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Inhibition of ultraviolet B-mediated activation of nuclear factor κB in normal human epidermal keratinocytes by green tea Constituent (-)-epigallocatechin-3-gallate

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Epigallocatechin-3-gallate (EGCG), the major constituent of green tea, possesses significant anti-inflammatory and cancer chemopreventive properties. Studies have shown the photochemopreventive effects of green tea and EGCG in cell culture, animal models, and human skin. The molecular mechanism(s) of photochemopreventive effects of EGCG are incompletely understood. We recently showed that EGCG treatment of the normal human epidermal keratinocytes (NHEK) inhibits ultraviolet (UV)B-mediated activation of the mitogen-activated protein kinase (MAPK) pathway. In this study, we evaluated the effect of EGCG on UVB-mediated modulation of the nuclear factor kappa B (NF-κB) pathway, which is known to play a critical role in a variety of physiological functions and is involved in inflammation and development of cancer. Immunoblot analysis demonstrated that the treatment of NHEK with EGCG (10–40 μM) for 24 h resulted in a significant inhibition of UVB (40 mJ/cm^2)-mediated degradation and phosphorylation of IκBα and activation of IKKα, in a dose-dependent manner. UVB-mediated degradation and phosphorylation of IκBα and activation of IKKα was also observed in a time-dependent protocol (15 and 30 min, 1, 2, 3, 6, 12 h post-UVB exposure). Employing immunoblot analysis, enzyme-linked immunosorbent assay, and gel shift assay, we demonstrate that EGCG treatment of the cells resulted in a significant dose- and time-dependent inhibition of UVB-mediated activation and nuclear translocation of a NF-κB/p65. Our data suggest that EGCG protects against the adverse effects of UV radiation via modulations in NF-κB pathway, and provide a molecular basis for the photochemopreventive effect of EGCG.
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Introduction

Solar ultraviolet (UV) radiation, particularly its UVB (290–320 nm) spectrum, is the primary cause of skin cancer and other cutaneous pathologies in the human population, more so in Caucasian individuals (Mukhtar and Elmets, 1996; Greenlee *et al*., 2000). Experimental studies have shown that solar UV radiation elicits many adverse biological effects in the skin, including hyperpigmentation, erythema, photoaging, immunosuppression, and cancer (Goihman-Yahr, 1996; Ananthaswamy *et al*., 1997; DeGruijil, 1999; Afaq and Mukhtar, 2001). UV radiation to mammalian skin is known to alter cellular function via DNA damage (Eller *et al*., 1997; Vink *et al*., 1997; Katiyar *et al*., 2000), generation of reactive oxygen species (ROS) (Morita *et al*., 1997; Scharffetter-Kochanek *et al*., 1997; Katiyar *et al*., 2001a, b), and the resultant alterations in a variety of signaling events (Gilchrest *et al*., 1996; Knebel *et al*., 1996; Rosette and Karin, 1996; Iordanov *et al*., 1997; Chen *et al*., 1999; Lefort *et al*., 2001; Pfundt *et al*., 2001). The endogenous antioxidant capacity of the skin is a major determinant in its response to UV-induced oxidative stress-mediated skin damage. However, an inefficient endogenous antioxidant ability of the skin as a result of increased generation of ROS leads to cutaneous damage. Regular intake of dietary antioxidants or treatment of the skin with products containing antioxidant ingredients may be a useful strategy for the prevention of UV-mediated cutaneous damages (Ames, 1983; Block, 1993; Afaq *et al*., 2002).

Green tea is the most popular beverage consumed by more than two-thirds of the world population and is a rich source of polyphenolic antioxidants. Based on several *in vitro* and *in vivo* studies, polyphenols present in green tea, most notably its major constituent epigallocatechin-3-gallate (EGCG) (Figure 1), have demonstrated remarkable antioxidant activity and photochemopreventive effects (Lu *et al*., 2000; Elmets *et al*., 2001; Kim *et al*., 2001; Katiyar *et al*. 2001a, b; Katiyar and Mukhtar, 2001; Nomura *et al*., 2001). We demonstrated that topical application of EGCG to human skin before a single UV exposure of 4 × minimal erythema dose markedly decreased UV-induced production of hydrogen peroxide (H_2O_2) and nitric oxide in a time-dependent manner (Katiyar *et al*., 2001b). EGCG

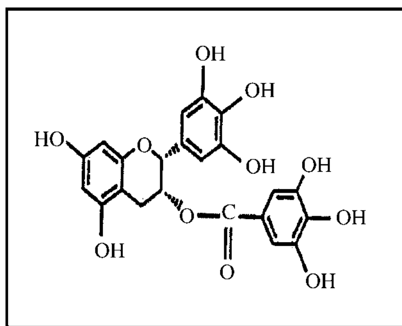

Figure 1 Chemical structure of EGCG

was also found to restore UV-induced decrease in glutathione level, afford protection against UV-induced alteration in glutathione peroxidase and catalase activities, and lipid peroxidation (Katiyar *et al.*, 2001b). Other studies from our laboratory have shown that green tea polyphenol (GTP) or EGCG protects against UV-mediated cutaneous damages in animal models (Katiyar and Mukhtar, 2001). Recently, we demonstrated that pretreatment of EGCG to normal human epidermal keratinocytes (NHEK) results in a significant inhibition of UVB-mediated increased production of H_2O_2 and activation of the mitogen-activated protein kinase (MAPK) signaling pathway (Katiyar *et al.*, 2001a). The molecular mechanism(s) by which EGCG imparts its chemopreventive effects against UV-mediated damages are not completely understood. Since nuclear factor kappa B (NF-κB) is regarded to play a critical role in the regulation of a variety of genes responsible for cell growth/cell death, we designed this study to define the involvement of the NF-κB pathway in the photochemopreventive effects of EGCG. The data from this study clearly demonstrated the critical role of the NF-κB pathway in the photochemopreventive effect of EGCG.

