Ultraviolet Radiation Induces Phosphorylation of the Epidermal Growth Factor Receptor

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Abstract

Ultraviolet light in solar radiation is responsible for more than 600,000 malignancies each year in the United States alone, making it the most efficient environmental carcinogen known. Ultraviolet radiation-induced direct DNA damage is thought to be responsible for its initiating properties, while the promotional aspects of such radiation are poorly defined and only recently gaining attention. We show here for the first time that physiologically relevant doses of ultraviolet radiation induce phosphorylation of the epidermal growth factor receptor in A431 keratinocytes at tyrosine sites within 30 min. Such alteration of this major signal transduction system is probably an important step in the ultraviolet radiation-induced, epidermal cell-signalling cascade.

Introduction

UV radiation is capable of inducing both acute and chronic effects in human skin. These include inflammation and hyperpigmentation, hyperplasia of the epidermis and, most importantly, melanoma and nonmelanoma skin cancer. UV acts in the skin, at least for nonmelanoma skin cancer, as a complete carcinogen, and it has been shown to act, with appropriate chemical agents, as both a tumor initiator and a tumor promoter (1, 2). We have been interested in the promotion-related effects of UV, because UV irradiation of intact skin produces many effects analogous to those produced by the classic chemical tumor promoter TPA (2). UV and TPA produce inflammation and proliferation in human and animal skin, both mediated in part by metabolites of arachidonic acid (3–5). Our previous investigations have shown that UV induces arachidonic acid release through phospholipase activation and eicosanoid production in murine and human fibroblasts and in human epidermal keratinocytes (6, 7). Others have reported similar observations (8, 9). A related early event in the cascade induced by TPA in cultured cells is the inhibition of EGF binding to its receptor (EGFR). This inhibition has been shown to be due to phosphorylation of EGFR at threonine sites by PKC, the receptor kinase for TPA (10). We have previously reported that UV induces a rapid inhibition of EGF binding in murine fibroblasts and human keratinocytes (11, 12). In other studies we examined the effect of UV on PKC activity and found that UV was capable of inducing activity of the kinase (13). This suggested that UV inhibition of EGF binding might be due to PKC phosphorylation of EGFR.

Materials and Methods

Cell Culture System. Cryopreserved stocks of human epidermoid carcinoma cells (A431) were obtained from the American Type Culture Collection, Rockville, MD. The cells were grown to subconfluence in DMEM supplemented with 10% bovine fetal calf serum, penicillin (75 units/ml), and streptomycin (25 mg/ml). The cells were grown in a humidified atmosphere of 5% CO2:95% air at 37°C. A431 cells in 10-cm dishes were treated with EGF, TPA, and DMSO and irradiated with UVB or sham irradiated in HBBS without phenol red.

Light Source and Irradiation. UVB irradiation was performed on monolayer cultures with dish lids removed, utilizing a light source composed of six Phillips TL20W/12 fluorescent Sun Tubes. The irradiance at a target distance of 25 cm was 12 W/m² measured with a Model IL700 International Light Research radiometer with a SEE 1240 probe. The spectral emission of the source was 290–360 nm with emission in the UVB range (290–320 nm) accounting for 60% of the irradiance.

Western Blotting. Cells were rinsed with ice-cold PBS and harvested in Laemmli buffer [143 mM Tris (pH 6.8):3% SDS:5% 2-mercaptoethanol:10% glycerol]. Equal amounts of protein from each sample were subjected to discontinuous polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) (14) in which the stacking gel was 4% acrylamide:bis(50:1) and the resolving gel was 8%. The separated proteins were then electrophoretically transferred overnight to a NC membrane. NC was washed and incubated with mouse anti-phosphotyrosine monoclonal IgG2b antibody (UBI, Lake Placid, NY). Proteins containing phosphotyrosine were visualized with a goat anti-mouse IgG antibody linked to horseradish peroxidase (UBI, Lake Placid, NY).

Immunoprecipitation of EGFR. A431 cells were washed with phosphate-free DMEM and then starved for 4 h in phosphate-free DMEM containing 5% dialyzed calf serum. This conditioned medium was removed, supplemented with 0.07 mCi/ml of 32Pi (Dupont, NEN Research Products, Boston, MA), and added back to the cells.

After 3 h the radiolabeled medium was removed, cells were washed twice with HBBS without Ca²⁺, and HBBS with Ca²⁺ was added to the dishes. This was followed by irradiation, sham irradiation, or treatment with EGF (150 ng/ml) (Sigma, St. Louis, MO).

At selected times after incubation at 37°C, cells were washed twice with ice-cold PBS and harvested on ice in enriched PBS solution containing 10 ng/ml of leupeptin, 10 ng/ml of pepstatin A, 20 ng/ml of aprotinin, and 1 mM PMSF. Then cells were centrifuged, and the supernatant was discarded and resuspended in RIPA buffer. All subsequent steps were performed at 4°C according to a procedure modified from that of Stoscheck and Carpenter (15).

The solution was centrifuged, and 15 µl of monoclonal mouse anti-human EGFR (UBI, Lake Placid, NY) were added per sample and incubated overnight. A 50% solution of Protein G (Sepharose Fast Flow 4B; Sigma Chemical Corp., St. Louis, MO) was added to each sample, and incubation continued for 2 h. Then the cell lysate was centrifuged, the supernatant was discarded, and the pellets were washed 4 times in RIPA buffer. The immunoprecipitated samples were boiled for 5 min in Laemmli buffer and then electrophoresed on a 7.5% SDS-PAGE gel.

