A Cell-Based Assay System for Monitoring NF-κB Activity in Human Epidermal Keratinocytes: A Screening Tool of the Antioxidants and Anti-inflammatories for Dermatological Purpose

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Abstract

A cell-based assay system for monitoring NF-κB activity was developed to determine the influence of activated NF-κB in human HaCaT cells. The pNF-κB-SEAP-NPT plasmid that permits expression of the secreted alkaline phosphatase (SEAP) reporter gene in response to the NF-κB activity and contains neomycin phosphotransferase (NPT) gene for the geneticin resistance in host cells was constructed and transfected into human keratinocyte cell line HaCaT.

Human HaCaT transfectant cells secreted the SEAP enzyme into the culture medium in a time-dependent manner until 72 h. NF-κB activities were measured in the SEAP reporter gene assay using a fluorescent detection method. The treatment of HaCaT cell transfectants with known antioxidants [e.g., N-acetyl-L-cysteine and vitamin C] showed inhibition of NF-κB activity in a time- and concentration-dependent manner. The phorbol 12-myristate 13-acetate (PMA) known as a stimulator of NF-κB expression demonstrated that it increased NF-κB activity in a time- and concentration-dependent manner.

This assay system could be used to determine the quantitative measurement of NF-κB activity in the human skin and allow the screening of anti-inflammatory agents from various synthetic chemicals and natural products for dermatological purpose.

Abbreviations used: NF-κB, nuclear factor kappa B; I-κB, Inhibitory kappa B; SEAP, secreted alkaline phosphatase; NPT, neomycin phosphotransferase; PCR, polymerase chain reaction; dNTP, deoxynucleoside triphosphates; DMEM, dulbecco's modified eagle medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MUP, 4-methylumbellifery phosphate; NAC, N-acetyl-L-cysteine; DMSO, dimethyl sulfoxide; PMA, phorbol 12-myristate 13-acetate.

INTRODUCTION

Environmental stresses have been shown to increase gene expression in mammalian cells. The inducible transcription factor NF-κB[2] is known to be a major role in the regulation of genes responsible for inflammatory and immune responses (1-4). In its latent form, NF-κB is bound to I-κB inhibitor proteins in the cytoplasm. Exposure of cell to various NF-κB activators [e.g., tumor necrosis factor, interleukin-I, lipopolysaccharides, and ultraviolet light] leads to the phosphorylation and degradation of the protein, I-κB. This enables the NF-κB to translocate into the nucleus where it binds to NF-κB binding sites (or its responsive elements) in promoter regions of target genes for the subsequent increase of their transcriptions involved in inflammatory and immune responses (5-12). For this reason, the

activation of NF-κB in skin cells is considered to be an important target for the therapeutic intervention.

Skin constitutes a primary target for many environmental stimuli, i.e., ultraviolet radiation and hazardous chemicals (13-15), frequently causing inflammation associated with NF-κB activity. Therefore, monitoring NF-κB activity in human skin cells could potentially provide an appropriate means for the screening of the anti-inflammatory agents for the dermatological purpose. To this end, we attempted to develop a stable assay system for the quantitative measurement of NF-κB activity in human skin cells. Specifically, we established a stable human HaCaT cells transfected with pNF-κB-binding site-SEAP-NPT plasmid designed for monitoring the NF-κB activity modulated by various synthetic chemicals or natural products. The plasmid permits expression of the reporter gene, i.e., secreted alkaline phosphatase (SEAP) gene (16-19) in response to the level of NF-κB activity and contains the neomycin phosphotransferase (NPT) gene for the dominant selective marker of geneticin activity resistance (20-22). Human HaCaT cell transfectants were found to release the SEAP enzyme into the culture medium in a time-dependent manner by fluorescent detection assay method. We have further tested whether this assay system could be used for the quantitative measurement of the level of NF-κB activity induced by known antioxidants such as N-acetyl-L-cysteine (NAC) (23-25) and vitamin C (26, 27), or NF-κB stimulator, the phorbol 12-myristate 13-acetate (PMA) (28, 29).

