoptosis. Keratinocytes were incubated with 30 μM H_2O_2 in the presence of indicated concentrations of E2, E2-BSA, BSA, or 17α-estradiol (17α-E2). After 5 h, apoptosis was analyzed by ELISA, and the results are shown as an enrichment factor which was defined as A_{405}–A_{492} of each culture divided by the absorbance difference of controls with medium alone. Values are mean ± SD of triplicate cultures. *$p < 0.05$ *versus* cultures with H_2O_2 alone, by one-way ANOVA with Dunnett's multiple comparison test. The data shown in the figure are representative of five separate experiments.

E2 increased Bcl-2 protein and mRNA levels, and counteracted the H_2O_2-induced reduction of Bcl-2 levels H_2O_2 decreased mRNA level of anti-apoptotic protein Bcl-2 in keratinocytes (**Fig 2*a***, lane 4). E2 alone increased bcl-2 mRNA level in the absence of H_2O_2 (lane 2), and treatment with H_2O_2 plus E2 led to the control level (lane 5). E2-BSA increased bcl-2 mRNA level (**Fig 2*a***, lane 3), and treatment with H_2O_2 plus E2-BSA led to the control level (lane 6). 17α-estradiol did not alter bcl-2 mRNA level either in the presence or absence of H_2O_2 (data not shown). We further analyzed bcl-2 mRNA level by a quantitative real-time reverse transcription–PCR (**Fig 2*b***), and obtained the results corresponding to those in semi-quantitative reverse transcription–PCR (**Fig 2*a***). In parallel with bcl-2 mRNA level, H_2O_2 treatment for 5 h reduced Bcl-2 protein level (**Fig 2*c***, lane 4). The reduction compared with controls was 29.1 ± 4.5% (n = 5) at 1.5 h, and was maximized to 50.3 ± 6.4% at 4 h, as examined by Bcl-2/GAPDH ratio. The H_2O_2-induced reduction of Bcl-2 protein level was associated with the reduction of mitochondrial membrane potential; 5 h of H_2O_2 treatment increased rhodamine 123 fluorescence negative cells from 14.5 ± 2.1% (n = 5) in controls to 55.6 ± 8.3% ($p < 0.05$, by paired t test). E2 or E2-BSA increased the Bcl-2 protein level in the absence of H_2O_2 (lanes 2 and 3), and treatment with H_2O_2 plus E2 or E2-BSA led to control levels (**Fig 2*c***, lanes 5 and 6). 17α-estradiol did not alter the Bcl-2 protein level either in the presence or absence of H_2O_2 (data not shown). On the other hand, E2, E2-BSA, or H_2O_2, either alone or in combination, did not alter the mRNA (**Fig 2*a***) and protein levels (data not shown) of another anti-apoptotic Bcl-2 family member Bcl-x_L or pro-apoptotic Bax, Bad, or Bak. Thus E2 or E2-BSA increased Bcl-2 mRNA and protein levels, and counteracted the H_2O_2-induced reduction of Bcl-2 levels. The inverse correlation of apoptosis rate with the Bcl-2 level indicates that the increase of Bcl-2 level by E2 may rescue keratinocytes from H_2O_2-induced apoptosis. This is supported by the transfection of *bcl-2* expression vector; *bcl-2*-overexpressed keratinocytes were resistant to the H_2O_2-induced apoptosis