Results

EGCG treatment inhibits UVB-induced activation and translocation of NF-κB in NHEK

Studies have shown that UV radiation results in an activation of NF-κB (Fisher *et al.,* 1996; Breuer-McHam *et al.,* 2001; Hong *et al.,* 2001). The aim of this study was to investigate whether pretreatment of NHEK with EGCG inhibits UVB-induced activation and nuclear translocation of NF-κB/p65. In the initial studies, we found that a UVB dose of 40 mJ/cm^2 significantly activates NF-κB in NHEK (data not shown). Therefore, in all subsequent experiments we used this dose of UVB exposure. For dose-dependent study with EGCG, an incubation time of 6 h after UVB exposure was chosen, because this time point allowed optimal and reproducible NF-κB/p65 activation and nuclear translocation (Figure 2b). Employing ELISA, EMSA, and immunoblot analysis, we investigated the effect of pretreatment of NHEK with EGCG against UVB-induced activation and nuclear translocation of NF-κB/p65. Employing ELISA, we found that pretreatment of NHEK with EGCG (10, 20, 40 μM) inhibited UVB-induced activation and nuclear translocation of NF-κB/p65 in a dose-dependent manner. EGCG was found to inhibit significantly UVB-induced NF-κB/p65 activation and nuclear translocation in a dose-dependent fashion (Figure 2a). In a time-dependent study, we observed that UVB-induced activation of NF-κB/p65 started as early at 30 min post-UVB, with the maximum activation at 6 h post-UVB, that persisted till 12 h post-UVB exposure. For a time-dependent study, we used 10 μM dose of EGCG, which was found to inhibit NF-κB/p65 activation and its nuclear translocation in an UVB-time-dependent manner (Figure 2b). We further confirmed our results by employing Western blot analysis. As is evident from Western blot analysis data and the relative density of bands, we found that EGCG pretreatment of NHEK resulted in a dose- and time-dependent inhibition of UVB-induced activation and translocation of NF-κB/p65 (Figure 3a, b). Further, we performed EMSA to investigate the effect of EGCG treatment on NHEK on UVB-induced NF-κB activation. As shown in Figure 4a, UVB treatment (Lane 8) resulted in a dramatic increase of NF-κB DNA-binding activity in comparison to control (Lane 4) and EGCG (10, 20 and 40 μM) alone treated (Lanes 5–7) groups. The induction of NF-κB DNA-binding activity coincided with the degradation of IκBα (Figure 5a) and activation of IKKα (Figure 7a). Pretreatment of NHEK with EGCG significantly inhibited UVB-induced NF-κB DNA-binding activity in a dose-dependent manner (Lanes 9–11). We also performed a time-dependent study using a single dose of EGCG (10 μM). Following 24 h of pretreatment, the cells were exposed to UVB, and at different time points, post-UVB exposure EMSA was done. We observed that UVB exposure resulted in an increased NF-κB DNA-binding activity in a time-dependent manner (Lanes 6, 8, 10, 12, 14, 16 and 18). Treatment of NHEK with EGCG resulted in a significant inhibition against UVB-induced NF-κB DNA-binding activity (Lanes 5, 7, 9, 11, 13, 15, 17 and 19) (Figure 4b). Further, to confirm our EMSA results, we performed supershift assay to investigate the effect of EGCG supplementation to NHEK on UVB-induced NF-κB/p65 activation with antibody (NF-κB/p65). We found that EGCG significantly inhibited UVB-induced activation and nuclear translocation of NF-κB/p65 (data not shown).

EGCG treatment inhibits UVB-induced degradation of IκBα in NHEK

UVB exposure to NHEK resulted in the degradation of IκBα protein, and because of this degradation NF-κB/p65 is activated and translocated into the nucleus (Figures 2–4). We were interested to see whether the degradation of IκBα is inhibited by EGCG, which will in turn inhibit the activation and translocation of NF-κB/p65. Western blot analysis and relative density of each