We report that this HaCaT cell-based reporter system in response to NF-κB activity could be used for the screening of anti-inflammatory agents for the dermatological purpose.

MATERIALS AND METHODS

Materials

The enzymes Hind III, Hinc II, Kpn I, Pst I, and Mlu I were purchased from New England Biolabs (Beverly, MA). Geneticin (antibiotic G-418 sulfate) and cell culture media were from Gibco BRL (Grand Island, NY). pNF-κB-SEAP plasmid and Great EscAPe Fluorescence detection kit were obtained from Clontech Laboratories (Palo Alto, CA). pCI-neo plasmid was from Promega (Piscataway, NJ). T4 DNA ligase was from United States Biochemicals (Cleveland, OH). Oligonucleotide PCR primers, 5'- C G A C G C G T C G C G C A G C A C C A T G G C C T G - 3' and 5'- C A C A C A A C C A A A A A A C A C A C G C T G C G C A G C - 3' for the amplification of neomycin resistance gene in pCI-neo plasmid were prepared on a DNA synthesizer (Applied Biosystems Inc., Model 380B) and purified by polyacrylamide gel electrophoresis. N-acetyl-L-cysteine (NAC), vitamin C, phorbol 12-myristate 13-acetate (PMA), and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (St. Louis, MO), and the other chemicals and solvents used in this study were from Aldrich Chemical Co. (Milwaukee, WI). Vitamin C and NAC were directly dissolved in the culture media and PMA first dissolved in DMSO and then made to a final concentration in culture media of 0.1 %.

Cell Culture

Human HaCaT cells were originally obtained from Dr. Norbert E. Fusenig (German Cancer Research Center, Heidelberg, Germany) and subcloned by this laboratory were used. Cell cultures were maintained at subconfluence in a 95 % air, 5 % CO_2 humidified atmosphere at 37 °C. The medium used for routine subcultivation was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were counted with a hemocytometer and the number of viable cells was determined by trypan blue dye exclusion. Transfected human

HaCaT cells were cultured as described with 500 μg/ml of geneticin (100 mg/ml) for selection and maintenance of stable transformants (20-22).

Plasmid Construction

The neomycin selectable marker (1369 bp) containing SV40 enhancer and early promoter, SV40 minimum origin of replication, coding region of neomycin phosphotransferase and synthetic polyadenylation signal sequence was generated from two primers by polymerase chain reaction (PCR). The PCR was carried out in pCl-neo vector (10 ng), pairs of primers (100 pmols of each), dNTPs (0.2 mM), reaction buffer (1 X), and Taq polymerase (2.5 units) in 100 μl of a final volume. The annealing temperature for PCR was 55 °C, and extension times were 90 s at 72 °C. PCR reactions were run for total of 30 cycles. The PCR products recovered from the gel were analyzed by restriction mapping with Hind III or Pst I, and then the recombinant plasmid pNF-κB-SEAP-NPT was prepared by ligating the Mlu I-linearized pNF-κB-SEAP plasmid with Mlu I-trimmed PCR products overnight at 4 °C. DH5α transformants with this recombinant plasmid were further analyzed with restriction fragments generated by Hind III, Mlu I or Kpn I to confirm that pNF-κB-SEAP-NPT (10 μl) was cloned in the correct orientation. DNA sequencing was also performed for the positive identification with Prism DyeDeoxy Termination Cycle sequencing kit and with automated DNA sequencer.

Cell Transfection

Transfection was carried out according to the procedure for stable transfection of adherent cells (30-32) with slight modifications. Briefly, the six-well Costar plates (35 mm, Cambridge, MA) were seeded with $3x10^5$ HaCaT cell per well in 2 ml of media and incubated overnight. Cells were rinsed twice with serum-free media and exposed with complexes containing pNF-κB-SEAP-NPT (6 μg/100 μl) and lipofectamine (25 μg/100 μl, Life Technologies) in 1 ml serum-free media for 2 h. After 48 h of incubation in a complete media without selective pressure, transfected cells were maintained with geneticin for selecting stably transfected cells.