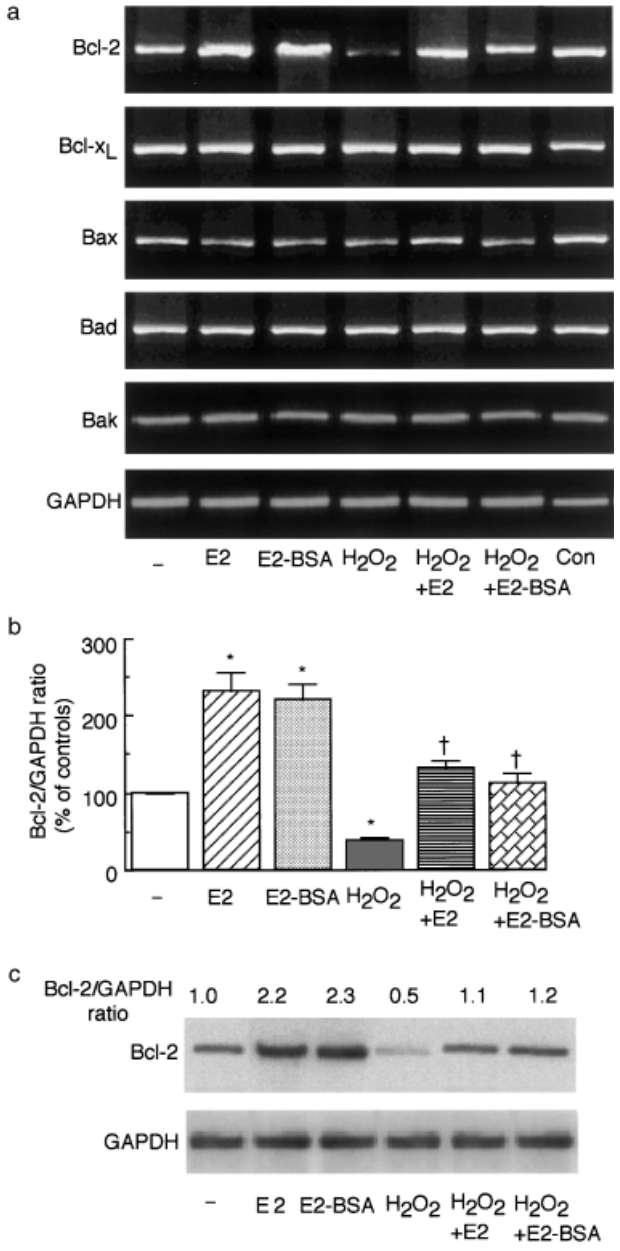

Figure 2. The effects of E2 or E2-BSA on Bcl-2 mRNA (*a,b*) and protein levels (*c*) in the presence or absence of H_2O_2. Keratinocytes were incubated with 30 μM H_2O_2 or with medium alone in the presence or absence of 10^{-8} M E2 or 10^{-7} M E2-BSA. After 5 h, total cellular RNA or proteins were isolated. (*a*) PCR was performed on cDNA from human spleen as a positive control (Con) in parallel. (*b*) bcl-2 mRNA level was measured by real-time quantitative reverse transcription–PCR, and was normalized to the level of GAPDH. (*c*) The band intensity ratio of Bcl-2 *versus* GAPDH in western blotting was corrected to that in controls (set as 1.0). The results shown in (*a,c*) are representative of five separate experiments. The results in (*b*) represent mean ± SEM of five separate experiments. $^*p<0.05$ *versus* controls, m $\dagger p<0.05$ *versus* H_2O_2 alone, by one-way ANOVA with Scheffe's multiple comparison test.

(**Fig 3**). We then examined if E2 may increase *bcl-2* promoter activity in the presence or absence of H_2O_2.

E2 enhanced bcl-2 promoter activity and counteracted the H_2O_2-induced reduction of the activity The promoter region of *bcl-2* gene contained two GC/GA-rich sites, which

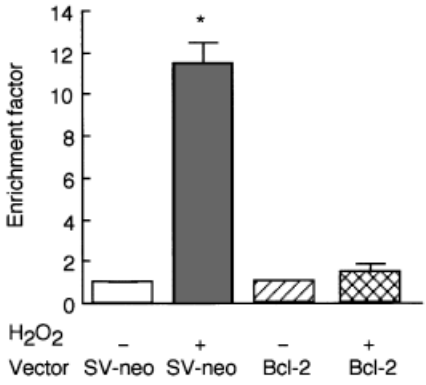

Figure 3. Effects of bcl-2 overexpression on H_2O_2-induced apoptosis. Keratinocytes transfected with empty vector (SV-neo) or bcl-2 expression vector were incubated with 30 μM H_2O_2 or medium alone. After 5 h, apoptosis was analyzed by ELISA as described in **Fig 1** legend. Values are mean ± SEM (n = 5). $^*p<0.05$ *versus* control cultures with medium alone, by paired t test.

may bind Sp1 and one element (TGACGTTA) homologous to consensus CRE (TGACGTCA), which may bind CREB (**Fig 4*a***). These elements may act as enhancer elements for *bcl-2* transcription (Dong *et al*, 1999). We transiently transfected *bcl-2* promoter linked to luciferase reporter into keratinocytes, and the promoter activity was evaluated by relative luciferase activity of the cell lysates. E2 alone increased *bcl-2* promoter activity (**Fig 4*b***, uppermost six columns). H_2O_2 reduced *bcl-2* promoter activity, and treatment with H_2O_2 plus E2 led to the basal level. Membrane-impermeable E2-BSA alone enhanced *bcl-2* promoter activity, and treatment with H_2O_2 plus E2-BSA led to the basal level, whereas 17α-estradiol did not alter *bcl-2* promoter activity either in the presence or absence of H_2O_2 (data not shown). The mutation of each of two Sp1 sites (**Fig 4*b***, second and third six columns) reduced the basal promoter activity slightly (about 20% each), but did not affect the E2-induced stimulation or H_2O_2-induced reduction of the activity. In contrast, the mutation of CRE (**Fig 4*b***, six columns at the bottom) mostly abrogated the basal promoter activity and completely abolished E2-induced stimulation and H_2O_2-induced reduction of the activity. These results suggest that CRE may be responsible for basal and E2-induced *bcl-2* promoter activities, and may be involved in H_2O_2-induced suppression of the activities. We then analyzed if E2 may promote CRE-dependent transcriptional activity in the presence or absence of H_2O_2.

E2 enhanced CRE-dependent transcriptional activity and counteracted the H_2O_2-induced reduction of the activity Keratinocytes were transiently transfected with luciferase reporter linked to four repeats of *bcl-2* promoter-derived CRE in front of the TATA box. The luciferase activity in enhancerless TATA-luciferase reporter-transfected keratinocytes was marginal, and was not altered by E2 or H_2O_2, either alone or in combination (data not shown). E2 alone increased CRE-dependent transcriptional activity (**Fig 5**). H_2O_2 reduced the CRE-dependent transcriptional activity, and treatment with H_2O_2 plus E2 led to the basal level. E2-BSA alone enhanced the CRE-dependent transcriptional activity and treatment with H_2O_2 plus E2-BSA led to the basal level (**Fig 5**), whereas 17α-estradiol did not alter CRE-dependent transcriptional activity, either in the presence or absence of H_2O_2 (data not shown). It is known that phosphorylation of CREB at Ser^{133} promotes its transcriptional activity on CRE (Kwok *et al*, 1994). The phosphorylation of CREB recruits transcriptional coactivator CREB binding protein. The association with CREB promotes the interaction of CREB