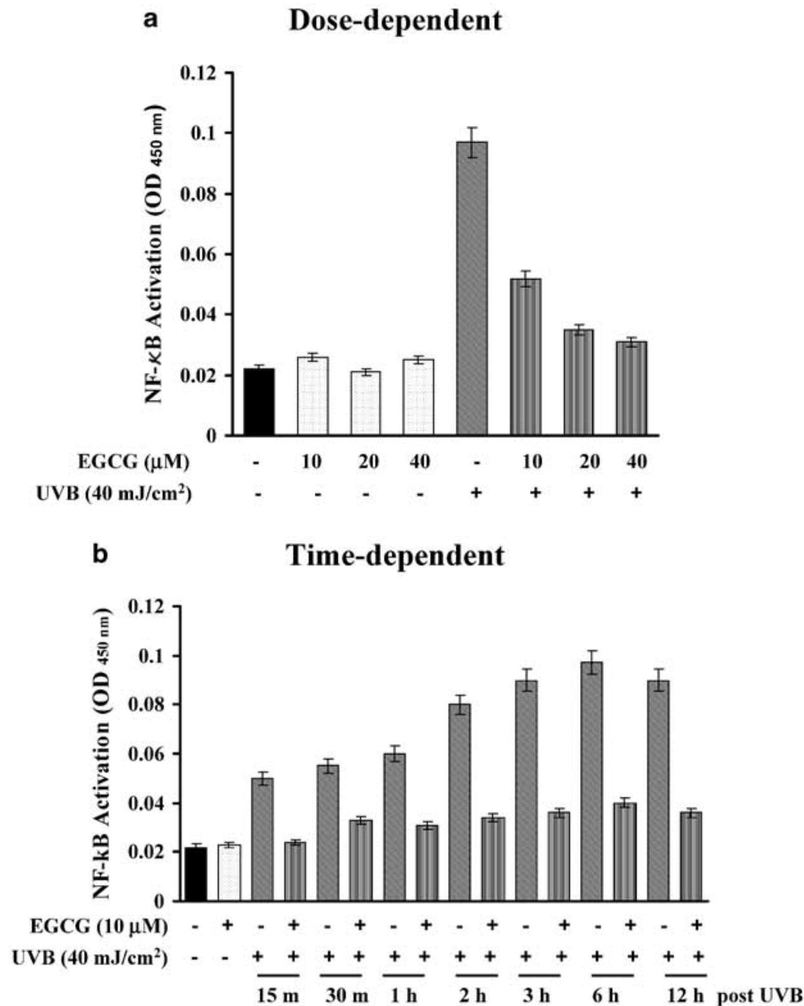

Figure 2 Inhibitory effect of EGCG on UVB-induced activation of NF-κB/p65. For dose-dependent studies, cells were pretreated with EGCG (10, 20 and 40 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps. At 6 h post-UVB, the cells were harvested and nuclear lysate was prepared (**a**). For time-dependent studies, cells were pretreated with EGCG (10 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps (**b**). At different time points, cells were harvested and nuclear lysate was prepared for enzyme-linked immunosorbent assay as detailed in 'Materials and methods'. The data shown here are from one representative experiment repeated two times with similar results

band revealed that treatment of NHEK with EGCG did not result in the degradation of IκBα protein (Figure 5a). When the cells were subjected to UVB exposure, it resulted in almost 55% degradation in IκBα protein, and this degradation of IκBα protein was almost completely inhibited in the keratinocytes pretreated with EGCG (10, 20, 40 μM), when compared to control. For the time-dependent study, we observed that pretreatment of NHEK with 10 μM EGCG resulted in significant inhibition against UVB-induced degradation of IκBα protein, as evident by Western blot analysis and the relative density of each band (Figure 5b).

EGCG treatment inhibits UVB-induced phosphorylation of IκBα in NHEK

NF-κB translocation to the nucleus is preceded by the phosphorylation and the proteolytic degradation of IκBα. We further determined whether UVB exposure affects the phosphorylation of IκBα protein. As shown by Western blot (Figure 6a, b), UVB exposure induced a prominent increase in the phosphorylation level of IκBα protein at Ser32. In the dose-dependent study, pretreatment of NHEK with EGCG (10, 20, 40 μM) resulted in a significant inhibition in UVB-mediated increase in the phosphorylation of IκBα protein. Western blot analysis and relative density of these bands showed that the phosphorylation of IκBα protein was almost completely inhibited at 20 and 40 μM concentration of EGCG (Figure 6a). In the time-dependent study, UVB-induced phosphorylation of IκBα protein was increased after 15 min post-UVB exposure and remained elevated till 12 h. We observed that pretreatment of NHEK with EGCG drastically decreased UVB-induced phosphorylation of IκBα protein, as evident by Western blot analysis and the relative density of the bands (Figure 6b).

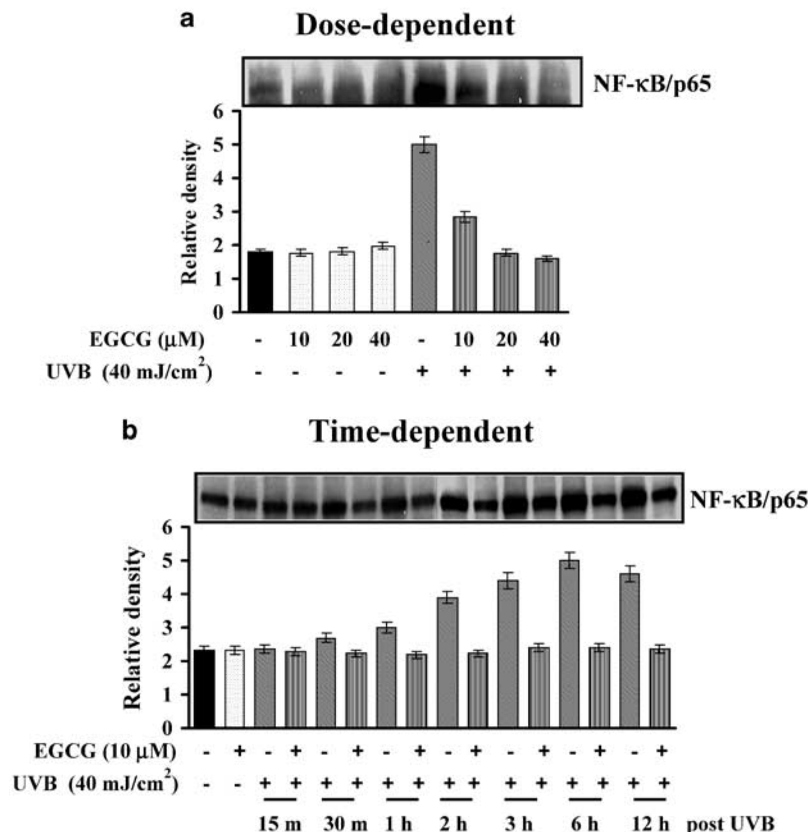

Figure 3 Inhibitory effect of EGCG on UVB-induced activation of NF-κB/p65. For dose-dependent studies, cells were pretreated with EGCG (10, 20 and 40 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps. At 6 h post-UVB, the cells were harvested and nuclear lysate was prepared (**a**). For time-dependent studies, cells were pretreated with EGCG (10 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps (**b**). At different time points, cells were harvested and nuclear lysate was prepared for Western blot analysis as detailed in 'Materials and methods'. The data shown here are from one representative experiment repeated three times with similar results

EGCG treatment inhibits UVB-induced activation of IKKα in NHEK

IκB kinase (IKK)α activity has been shown to be necessary for IκBα protein phosphorylation/degradation induced by UVB radiation. To evaluate the possible inhibitory mechanism of EGCG on IκBα protein degradation, we measured IKKα protein level. Western blot analysis showed that UVB (40 mJ/cm^2) radiation resulted in the activation of IKKα in NHEK. Pretreatment of NHEK with EGCG prior to UVB significantly inhibits UVB-induced activation of IKKα in a dose-dependent manner (Figure 7a). For the time-dependent study, we observed that 10 μM dose of EGCG was found to significantly inhibit UVB-induced activation of IKKα in a time-dependent manner (Figure 7b).