Reporter (SEAP) Gene Assay

Single cell-derived stable transfectants ($3x10^6$ cells) were plated in 5 ml of T-25 flask, and the media was decanted 24 h later. At this time, cells were washed twice with phosphate-buffered saline (PBS), and incubations were initiated by addition of new media. Chemicals were added to the culture medium after 24 h of incubations. Aliquots (25 μl) of medium from a control or chemical-treated cultures were taken at 0, 3, 20, 24, 48, and 72 h, heated at 65 °C for 5 min to eliminate the endogeneous alkaline phosphatase activity, and used immediately or stored at –20 °C. Mixtures, containing dilution buffer (25 μl), assay buffer (97 μl), culture media (25 μl), and 4-methylumbellifery phosphate (MUP, 1 mM, 3 μl) in the 96 well plates were incubated for 60 min in the dark at room temperature. Fluorescence from the product of the SEAP/MUP were measured using a 96 well plate fluorometer (Molecular Devices, F max) by excitation at 360 nm and measuring light emission at 449 nm .

RESULTS AND DISCUSSION

To measure the quantitative changes of NF-κB expression in human skin cells induced by external stimuli, a stable assay system was developed using human HaCaT cells transfected with the pNF-κB-SEAP-NPT plasmid. The plasmid permits expression of the secreted alkaline phosphatase (SEAP) reporter gene in response to the NF-κB activity and contains neomycin phosphotransferase (NPT) gene for the geneticin resistance in host cells

(Fig. 1). The HaCaT cell line is a human skin equivalent, which spontaneously transformed and immortalized human keratinocytes and has been shown to be normal differentiation and similar to normal keratinocyte cell line (33, 34). Therefore, this cell line was selected to monitor the activation of NF-κB in human keratinocytes.

Several assay methods have been used for measuring the cellular or tissue-level NF-κB activity such as electrophoretic mobility shift assay, nuclear translocation of p65, and transactivation assays for the measurement of NF-κB regulated protein expression (35). The secreted nature of SEAP provides several advantages for the choice of this enzyme as a transcription reporter (18, 19). It is not necessary to prepare cell lysates to assay for the enzyme activity, and the kinetics of reporter gene expression can be simply measured by repeatedly sampling the cell culture. Since, unlike most endogenous alkaline phosphatases, SEAP is extremely heat-stable, background noise due to endogenous alkaline phosphatases could be virtually eliminated by preheating the aliquots of culture medium at 65 °C.

The full-length DNA fragment of neomycin selectable marker containing SV40 enhancer and early promoter, neomycin phosphotransferase, and poly (A) tail was recloned from the pCl-neo plasmid by PCR using the primers (see Materials and Methods). Electrophoretic analysis of the PCR product treated with Hind III or Pst I confirmed the presence of expected subfragments of the 1369 bp full length marker, 960 and 410 bp for Hind III, and 730 and 640 bp for Pst I (Fig. 2A). The intact neomycin selectable marker fragment and pNF-κB-SEAP plasmid were treated with Mlu I to make cohesive-ended and linearized, respectively and recovered from the gel. These two DNA fragments were ligated to form a pNF-κB-SEAP-NPT plasmid and the resulting construct was tested for the right orientation of the marker fragment by restriction mapping with Mlu I, Kpn I and Hind III (Fig. 2B). The chosen pNF-κB-SEAP-NPT was transfected into human HaCaT cells by 2 h exposure of the cell monolayer to DNA complexes formed with lipofectamine reagent in a serum-free media.