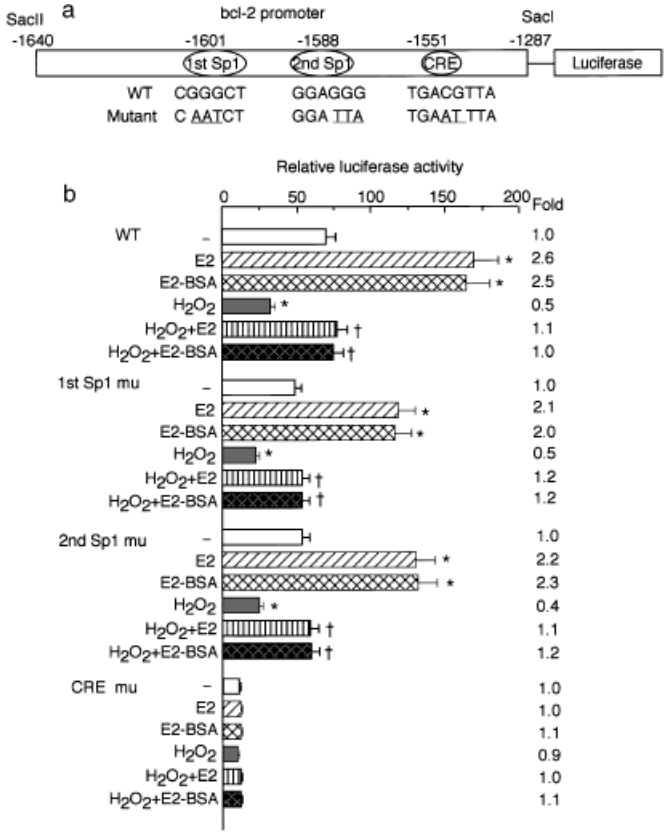

Figure 4. The effects of E2 on wild-type (WT) or mutated bcl-2 promoter activities in the presence or absence of H_2O_2. (*a*) Schematic representation of human *bcl-2* promoter. The locations of putative two Sp1-binding sites and one CRE are shown with their sequences, and substituted bases for mutation are underlined. The nucleotide positions are relative to the transcriptional start site. (*b*) Keratinocytes were transiently transfected with WT or mutated pbcl-2 luc together with pCH110, and incubated with 30 μM H_2O_2 or with medium alone in the presence or absence of 10^{-8} M E2 or 10^{-7} M E2-BSA for 5 h. Relative luciferase activities normalized to β-galactosidase activities are shown. The data are mean ± SEM (n = 4). Values at right indicate the fold induction *versus* basal promoter activity. $^*p < 0.05$ *versus* values with medium alone, $\dagger p < 0.05$ *versus* values with H_2O_2 alone, by one-way ANOVA with Scheffe's multiple comparison test.

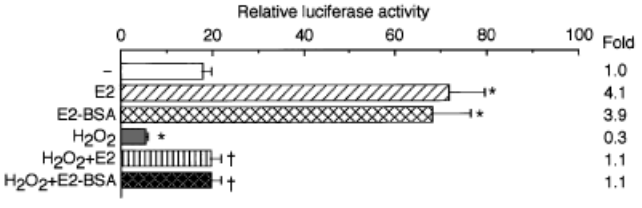

Figure 5. The effects of E2 or E2-BSA on CRE-dependent transcriptional activity. Keratinocytes were transiently transfected with p4xCRE-TATA-luc together with pCH110. The cells were incubated with 30 μM H_2O_2 or with medium alone in the presence or absence of 10^{-8} M E2 or 10^{-7} M E2-BSA for 5 h. The results are shown as relative luciferase activities normalized to β-galactosidase activities, and represent mean ± SEM (n = 4). Values at right indicate the fold induction *versus* basal activity. $^*p < 0.05$ *versus* values with medium alone, $\dagger p < 0.05$ *versus* values with H_2O_2 alone, by one-way ANOVA with Scheffe's multiple comparison test.

binding protein with basal transcription factor TFIIB, and thus facilitates CREB-mediated transcription. We then examined if E2 may stimulate the CREB phosphorylation in keratinocytes in the presence or absence of H_2O_2.

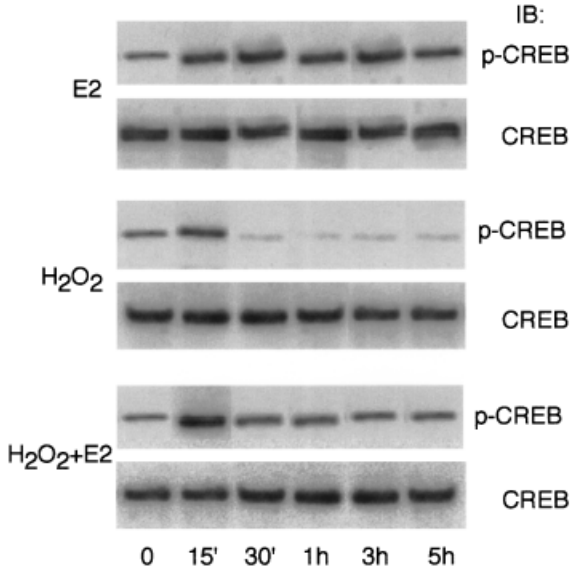

Figure 6. The effect of E2 on CREB phosphorylation in the presence or absence of H_2O_2. Keratinocytes were incubated with 30 μM H_2O_2 or with medium alone in the presence or absence of 10^{-8} M E2. At indicated time-points, whole cell lysates were obtained and probed with an antibody against phosphorylated CREB (upper panel). A parallel blot was probed with an antibody against total CREB (lower panel). The results shown in the figure are representative of four separate experiments.

E2 enhanced CREB phosphorylation and counteracted the H_2O_2-induced suppression of the phosphorylation The amounts of phosphorylated or total CREB were analyzed by western blotting. E2 alone increased the amount of phosphorylated CREB at 15 min, and the increased level was maintained up to 3 h, followed by the slight reduction at 5 h, whereas the amount of total CREB was not altered over the incubation period (**Fig 6**, uppermost two panels). In H_2O_2-treated cells (**Fig 6**, middle two panels), the amount of phosphorylated CREB transiently increased at 15 min, however, became less than basal level at 30 min, and the decreased level was maintained until 5 h, whereas the amount of total CREB did not change over the incubation period. Treatment with H_2O_2 plus E2 resulted in the basal level of phosphorylated CREB (**Fig 6**, two panels at the bottom). E2-BSA alone increased the amount of phosphorylated CREB, and counteracted the H_2O_2-induced reduction of phosphorylated CREB without altering the amount of total CREB, whereas 17α-estradiol did not alter the amount of phosphorylated CREB either in the presence or absence of H_2O_2 (data not shown).