Discussion

Based on considerable evidence accumulated within the last few years, currently much attention is focused on the use of naturally occurring botanicals for their potential preventive effect against UV radiation-mediated damages, which we refer to as 'photochemopreventive effects' (Ahmad and Mukhtar, 2001). In fact, in recent years, a variety of naturally occurring agents, which are antioxidant in nature, are being widely used in skin care products and even in customized diets and beverages. Among these botanicals, green tea, obtained from the tea leaves of the plant *Camellia sinensis*, a popular beverage consumed worldwide, contains many polyphenolic flavonoids. Green tea is being supplemented in a variety of skin care products such as lotion, sunscreens, shampoos, and other dietary items such as cookies, candies, ice creams, etc.

Solar UV radiation is the most prominent and ubiquitous carcinogen in our environment and skin is its major damaging target. Studies in animal models have shown that UV radiation can act both as a tumor initiator and tumor promoter (Kligman *et al*., 1980; Katiyar *et al*., 1997a). Solar UV radiation is a potent inducer of ROS, which are responsible for different types of skin manifestation via different pathways/

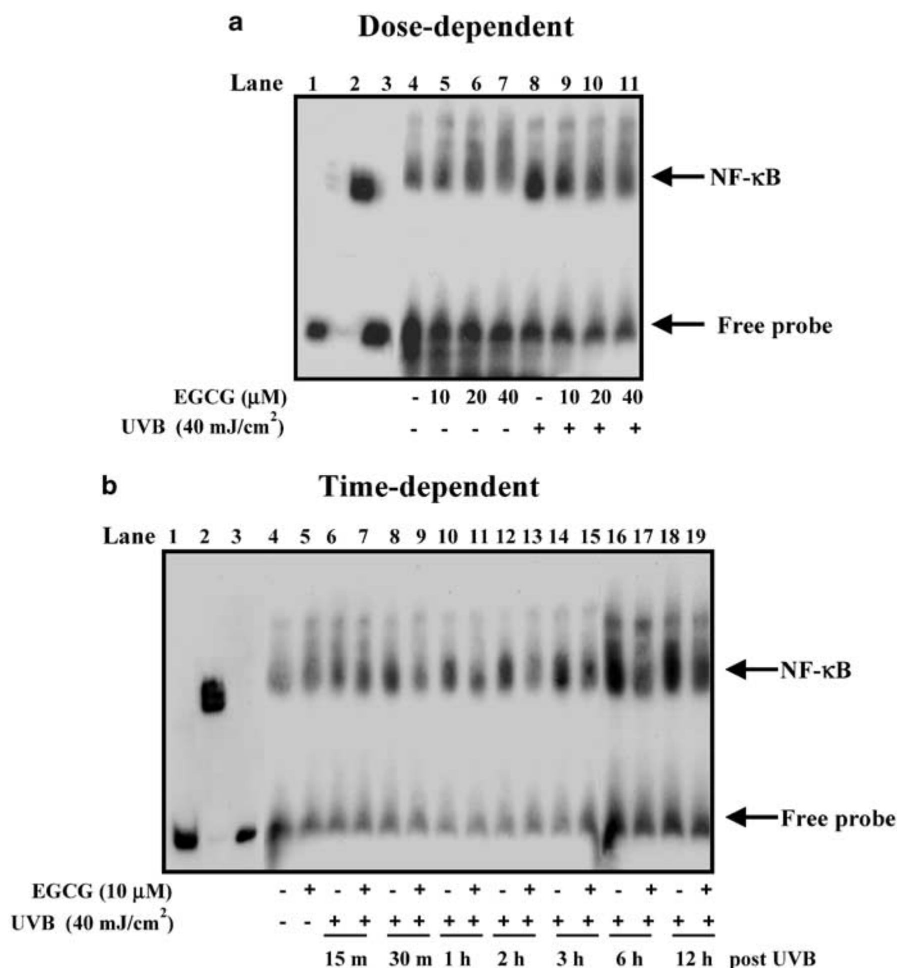

Figure 4 Inhibitory effect of EGCG on UVB-induced activation of NF-κB. For dose-dependent studies, cells were pretreated with EGCG (10, 20 and 40 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm²) delivered through FS40 lamps. At 6 h post-UVB, the cells were harvested and nuclear lysate was prepared (**a**). For time-dependent studies, cells were pretreated with EGCG (10 μM) for 24 h after which the medium was removed and cells were washed once with PBS and than fresh PBS was added and cells were exposed to UVB (40 mJ/cm²) delivered through FS40 lamps (**b**). At different time points, cells were harvested and nuclear lysate was prepared for electrophoretic mobility shift assay as detailed in 'Materials and methods'. The data shown here are from one representative experiment repeated two times with similar results. Lane 1: biotin-EBNA control DNA only; lane 2: biotin-EBNA control DNA and the EBNA extract; lane 3: biotin-EBNA control DNA and the EBNA extract and a 200-fold molar excess of unlabeled EBNA DNA

mechanisms. These ROS cause modifications of protein that results in functional changes of enzymatic proteins (Stadtman, 1992; Katiyar *et al.* 2001b). ROS are responsible for photoxidative damage on nucleic acids, lipids, and activation of transcription factors that control the expression of genes involved in tumor promotion (Goihman-Yahr, 1996; Naylor, 1997; Griffiths *et al.*, 1998). UV radiation in the keratinocytes is directly involved in cyclobutane pyrimidine dimer formation, which is implicated in photocarcinogenesis (Kripke *et al.*, 1992; Burren *et al.*, 1998). Our studies have shown that GTP or EGCG prevents against UV radiation-mediated damages in *in vitro* and *in vivo* models as well as in human skin (Katiyar *et al.*, 1997b, 2001a, b; Katiyar and Mukhtar, 2001). However, the mechanism of the photochemopreventive effect of EGCG is not clear.