To test whether the human HaCaT transfectant cells secreted the reporter enzyme, SEAPs were assayed using a fluorescent detection method that can detect as little as 10^{-12} g of the enzyme secreted in the culture media. SEAPs can be detected as early as 3 h after incubation and these cells continuously released the enzymes into the culture medium in a time-dependent manner until 72 h (Figs. 3A and 3B). Antioxidants, e.g., vitamin C and N-acetyl-L-cysteine (NAC) have been known as inhibitors of NF-κB induction (23-27). Treatment of transfected cells with vitamin C (10 mM) and NAC (10 mM) inhibited the NF-κB activation up to 25 % and 50 % compared to a control, respectively and this inhibition increased in a concentration-dependent manner as measured by fluorescent detection assay method (Figs. 3A and 3B). On the other hand, phorbol 12-myristate 13-acetate (PMA, 600 nM) known as a stimulator of NF-κB activation (28, 29) demonstrated that it increased the reporter enzyme activity up to 70 % compared to a control in a concentration-dependent manner (Fig. 4). These results confirmed that the pNF-κB-SEAP-NPT-transfected human HaCaT cells were stable and consistent in response to the environmental stimuli. Therefore, this assay system could be useful for the determination of quantitative measurement of NF-κB activity in the human keratinocytes and the screening of anti-inflammatory agents in vitro for dermatological purpose.

Acknowledgment

This research was supported by a grant of the Korea Health 21 R & D Project, Ministry of Health & Welfare, Republic of Korea (HMP-98-D-0049).