PKA may be responsible for the CREB phosphorylation by E2 It is known that CREB is phosphorylated at Ser^{133} by a variety of serine/threonine kinases, such as cAMP-dependent PKA, protein kinase C, Akt, Ca^{2+}/calmodulin-dependent protein kinase type IV, mitogen-activated protein kinase (MAPK)-activated protein (MAPKAP) kinase-2, $p70^{S6K}$, or $p90^{rsk}$ (Tan *et al*, 1996). We then analyzed which of the kinases may be responsible for the E2-induced CREB phosphorylation, using specific kinase inhibitors. The concentrations of the inhibitors used were decided on a basis of the reported IC_{50} values (Chijiwa *et al*, 1990; Flamigni *et al*, 1997; Begemann *et al*, 1998; Horstmann *et al*, 1998; Sharma *et al*, 1998; Miura *et al*, 1999; Chen *et al*, 2000). These kinase inhibitors did not alter the amount of total CREB in keratinocytes treated with medium alone or in the presence of E2, H_2O_2, or both (data not shown). H-89, a specific PKA inhibitor (**Fig 7*a***, lane 2), and SQ22536, an inhibitor of AC, which catalyzes the synthesis of cAMP (**Fig 7*a***, lane 3), reduced the amount of phosphorylated CREB in

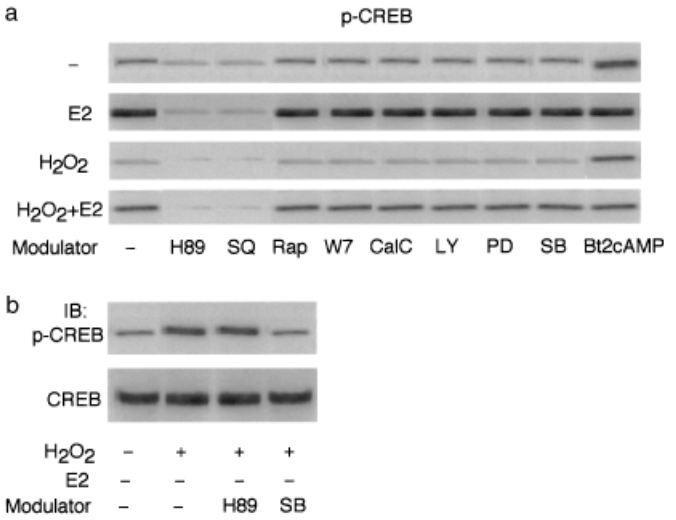

Figure 7. The inhibition by signal inhibitors on CREB phosphorylation in the presence or absence of E2, H_2O_2, or both (*a*), and on H_2O_2-induced transient CREB phosphorylation (*b*). (*a*) Keratinocytes were preincubated with 1 μM H-89, 200 μM SQ22536 (SQ), 0.1 μM rapamycin (Rap), 50 μM W-7, 1 μM calphostin C (CalC), 10 μM LY294002 (LY), 10 μM PD98059 (PD), or 10 μM SB202190 (SB) for 10 min, then incubated with medium alone or with 30 μM H_2O_2 in the presence or absence of 10^{-8} M E2 or 1 mM dibutyryl cAMP (Bt2cAMP) for another 30 min. The whole cell lysates were probed for phosphorylated CREB. (*b*) Keratinocytes were preincubated with 1 μM H-89 or 10 μM SB202190 (SB) for 10 min, then incubated with 30 μM H_2O_2 for 15 min. The cell lysates were immunoblotted for phosphorylated or total CREB. The results shown are representative of five separate experiments.

keratinocytes treated with medium alone, E2, H_2O_2, or H_2O_2 plus E2. These results suggest that PKA may mediate both constitutive and E2-induced CREB phosphorylation, and that cAMP may be required for the activity of PKA. This is supported by the fact that the cAMP analog, dibutyryl cAMP, increased the amount of phosphorylated CREB in keratinocytes treated with medium alone or H_2O_2 (**Fig 7*a***, first and third panels, lane 10). Dibutyryl cAMP, however, did not further increase the amount of phosphorylated CREB in keratinocytes treated with E2 or H_2O_2 plus E2 (**Fig 7*a***, second and fourth panels, lane 10), indicating that CREB phosphorylation by E2 and that by dibutyryl cAMP may occur via a common mechanism, both through the activation of PKA. The amount of phosphorylated CREB in H-89 or SQ22536 plus H_2O_2-treated cells (**Fig 7*a***, third panel, lanes 2 and 3) was lower than that in cells treated with H-89 or SQ22536 alone (**Fig 7*a***, first panel, lanes 2 and 3). These results suggest that H_2O_2-induced reduction of phosphorylated CREB may occur independently from the inhibition of PKA or AC. On the other hand, irrespective of the presence of H_2O_2 or E2, the amount of phosphorylated CREB was not reduced by rapamycin, W-7, or calphostin C (**Fig 7*a***, lanes 4–6), which inhibits $p70^{S6K}$, Ca^{2+}/calmodulin-dependent protein kinase type IV, or protein kinase C, respectively, indicating that these kinases may not be involved in constitutive or E2-induced CREB phosphorylation. In addition, the amount of phosphorylated CREB was not reduced by LY294002, PD98059, or SB202190 (**Fig 7*a***, lanes 7–9), which inhibit phosphatidylinositol-3-OH kinase (PI-3K)/Akt, MAPK or ERK kinase (MEK)1/ERK/$p90^{rsk}$, or p38 MAPK/MAPKAP kinase-2 signaling pathways, respectively. These results indicate that Akt, $p90^{rsk}$, or MAPKAP kinase-2 may not be involved in constitutive or E2-induced CREB phosphorylation. In contrast, H_2O_2-induced transient increase of phosphorylated CREB at 15 min (**Fig 7*b***, lane 2) was suppressed by SB202190 (lane 4), and not by H-89 (lane 3) or other kinase inhibitors (data not shown), indicating that the transient CREB phosphorylation by H_2O_2 may be mediated by p38 MAPK/MAPKAP kinase-2 pathway, and not by cAMP/PKA pathway.