In the present investigation, the effect of EGCG on the pattern of NF-κB/p65 activation and its translocation induced by UVB radiation in NHEK was defined in order to assess its mechanism of action. The NF-κB transcription factor has emerged as a central component of the inductile cellular signaling machinery that serves as an important regulatory role in inflammation, immunity, cell proliferation, and oncogenesis (Thanos and Maniatis, 1995; Baeuerle and Baltimore, 1996; Baldwin, 1996; Maniatis, 1997). NF-κB is activated by oxidant including H_2O_2 (Ikeda *et al.*, 2002) and by agents that generate ROS such as UV radiation (Vile *et al.*, 1995; Legrand-Poels *et al.*, 1998; Helenius *et al.*, 1999), phorbol myristate acetate (Haas *et al.*, 1998; Majumdar *et al.*, 2002), and tumor necrosis factor-α (TNF-α) (Schreck *et al*, 1991; Devary *et al*, 1993). Compounds that scavenge ROS inhibit NF-κB activa-

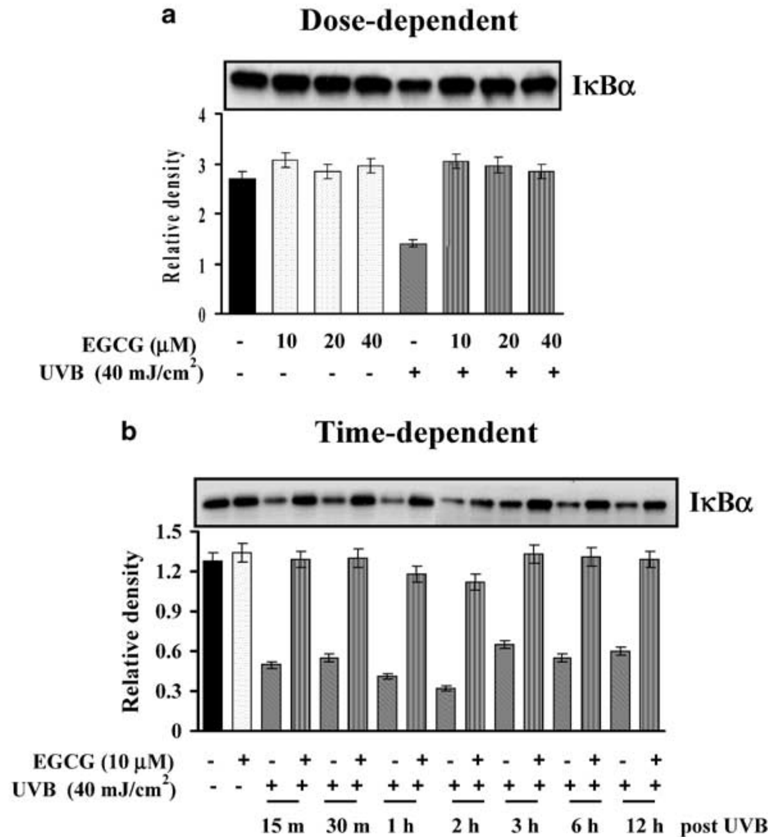

Figure 5 Inhibitory effect of EGCG on UVB-induced degradation IκBα. For dose-dependent studies, cells were pretreated with EGCG (10, 20 and 40 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps. At 6 h post-UVB, the cells were harvested and cytosolic lysate was prepared (**a**). For time-dependent studies, cells were pretreated with EGCG (10 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps (**b**). At different time points, cells were harvested and cytosolic lysate was prepared for Western blot analysis as detailed in 'Materials and methods'. The data shown here are from one representative experiment repeated two times with similar results

tion (Saliou *et al.,* 1999). Studies have shown that EGCG possesses several-fold higher antioxidant activity than vitamin C and vitamin E (Rice-Evans, 1999). Previously, we have demonstrated that UVB exposure to NHEK cells results in an increased production of H_2O_2, which in turn activates MAPK signaling pathways. Supplementation of EGCG to NHEK prior to UVB exposure significantly inhibits UVB-induced increased production of H_2O_2 and activation of MAPK signaling pathways (Katiyar *et al*., 2001a). Ahmad *et al.* (2000) have demonstrated that EGCG inhibited constitutive expression and TNF-α-mediated activation of NF-κB in cancer cells (A431) more efficaciously than in normal cells (NHEK). EGCG inhibited TPA-induced transcriptional activity of NF-κB and its DNA binding in JB6 mouse epidermal cell lines, which could be correlated with the blockage of phosphorylation of IκBα at Ser32 (Nomura *et al*., 2000). EGCG also inhibited TNF-α gene expression and okadaic acid-induced DNA binding of NF-κB and AP-1 transcriptional factors in KATO III cells (Okabe *et al*., 1999) and in RAW 264.7 cell lines stimulated with lipopolysaccharides (Yang *et al*., 1998).