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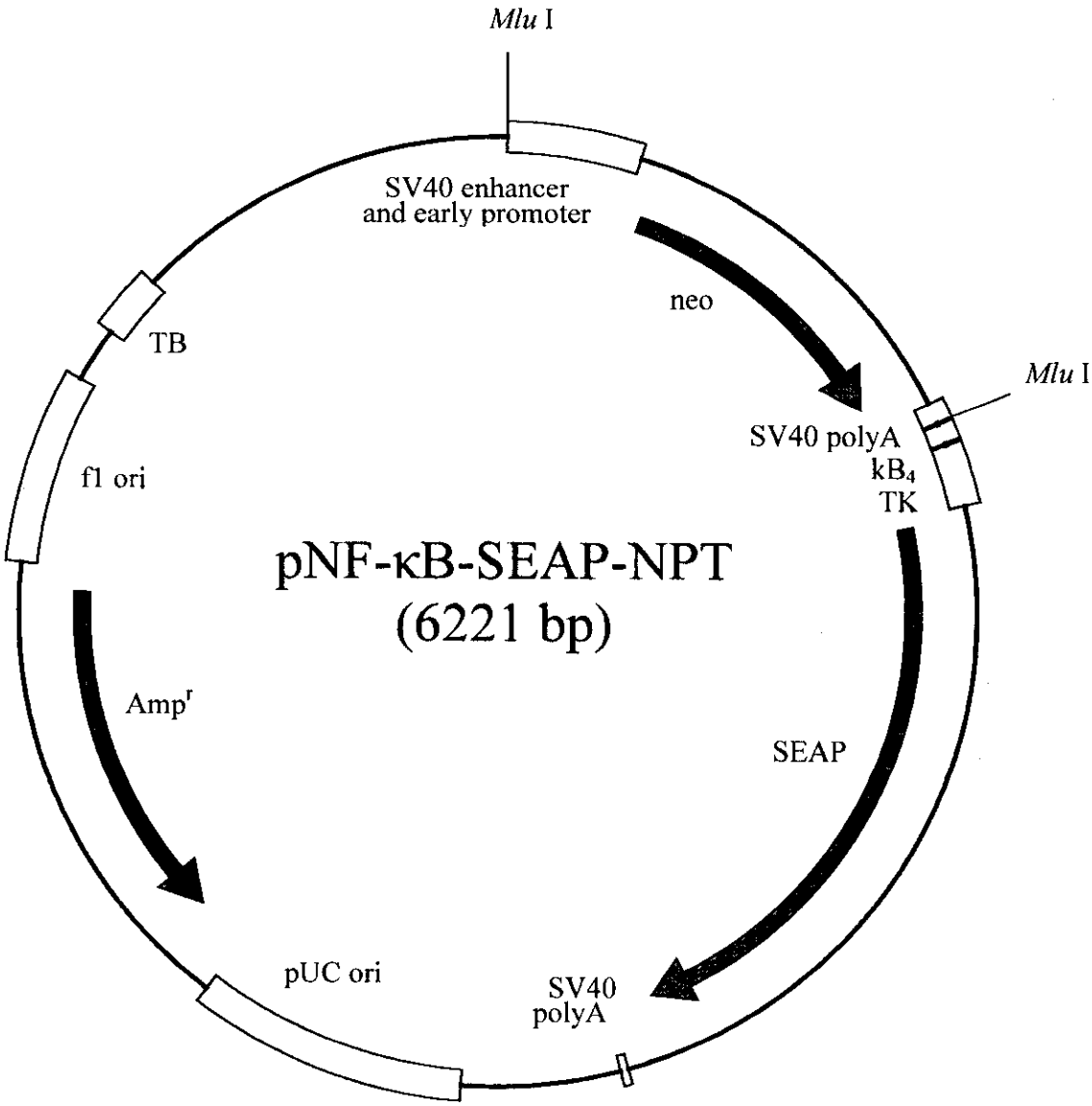
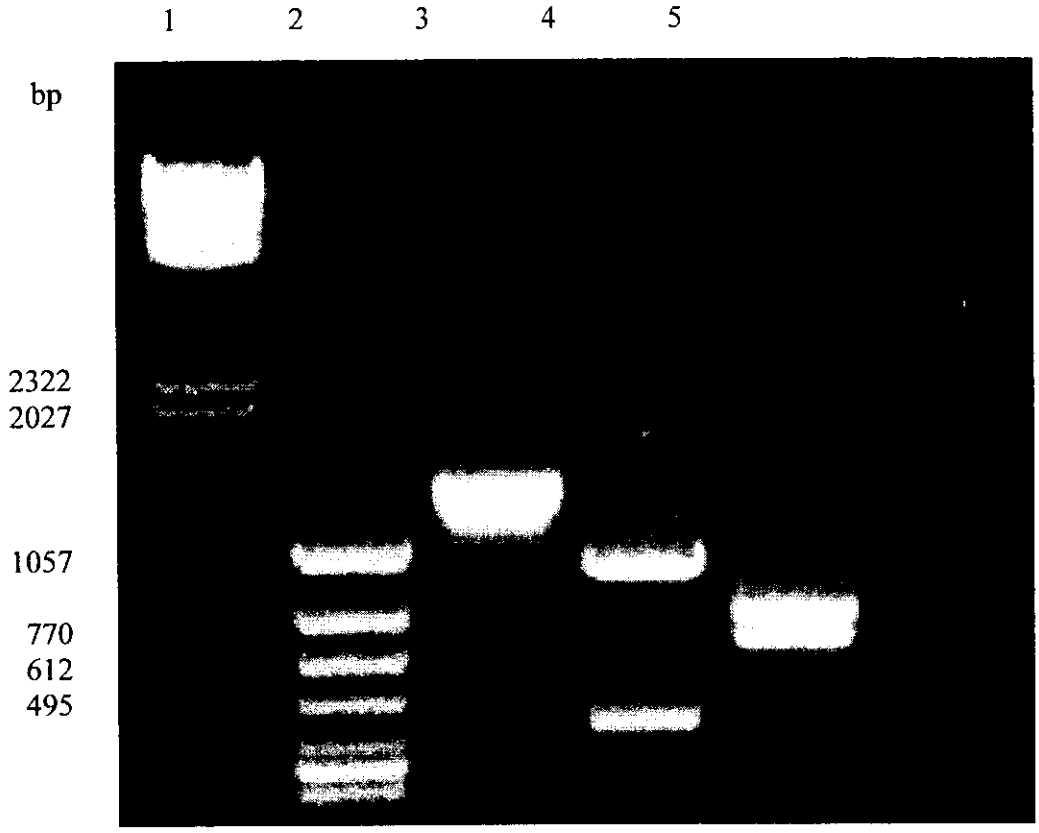