The dried gel was exposed at −70°C to Kodak X-OMat AR film. The intensity of the bands obtained after autoradiography was quantified photodensitometrically with a computerized laser densitometer (Version 3.2, Image Quant, Series 300 densitometer; Molecular Dynamics, Sunnyvale, CA).
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coupled to an on-line 130A separation system (RP-HPLC) using precolumn derivatization followed by subsequent analysis of amino acids. Solutions of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) were used as internal standards.

Elution absorbance data were collected using a 760 interface and XTRA CHROM II data system (PE Nelson, Cupertino, CA). The derivatized phosphoamino acids were quantitated by comparing the sample absorbance peak areas.

Results and Discussion

We examined the effect of UVB (290–320 nm) on EGFR in A431 cells, which are derived from a human squamous cell carcinoma and which express high levels of EGFR. Cells were irradiated with UVB (600 J/m²), and extracted proteins were analyzed by immunoblotting with a monoclonal anti-phosphotyrosine antibody. As seen in Fig. 1, an immunoreactive protein with a molecular weight of approximately 170,000 was noted, corresponding to EGFR. A similar band was seen in EGF-treated but not sham-irradiated or TPA-treated cells. Preliminary studies revealed that this phosphorylation was maximal at 30 min postirradiation (data not shown).

We then examined specific characteristics of this phosphorylation. Cells were prelabeled with [32p]orthophosphate and then irradiated with 400 and 800 J/m². Cell extracts were immunoprecipitated with monoclonal anti-EGFR antibody and separated by gel electrophoresis. A phosphorylated M, 170,000 protein was identified in irradiated but not sham-treated cells (Fig. 2). A similar band was noted in EGF-treated cells. Densitometric analysis of the autoradiograms revealed a dose-related phosphorylation of EGFR, with 400 J/m² inducing twice and 800 J/m² almost 3 times the level of sham-irradiated cells.

A time course study shown in Fig. 3 revealed that the phosphorylation of the immunoprecipitated receptor in UVB-treated cells peaked somewhat later and persisted longer than in EGF-treated cells. To more clearly define the amino acid identity of the phosphorylated site induced by UVB, the immunoprecipitated receptor was hydrolyzed, and phosphorylated amino acids were identified by RP HPLC after derivatization (Fig. 4). UVB markedly increased the quantity of phosphorylated (p-) tyrosine residues in the EGFR hydrolysates, but had no effect on p-serine or p-threonine content. EGF
treatment resulted in a similar increase in p-tyrosine but unlike UVB also increased p-threonine, consistent with data reported previously in this cell type (17).

Others have examined the effect of UVB on EGFR. Brooks et al. (18) reported that UVB-induced inhibition of 125I-EGF binding in murine fibroblasts and keratinocytes was not blocked by depletion of PKC with TPA treatment and was not associated with phosphorylation of the Mr 80,000 protein substrate of PKC. They concluded that the UVB-induced inhibition, therefore, was by a PKC-independent mechanism. Our finding of tyrosine rather than serine or threonine phosphorylation in UVB-treated cells supports their view. We have also reported that neither TPA-induced depletion of PKC nor treatment with the PKC inhibitors H7 and staurosporine affected UVB-induced inhibition of 125I-EGF binding in C3H10T1/2 cells (17).

When EGF binds to EGFR, receptor dimerization and autophosphorylation of tyrosine sites occur, which condition leads to phosphorylation of substrates such as phospholipase C. This begins a cascade of signal transduction which results in proliferation of most cells. Interestingly enough, this cascade includes PKC activation which probably acts to exert a later, negative control on EGFR activity (4). The phosphorylation of proteins in A431 human tumor cells. Cell, 24: 741-752, 1981.

The mechanism by which UVB stimulated phosphorylation of EGFR at this point undefined. TGF-α, which also binds to EGFR, has been shown to be secreted by UVB-treated cultured cells. In their studies, Brooks et al. (18) investigated the effect of UVB-induced, soluble, excised factors on EGF binding and found no effect, suggesting that UVB-induced inhibition of binding was not through TGF-α production. UVB-induced phosphorylation is rapid, occurring within 30 min of irradiation. This suggests a direct photon-membrane interaction. It is possible that UVB causes dimerization and autophosphorylation of EGFR through a photochemical reaction. Activation of other tyrosine kinases, however, cannot be ruled out.

The doses of radiation capable of inducing receptor phosphorylation (400 J/m² measured as described) can be received at the skin surface in New York City in July within 5 to 10 min of outdoor activity. Several consequences of UVB-induced EGFR phosphorylation may be physiologically important to the epidermal responses observed after irradiation. For example, activation of tyrosine kinase may be a critical early event in the induction of phospholipase activity, which is involved in the UV-induced inflammatory response (4). Postinflammatory hyperplasia and tanning may also be linked to kinase activation.

In addition, the ability of UVB to alter such an important membrane signal transducer as EGFR suggests that membrane second messengers are intimately involved in the cascade of events that leads to late effects of solar damage in human skin like carcinogenesis and photocaging. Finally, the effect of UVB on EGFR may play a role in the mechanism by which UVB phototherapy benefits psoriasis, a disease with abnormal, excessive EGFR expression (20).

References
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