In the present study, we have demonstrated that NF-κB is activated in NHEK upon exposure to UVB radiation and is translocated to the nucleus when measured by ELISA, NF-κB/p65 DNA-binding by EMSA, and protein expression by Western blot analysis (Figures 2–4). UVB exposure also resulted in an increased phosphorylation (Figure 6a, b) and degradation of IκBα protein (Figures 5a, b). Our results showed a positive correlation between NF-κB/p65 activation and its translocation to the nucleus (Figures 2–4), and phosphorylation and degradation of IκBα in the cytoplasm in NHEK after UVB exposure (Figures 5 and 6). Interestingly, we found that pretreatment of NHEK with EGCG prior to UVB radiation significantly inhibited UVB-induced NF-κB activation (Figures 2–4), and phosphorylation and degradation of IκBα protein (Figures 5 and 6) in a dose- and time-dependent manner. As EGCG blocks IκBα phosphorylation and degradation, this study suggests that the effects of EGCG on NF-κB/p65 are through inhibition of phosphorylation and subsequent proteolysis of IκBα. It is well documented that through a protein–protein interaction, IκBα is bound to NF-κB/p65 and thus prevents migration of

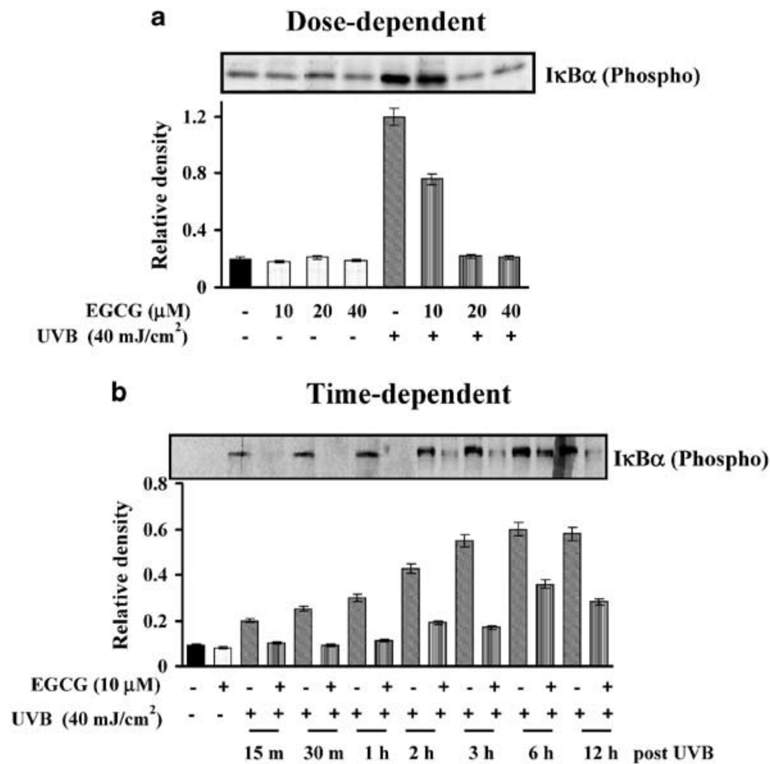

Figure 6 Inhibitory effect of EGCG on UVB-induced phosphorylation of IκBα. For dose-dependent studies, cells were pretreated with EGCG (10, 20 and 40 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps. At 6 h post-UVB, the cells were harvested and cytosolic lysate was prepared (**a**). For time-dependent studies, cells were pretreated with EGCG (10 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps (**b**). At different time points, cells were harvested and nuclear lysate was prepared for Western blot analysis as detailed in 'Materials and methods'. The data shown here are from one representative experiment repeated three times with similar results

NF-κB/p65 into the nucleus (Baeuerle and Baltimore, 1996; Ghosh *et al*., 1998). The IκB is made up of ankyrin repeats, and these ankyrin repeats bind to actin cytoskeleton, both by preventing the migration of NF-κB from the cytoplasm to the nucleus and by blocking the nuclear localization signal of NF-κB (Baeuerle and Baltimore, 1988; Yamazaki *et al*., 2001; Garg and Aggarwal, 2002). Phosphorylation of IκBα, on serine residues 32 and 36, an inhibitory subunit of NF-κB by cytokine-activated IKK, and this phosphorylation precedes rapid degradation of IκBα, which in turn activates NF-κB (Baldwin, 1996; Maniatis, 1997).

The IKK complex is believed to be an important site for integrating signals that regulate the NF-κB pathway. In the present study, we observed that UVB exposure to NHEK resulted in an increase of IKKα protein expression (Figure 7). Further, we found that supplementation of EGCG to NHEK prior to UVB irradiation resulted in a marked inhibition against UVB-induced activation of IKKα protein in a dose- and time-dependent manner (Figures 7a, b). It is only when IκBα is degraded and then NF-κB is transported into the nucleus (Baeuerle and Baltimore, 1996; Stancovski and Baltimore, 1997). At present, there is no information that once NF-κB is inside the nucleus how long it continues to function. However, without any doubt, IκB is the target gene of NF-κB, and an increase in IκB may sequester NF-κB back into the cytoplasm. Recent studies have shown that NF-κB activation plays an important role in cell survival by its ability to block or reduce apoptosis in different cell types, including keratinocytes, apparently by enhancing the expression of antiapoptotic factors (Kaufman and Fuchs, 2000). Fisher *et al*. (1996) have demonstrated the maintenance of NF-κB and AP-1 activation for a longer period (8–24 h) in human skin exposed to UVB. We have demonstrated that topical application of EGCG to human and mouse skin before UV exposure markedly decreased UV-induced production of H_2O_2 and nitric oxide both in the epidermis and dermis, influx of inflammatory cells, myleoperoxidase activity, alteration in endogenous antioxidant, and induction of lipid peroxidation (Katiyar *et al*., 2001b).