Fig. 1. Diagram of pNF-κB-SEAP-NPT plasmid

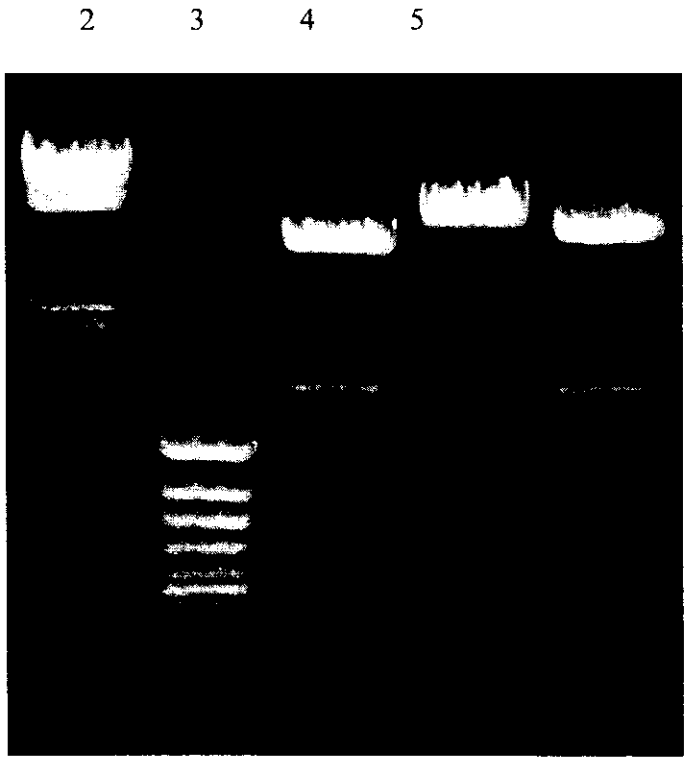
A) PCR amplification of neomycin phosphotransferase gene (NPT)



Lane 1 ; Hind III digest of λ phage DNA as molecular weight markers
Lane 2 ; Hinc II digest of ϕX174 molecular weight markers
Lane 3 ; PCR product of NPT, 1369 bp
Lane 4 ; Hind III digest of NPT, 960 bp and 410 bp
Lane 5 ; Pst I digest of NPT, 730 bp and 640 bp

Fig. 2. Electrophoretic analysis of pNF-κB-SEAP-NPT

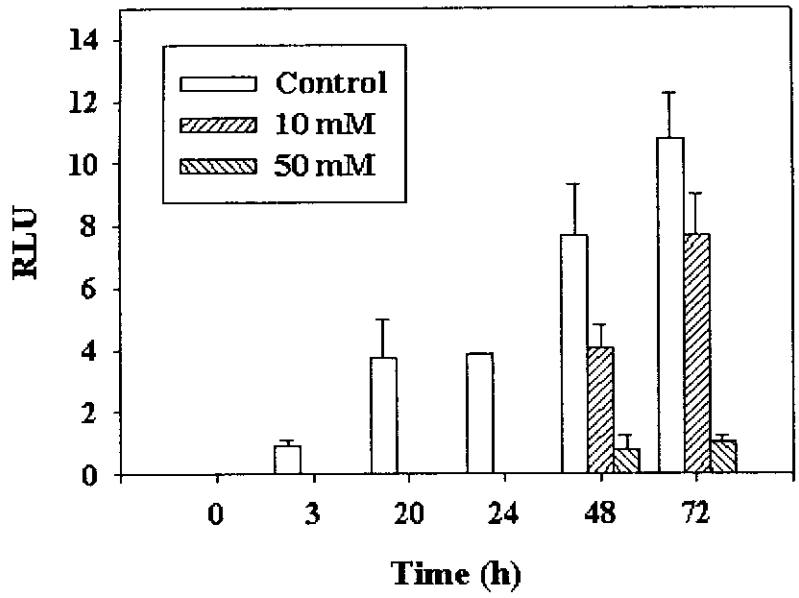
B) Restriction fragments of pNF-κB-SEAP-NPT



Lane 1 ; Hind III digest of λ phage DNA
Lane 2 ; Hinc II digest of ϕX174
Lane 3, 4, 5 ; Mlu I, Kpn I, and Hind III digest of the recombinant plasmid, respectively