cAMP signal induced by E2 may promote CREB-dependent Bcl-2 expression and generate apoptosis resistance We then analyzed if E2-induced Bcl-2 expression and apoptosis resistance may be suppressed by PKA inhibitor, in parallel with the inhibition of CREB phosphorylation. H-89 reduced CRE-dependent transcriptional activity (**Fig 8*a***), bcl-2 promoter activity (**Fig 8*b***), Bcl-2 protein level (**Fig 8*d***), and increased apoptosis rate (**Fig 8*c***) in keratinocytes treated with medium alone, E2, H_2O_2, or E2 plus H_2O_2 (lanes 2, 5, 8, and 11, respectively). An AC inhibitor SQ22536 also showed the inhibitory effects similar to those of H-89 (data not shown). Dibutyryl cAMP increased CRE-dependent transcriptional activity (**Fig 8*a***), bcl-2 promoter activity (**Fig 8*b***), and Bcl-2 protein level (**Fig 8*d***) in keratinocytes treated with medium alone or H_2O_2 (lanes 3 and 9, respectively), and suppressed apoptosis in H_2O_2-treated cells (**Fig 8*c***, lane 9). Dibutyryl cAMP, however, did not further increase CRE-dependent transcriptional activity, bcl-2 promoter activity, and Bcl-2 protein level in keratinocytes treated with E2 or E2 plus H_2O_2 (lanes 6 and 12, respectively), and did not reduce the apoptosis rate in these cells, indicating a common mechanism for Bcl-2 expression by E2 and that by dibutyryl cAMP. These results indicate that cAMP/PKA signaling pathway may be required for both constitutive and E2-induced Bcl-2 expression and may be responsible for the apoptosis resistance.

We then examined if E2 may generate cAMP signal in keratinocytes. At 30 min of incubation, E2 increased intracellular cAMP level 4.1-fold of controls (**Fig 9*a***), which was associated with the increase of PKA activity (**Fig 9*b***). H_2O_2 did not alter cAMP level or PKA activity either in the presence or absence of E2. E2-BSA increased cAMP level and PKA activity either in the presence or absence of H_2O_2 (**Fig 9*a,b***), whereas 17α-estradiol did not alter cAMP level or PKA activity (data not shown). These results suggest that E2 may increase cAMP level and resultantly promote PKA activity in keratinocytes, whereas these may not be altered by H_2O_2.

Anti-sense GPR30 suppressed E2-induced cAMP signal, CREB phosphorylation, and Bcl-2 expression As membrane-impermeable E2-BSA generated cAMP signal as well as E2, E2 may act on the surface of keratinocytes. It is reported that E2 induces cAMP signal by interacting with membrane-localized ERα, ERβ (Razandi *et al*, 1999), or GPR30 (Filardo *et al*, 2002). As examined by reverse transcription–PCR, keratinocytes expressed ERβ and GPR30 mRNA, but not that of ERα (**Fig 10*a***), indicating that E2 may interact with membrane-localized ERβ or GPR30 and thus generate cAMP signal. As specific antibody against GPR30 cannot be obtained, we analyzed if ERβ or GPR30 anti-sense oligonucleotides may inhibit E2-induced cAMP signal. GPR30 anti-sense did but ERβ anti-sense did not suppress the E2-induced cAMP signal (**Fig 10*b***), CREB phosphorylation (**Fig 10*c***), Bcl-2 expression (**Fig 10*d***), and apoptosis resistance (**Fig 10*e***). E2-BSA-induced cAMP signal, CREB phosphorylation, Bcl-2 expression, and apoptosis resistance were also suppressed by GPR30 anti-sense but not by ERβ anti-sense (data not shown). These results suggest that cell surface GPR30 may be involved in E2 or E2-BSA-induced cAMP signal and resultant CREB phosphorylation, Bcl-2 expression, and apoptosis resistance, whereas ERβ may not be involved in these effects.

DISCUSSION

In this study, E2 protected keratinocytes from H_2O_2-induced apoptosis by promoting Bcl-2 expression. The *bcl-2* transcription