In summary, our results suggested that pretreatment of NHEK to EGCG before UVB exposure resulted in a significant dose- and time-dependent inhibition of UVB-mediated activation and nuclear translocation of NF-kB/p65, phosphorylation and degradation of IκBα, and activation of IKKα. Thus, inhibition of IKKα protein expression and IκBα phosphorylation and degradation is involved in EGCG-mediated inhibition of UVB-induced NF-κB/p65 activation and its nuclear

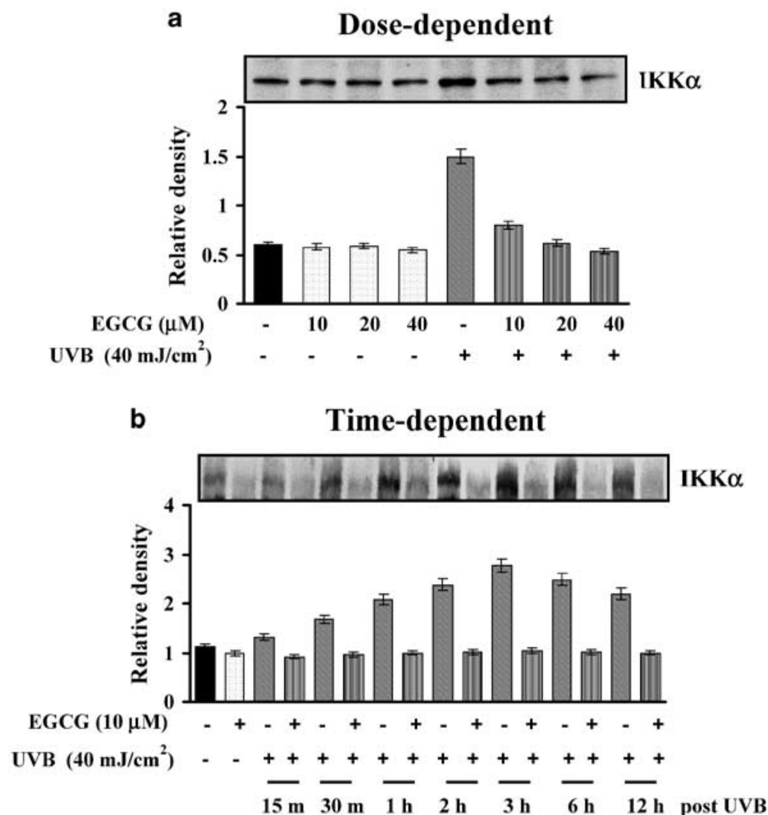

Figure 7 Inhibitory effect of EGCG on UVB-induced activation of IKKα. For dose-dependent studies, cells were pretreated with EGCG (10, 20 and 40 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps. At 6 h post-UVB, the cells were harvested and cytosolic lysate was prepared (**a**). For time-dependent studies, cells were pretreated with EGCG (10 μM) for 24 h after which the medium was removed and cells were washed once with PBS, and then fresh PBS was added and cells were exposed to UVB (40 mJ/cm^2) delivered through FS40 lamps (**b**). At different time points, cells were harvested and cytosolic lysate was prepared for Western blot analysis as detailed in 'Materials and methods'. The data shown here are from one representative experiment repeated two times with similar results

translocation. Therefore, these results suggest that green tea antioxidant EGCG has the potential to protect against the adverse effects of UVB radiation via modulation in the NF-κB pathway, which in turn may protect the cellular targets against UVB-induced damage. Our results provide a molecular basis for the photochemopreventive effect of EGCG and suggest that green tea may be a useful agent against UVB-induced damage for human skin.

Materials and methods

Materials

A purified preparation of EGCG (>98% pure) was kindly provided by Dr Yukihiko Hara of Mitsui Norin Co. Ltd (Shizuoka, Japan). IκBα and IκBα (phospho) antibodies were obtained from New England Biolabs, Inc. (Beverly, MA, USA). NF-κB/p65 antibody was procured from Geneka Biotechnology Inc. (Montreal, Canada). IKKα antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Life Science Inc. (Arlington Heights, IL, USA). TransAM NF-κB/p65 ELISA kit was purchased from Active Motif North America (Carlsbad, CA, USA). Lightshift™ chemiluminiscent EMSA kit was obtained from Pierce (Rockford, IL, USA). The DC BioRad Protein assay kit was purchased from BioRad Laboratories (Herculus, CA, USA). Novex precast Tris-Glycine gels were obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

The NHEK were prepared from neonatal foreskin specimens as described earlier (Katiyar *et al*., 2001a) and primary cultures were maintained in keratinocyte-SFM medium (Life Technologies, Grand Island, NY, USA) supplemented with 0.1 mM calcium, 0.2% (v/v) bovine pituitary extract, EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (5×10^{-7} M), ethanolamine (1×10^{-4} M), phosphoethanolamine (1×10^{-4} M), and amino acid. The cells were maintained at 95% humidity in 5% CO_2 environment at 37°C.

Treatment of cells

EGCG dissolved in PBS (10 mM; pH 7.4) was employed for the treatment of cells. For dose-dependent studies, the cells (70–80% confluent) were treated with EGCG (10, 20 and 40 μM) for 24 h in keratinocyte-SFM medium, after which the medium

was removed and cells were washed with PBS, and then fresh PBS-was added and these EGCG-pretreated cells were exposed to UVB (40 mJ/cm^2) delivered through a bank of FS40 lamps (Westinghouse, Pittsburgh, PA, USA) that emit an energy spectrum with high fluence in the UVB region with a peak at 313 nm. The UV radiations, which are normally not present in natural solar light, were filtered out using Kodacel cellulose film as described earlier (Katiyar *et al*., 2001a). After filtration with a Kodacel film, the majority of the resulting wavelengths of UV radiation were in the UVB range (290–320). The emitted UVB dose was regularly quantitated with an IL-443 phototherapy UVB radiometer (International Light, Newburyport, MA, USA) equipped with an IL SED 240 detector fitted with a W side angle quartz diffuser and an SC5 280 filter. At 6 h post-UVB exposure, the cells were harvested and nuclear and cytosolic lysates were prepared as discussed below. For time-dependent studies, the cells (60–70% confluent) were treated with EGCG (10 μM) for 24 h after which the medium was removed and cells were washed with PBS, and then fresh PBS was added and these EGCG-pretreated cells were exposed to UVB (40 mJ/cm^2). At different time points post-UVB (15 and 30 min, 1, 2, 3, 6, 12 h) exposure, cells were harvested and nuclear and cell lysates were prepared.