Fig. 2. Electrophoretic analysis of pNF-κB-SEAP-NPT

A) Vitamin C effect



B) N-acetyl-L-cysteine effect

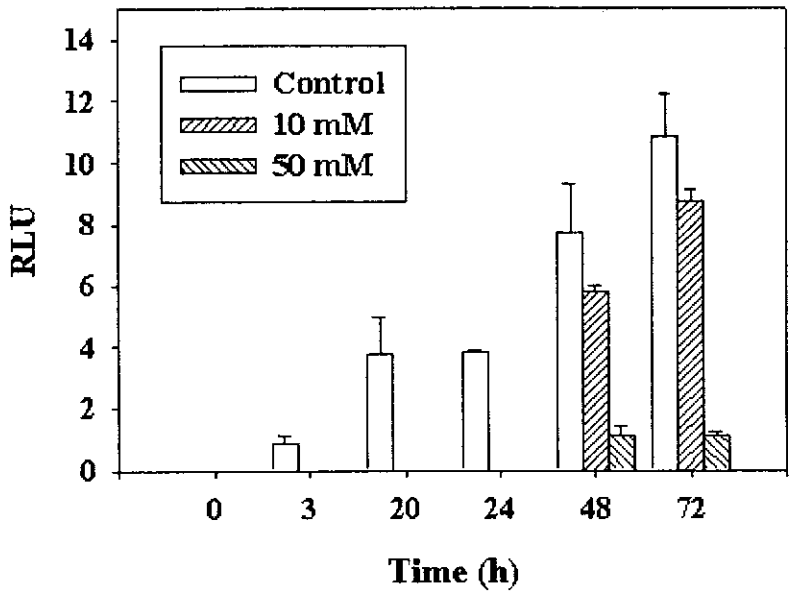
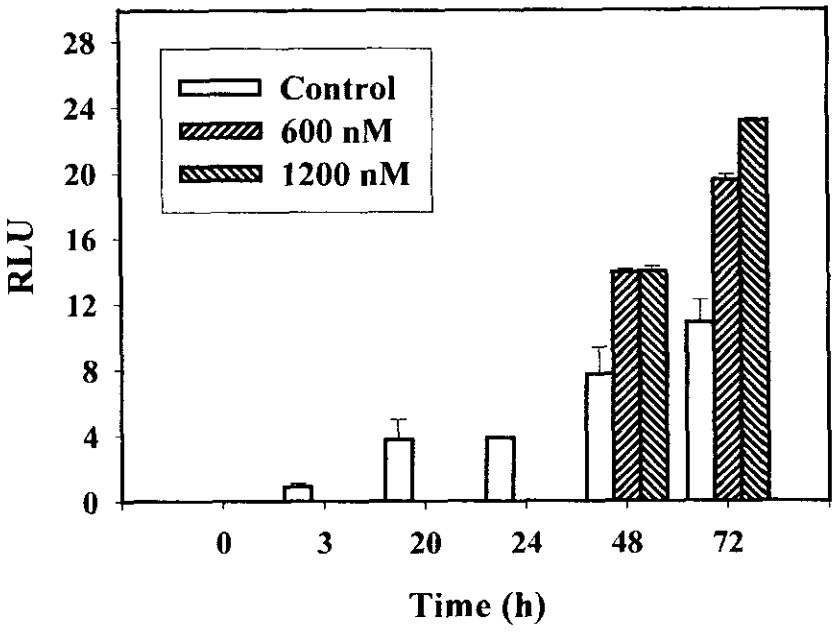


Fig. 3. Dose-dependent downregulation of cellular NF-κB activity by antioxidants in transfectant HaCaT cells

Fig. 4. Dose-dependent upregulation of cellular NF-κB activity by PMA in transfectant



HaCaT cells.

Chemicals were added to the culture medium on 24 h of incubation and SEAP activities were measured 24 and 48 h after exposure of chemicals, respectively. Each value represents the mean ± SE of three determinations. RLU stands for relative light units.