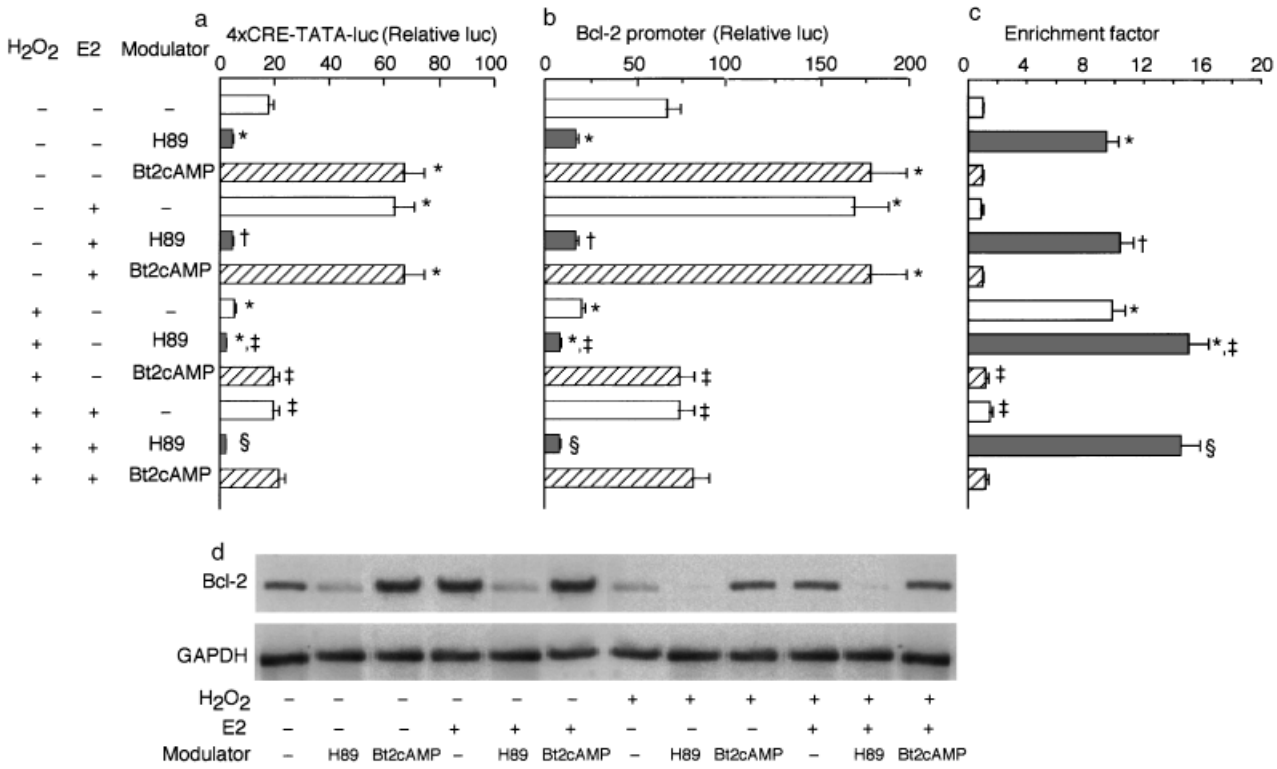

Figure 8. The inhibition by PKA inhibitor on CRE-dependent transcriptional activity (*a*), bcl-2 promoter activity (*b*), apoptosis resistance (*c*), and Bcl-2 protein level (*d*) in the presence or absence of E2, H_2O_2, or both. (*a,b*) Keratinocytes were transiently transfected with p4xCRE-TATA-luc (*a*) or pbcl-2 luc (*b*), together with pCH110, then preincubated with 1 μM H-89 for 10 min, then incubated with medium alone or with 30 μM H_2O_2 in the presence or absence of 10^{-8} M E2 or 1 mM dibutyryl cAMP (Bt2cAMP) for another 5 h. Luciferase activities of the cell lysates were analyzed and were normalized to β-galactosidase activities. (*c,d*) Keratinocytes without transfection were incubated as above, and apoptosis (*c*) or Bcl-2 protein level (*d*) were analyzed. (*a–c*) Values are mean ± SEM of five separate experiments. *$p < 0.05$ *versus* values with medium alone, †$p < 0.05$ *versus* values with E2 alone, ‡$p < 0.05$ *versus* values with H_2O_2 alone, §$p < 0.05$ *versus* values with H_2O_2 plus E2, by one-way ANOVA with Scheffe's multiple comparison test. The results shown in (*d*) are representative of five separate experiments.

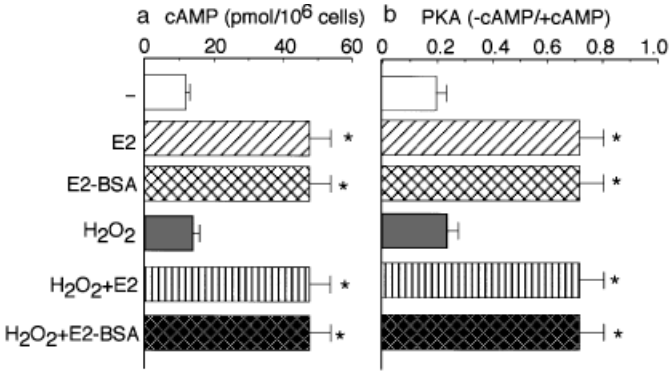

Figure 9. The effects of E2 or E2-BSA on the intracellular cAMP level (*a*) and PKA activity (*b*) in the presence or absence of H_2O_2. Keratinocytes were incubated with 30 μM H_2O_2 or with medium alone in the presence or absence of 10^{-8} M E2 or 10^{-7} M E2-BSA for 30 min. The intracellular cAMP level (*a*) or PKA activity of the cell lysates (*b*) were analyzed. The data represent the mean ± SEM of four separate experiments. *$p < 0.05$ *versus* values with medium alone, by one-way ANOVA with Scheffe's multiple comparison test.

was mediated via the phosphorylation of CREB by PKA, and PKA was activated by cAMP signal. It is reported that cAMP-elevating stimuli such as hormones or cytokines induce Bcl-2 expression and apoptosis resistance in a variety of cell types (Chen *et al*, 2001; Hui *et al*, 2003; Koriyama *et al*, 2003). cAMP is generated by the stimulation of G-protein-coupled receptors linked to the activation of AC. In keratinocytes, E2 appeared to generate the cAMP signal via cell surface G-protein-coupled receptor, GPR30. That is also supported by a previous study on breast cancer cells (Filardo *et al*, 2002). In the Filardo *et al* (2002) study, E2 induced cAMP signal in GPR30-positive SKBR3 cells, whereas it did not in GPR30-negative MDA-MB-231 cells, and transfection of GPR30 into MDA-MB-231 restored the ability to generate cAMP signal by E2. Besides E2 generated cAMP signal independently from ERβ in their study, which is also consistent with our present results (**Fig 10**). It is reported that E2 induced *bcl-2* transcription dependently on cAMP signal in MCF-7 cells (Dong *et al*, 1999). E2 also increased the amounts of phosphorylated CREB and CREB binding protein in the nuclei of rat hippocampal neurons, and these effects were dependent on PKA activity (Murphy and Segal, 1997). These studies, however, did not clarify if GPR30 may mediate the effects of E2. Besides it has not been studied if GPR30 may regulate apoptosis or Bcl-2 expression via CREB phosphorylation. Thus this is the first study that supports GPR30-mediated Bcl-2 expression and apoptosis resistance. GPR30 is a 375 amino acid G-protein-coupled receptor with seven transmembrane domains (Carmeci *et al*, 1997). The ligand for GPR30 has not been identified yet, but may be a certain peptide or glycoprotein (Carmeci *et al*, 1997). GPR30 mRNA is expressed ubiquitously in brain, lung, liver, prostate, or colon (Owman *et al*, 1996). Thus GPR30 may generally induce apoptosis resistance in a variety of tissues, and this possibility should further be examined.