Preparation of cytosolic and nuclear lysates

Following treatment of cells with EGCG and/or UVB, the medium was aspirated and the cells were washed twice in PBS (10 mM, pH 7.4). The cells were incubated in 0.4 ml ice-cold lysis buffer (HEPES (10 mM, pH 7.9), KCl (10 mM), EDTA (0.1 mM), EGTA (0.1 mM), DTT (1 mM), PMSF (1 mM)) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA, USA) for 15 min, after which 12.5 μl of 10% Nonidet P-40 was added and the contents were mixed on a vortex and then centrifuged for 1 min (14 000 *g*) at 4°C. The supernatant was saved as cytosolic lysate and stored at –80°C. The nuclear pellet was resuspended in 50 μl of ice-cold nuclear extraction buffer (HEPES (20 mM, pH 7.9), NaCl (0.4 M), EDTA (1 mM), EGTA (1 mM), DTT (1 mM), PMSF (1 mM)) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA, USA) for 30 min with intermittent mixing. The tubes were centrifuged for 5 min (14 000 *g*) at 4°C, and the supernatant (nuclear extract) was stored at −80°C. The protein concentration was determined by the DC BioRad assay using the manufacturer's protocol (BioRad Laboratories, Herculus, CA, USA).

Enzyme-linked immunosorbent assay

The commercially available Trans-AM kit that was employed for the assay of NF-κB/p65 uses an oligonucleotide containing an NF-κB consensus site (5′-GGGACTTTCC-3′) that binds to the nuclear extract and can detect NF-κB, which can recognize an epitope on p65 activated and bound to its target DNA. In the absence of competitive binding with the wild-type or mutated consensus oligonucleotide, 30 μl of binding buffer was added to each well in duplicate. Alternatively, 30 μl of binding buffer containing 20 pmol (2 μl) of appropriate oligonucleotide, in duplicate, was added to the corresponding well. Nuclear lysate protein (10 μg) of each sample diluted in 20 μl lysis buffer was loaded per well. For positive control, 20 μl of lysis buffer containing 1 μl of control cell extract per well, was used and for blank 20 μl of lysis buffer per well was used. The plate was sealed with the adhesive film and incubated for 1 h at room temperature with mild agitation (100 r.p.m. on a rocking platform), after which the well was washed three times with 200 μl of 1X wash buffer, and 100 μl of diluted primary antibody (1:1000 dilution in 1X antibody-binding buffer) was added to each well and incubated at room temperature for 1 h without agitation. The wells were washed again three times with 1X wash buffer, and 100 μl of diluted HRP-conjugate antibody (1:1000 dilution in 1X antibody-binding buffer) was added to each well and incubated for 1 h. The wells were again washed four times with 1X wash buffer followed by the addition of 100 μl of developing solution. The content was incubated for 5 min at room temperature. This was followed by the addition of 100 μl of stop solution to each well, and the absorbance was read within 5 min at 450 nm.

Electrophoretic mobility shift assay (EMSA)

EMSA for NF-κB was performed using lightshift™ chemiluminiscent EMSA kit (Pierce, Rockford, IL, USA) by following the manufacturer′s protocol. To start with, DNA was biotin labeled using the Biotin 3′ end labeling kit (Pierce, Rockford, IL, USA). Briefly, in a 50 μl reaction buffer, 5 pmol of double-stranded NF-κB oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ was incubated in a microfuge tube with 10 μl of 5X TdT (terminal deoxynucleotidyl transferase) buffer, 5 μl of 5 μM biotin-N4-CTP, 10 U of diluted TdT, and 25 μl of ultrapure water at 37°C for 30 min. The reaction was stopped with 2.5 μl of 0.2 M EDTA. To extract labeled DNA, 50 μl of chloroform : isoamyl alcohol (24 : 1) was added to each tube and centrifuged briefly at 13 000 *g*. The top aqueous phase containing the labeled DNA was removed and saved for binding reactions. Each binding reaction contained 1X-binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol, pH 7.5), and 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl poly (dI-dC), 0.05% NP-40, 5 μg of nuclear extract, and 20–50 fm of biotin end-labeled target DNA. The contents were incubated at room temperature for 20 min. To this reaction mixture was added 5 μl of 5X loading buffer, subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. When the transfer was complete, DNA was crosslinked to the membrane at 120 mJ/cm^2 using a UV crosslinker equipped with 254 nm bulbs. The biotin end-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to X-ray film (XAR-5 Amersham Life Science Inc., Arlington Heights, IL, USA) and developed using a Kodak film processor.

Western blot analysis

For Western analysis, 25–50 μg of protein was resolved over 8–12% PAGE and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk, 1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature followed by incubation with appropriate monoclonal primary antibody in blocking buffer for 1 h to overnight at 4°C. This was followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase (Amersham Life Sciences, Inc.) for 1 h and then washed several times, and detected by chemiluminescence (ECL kit, Amersham Life Sciences, Inc.) and autoradiography using XAR-5 film obtained from Eastman Kodak Co. (Rochester, NY, USA). Densitometric measurements of the band in Western blot analysis were performed using the digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT, USA).

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