As activation of GPR30-generated cAMP signal, GPR30 may possibly be linked to AC via G_s. It is hypothesized that the binding of E2 may activate GPR30 and induce the dissociation of the receptor-coupled G-protein heterotrimers, possibly $G_s\alpha\beta\gamma$, into

$G_s\alpha$ and $G\beta\gamma$, the former of which may stimulate AC catalytic subunit. E2 stereoisomer, 17α-estradiol did not induce cAMP signal in keratinocytes, which is consistent with a previous study on GPR30-transfected MDA-MB-231 cells (Filardo *et al*, 2002). We also found that ER antagonists, ICI 182 780 and 4-hydroxytamoxifen induced cAMP signal, CREB phosphorylation, bcl-2 expression, and prevented H_2O_2-induced apoptosis in keratinocytes, and these effects were suppressed by anti-sense GPR30 (data not shown). Filardo *et al* (2002) also reported that these anti-estrogens generated cAMP signal in GPR30-transfected MDA-MB-231 cells. Thus tamoxifen derivatives or ICI compounds may act as agonists for membrane GPR30, whereas 17α-estradiol may not.

The phosphorylation of CREB at Ser^{133} is induced by various signaling pathways activating serine/threonine kinases, such as PI-3K/Akt, phospholipase C/protein kinase C, MEK/ERK/$p90^{rsk}$, PI-3K/$p70^{S6k}$, p38 MAPK/MAKAP kinase-2 pathways, or Ca^{2+}/calmodulin-dependent protein kinase IV, in addition to cAMP/PKA pathway (Tan *et al*, 1996). Cell types or stimuli may influence which of these signals dominantly induces CREB phosphorylation. In keratinocytes, PKA may mainly mediate the constitutive and E2-induced CREB phosphorylation. In rat cortical neurons, E2 enhanced Bcl-2 expression via PI-3K/Akt-mediated CREB phosphorylation (Honda *et al*, 2001). The activation of the PI-3K/Akt pathway by E2, however, is rather unlikely in keratinocytes lacking ERα as PI-3K can be activated by E2-bound ERα but not by ERβ (Simoncini *et al*, 2000). In the cell types other than keratinocytes, E2 induced intracellular Ca^{2+} signal (Benten *et al*, 2001), activated phospholipase C/protein kinase C pathway (Le Mellay *et al*, 1999), or activated the src/Ras/MEK/ERK pathway (Migliaccio *et al*, 1996), all of which may be relevant to CREB phosphorylation. In keratinocytes, however, these E2-induced signals may not occur, or may be insufficient, if any, for CREB-dependent *bcl-2* transcription, possibly because the magnitude of the signals may be small and/or their duration may be short. Previous study also reported that CREB-dependent gene transcription needs sustained CREB phosphorylation in the order of hours (Schultz *et al*, 1999). H_2O_2 induced transient CREB phosphorylation (⩽15 min), and this appeared to be mediated by the p38 MAPK/MAPKAP-kinase 2 pathway. The transient activation of p38 MAPK by H_2O_2 was also reported in rat pheochromocytoma PC12 cells (Zhang and Jope, 1999), and may be a protective response to stress, however, without a parallel increase in Bcl-2 expression. On the contrary, more than 30 min of incubation with H_2O_2 reduced the amount of phosphorylated CREB in keratinocytes (**Fig 6**), whereas H_2O_2 did not suppress PKA activity (**Fig 9**). It is thus indicated that H_2O_2 may reduce the amount of phosphorylated CREB by a PKA-independent mechanism; H_2O_2 may activate serine/threonine phosphatases that dephosphorylate and inactivate the phosphorylated CREB. It is reported that protein phosphatase 1 directly dephosphorylates the phosphorylated CREB and that protein phosphatase 2B enhances the activity of protein phosphatase 1 by dephosphorylating and inactivating an inhibitory subunit of protein phosphatase 1 (Bito *et al*, 1996). In keratinocytes, H_2O_2 may activate either or both of these phosphatases, and thus reduce the amount of phosphorylated CREB. Previous studies also suggested that H_2O_2 may activate protein phosphatase 1 in murine cerebellar granule

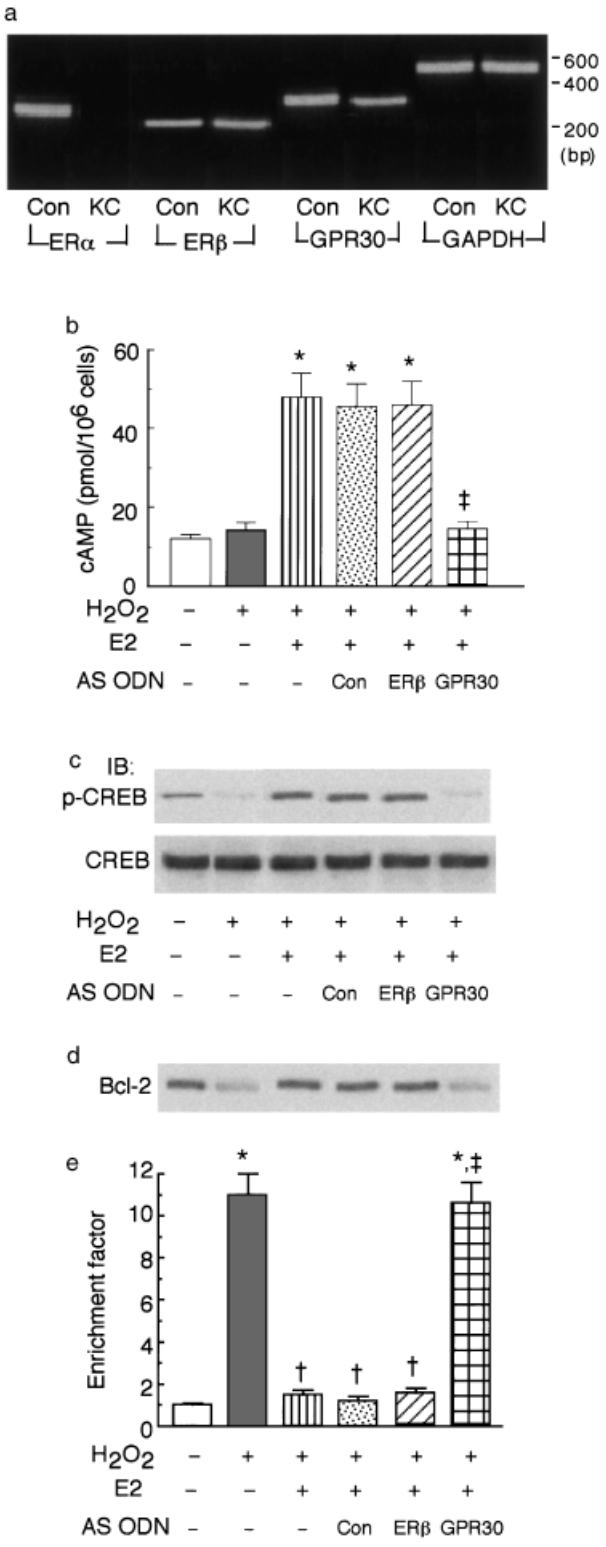

Figure 10. ERβ and GPR30 mRNA expression in keratinocytes (*a*) and the inhibition by GPR30 anti-sense oligonucleotide on E2-induced cAMP signal (*b*), CREB phosphorylation (*c*), Bcl-2 protein expression (*d*), and apoptosis resistance (*e*). (*a*) PCR was performed with cDNA from keratinocytes (KC) and from breast cancer MCF-7 cells as positive controls (Con). (*b–d*) Keratinocytes were transfected with 0.2 μM of indicated anti-sense (AS) or control scrambled oligonucleotides (ODN) for 4 h. The medium was changed and the cells were incubated with 30 μM H_2O_2 or with medium alone in the presence or absence of 10^{-8} M E2. The intracellular cAMP level (*b*) and CREB phosphorylation (*c*) were analyzed at 30 min, whereas Bcl-2 protein level (*d*) and apoptosis (*e*) were evaluated at 5 h. (*b,e*) Values are mean ± SEM of five separate experiments. *$p < 0.05$ *versus* values with medium alone, †$p < 0.05$ *versus* values with H_2O_2 alone, ‡$p < 0.05$ *versus* values with H_2O_2 plus E2, by one-way ANOVA with Scheffe's multiple comparison test. The results shown in (*a,c,d*) are representative of five separate experiments.

neurons (See and Loeffler, 2001) or sheep erythrocytes (Bize and Dunham, 1995; Bize *et al*, 1998).

In addition to CRE, two GC/GA-rich elements on the *bcl-2* promoter acted as E2-inducible enhancer elements in MCF-7 cells (Dong *et al*, 1999). These two elements, however, were not responsible for E2-induced *bcl-2* transcription in keratinocytes (**Fig 4**). The difference is possibly due to the absence of ERα in keratinocytes. E2-bound ERα can interact with Sp1 and enhance its DNA binding and transcriptional activity, however, ERβ cannot promote the activity of Sp1 (Safe, 2001; Kanda and Watanabe, 2003c).

E2 is reported to suppress apoptosis independently of Bcl-2 in the cell types different from keratinocytes. E2 enhanced the expression of another anti-apoptotic protein Bcl-x_L in cultured hippocampal neurons (Pike, 1999) or suppressed the expression of pro-apoptotic Bak in MCF-7 cells (Leung and Do Linh Wang, 1998). At least in mRNA and protein levels, E2 did not alter the expression of these anti-apoptotic or pro-apoptotic proteins in keratinocytes (**Fig 2**). E2 also inhibits membrane phospholipid peroxidation and acts as an anti-oxidant (Sugioka *et al*, 1987). E2 suppressed H_2O_2-induced apoptosis in murine hippocampal HT22 cells via its anti-oxidant capacity (Behl *et al*, 1995, 1997). In Behl *et al*'s (1995) study, however, higher concentrations of E2 (about 10^{-5} M) are needed for the anti-apoptotic effect and 17α-estradiol also suppressed H_2O_2-induced apoptosis via anti-oxidant capacity, which is inconsistent with our present results (**Fig 1**). Thus the anti-oxidant activity of E2 may not be relevant to the anti-apoptotic effect exerted at 10^{-8} M in this study.

Our present results support that E2 may rescue keratinocytes from oxidative stress-induced apoptosis. H_2O_2 is generated in keratinocytes by UVB irradiation, and acts as an apoptotic mediator (Peus *et al*, 1999). Our results thus indicate that E2 may protect keratinocytes from UVB-induced apoptosis and thus can be prophylactically applied to the skin before sun exposure. Bianchi *et al* (1994) reported that skin inflammatory diseases such as psoriasis or lichen planus were associated with abnormally increased apoptosis and the reduction of Bcl-2 levels in basal keratinocytes, and suggested a central role for apoptosis in the pathogenesis of these diseases. Thus topical application of E2 may be therapeutically effective for these skin lesions by restoring Bcl-2 levels and by preventing the aberrant apoptosis in the lesional keratinocytes.

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