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OXIDATIVE INHIBITION OF RECEPTOR TYPE PROTEIN TYROSINE PHOSPHATASE KAPPA BY ULTRAVIOLET IRRADIATION ACTIVATES EGFR IN HUMAN KERATINOCYTES

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Abstract

Ultraviolet (UV) irradiation rapidly increases tyrosine phosphorylation (i.e. activates) of epidermal growth factor receptors (EGFR) in human skin. EGFR-dependent signaling pathways drive increased expression of matrix-metalloproteinases, whose actions fragment collagen and elastin fibers, the primary structural protein components in skin connective tissue. Connective tissue fragmentation, which results from chronic exposure to solar UV irradiation, is a major determinant of premature skin aging (photoaging). UV irradiation generates reactive oxygen species, which readily react with conserved cysteine residues in the active site of protein tyrosine phosphatases (PTP). We report here that EGFR activation by UV irradiation results from oxidative inhibition of receptor type PTP-kappa (RPTP-κ). RPTP-κ directly counters intrinsic EGFR tyrosine kinase activity, thereby maintaining EGFR in an inactive state. Reversible, oxidative inactivation of RPTP-κ activity by UV irradiation shifts the kinase-phosphatase balance in favor of EGFR activation. These data delineate a novel mechanism of EGFR regulation and identify RPTP-κ as a key molecular target for anti-oxidant protection against skin aging.

INTRODUCTION

Skin is the largest human organ, and only organ directly exposed to the environment. Acute exposure to ultraviolet (UV) irradiation from the sun is harmful to skin, causing sunburn, immune suppression, DNA damage, and connective tissue degradation (1). Accumulated damage, resulting from chronic sun exposure, causes skin cancer and premature skin aging (photoaging) (2). Approximately one million individuals in the USA develop skin cancer each year, and essentially all people experience photoaging to some degree, depending on the amount of sun exposure and skin pigmentation (pigmentation is protective) (3).

Epidermal growth factor receptor (EGFR) is a ubiquitously-expressed, cell surface, transmembrane receptor that possesses intrinsic protein tyrosine kinase activity. Functional activation of EGFR results from increased phosphorylation of specific tyrosine residues in its C-terminal cytoplasmic domain. Tyrosine phosphorylation is catalyzed by intrinsic tyrosine kinase activity. Phosphotyrosine residues function as binding sites for assembly of protein complexes, which initiate down-stream signaling pathways that regulate cellular function (4). EGFR is highly expressed in human skin cells (keratinocytes) *in vivo* and *in vitro* (5,6). Emerging evidence indicates that EGFR is a critical functional mediator of cellular responses to a diverse array of extracellular stimuli, including ligands for other cell surface receptors. (7,8). Hypertyrosine phosphorylation (i.e. activation) of EGFR in response

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to non-cognate ligands involves release of surface membrane bound EGF family ligands, as well as other mechanisms (9-15).

UV irradiation rapidly increases EGFR tyrosine phosphorylation in human keratinocytes *in vivo* and in culture (5). This EGFR activation is necessary for induction of signaling pathways (termed the mammalian UV response), including MAP kinases, PI-3 kinase/Akt, and PLC/PKC (16,17). These signaling pathways induce a variety of transcription factors and their target genes, including AP-1 and matrix metalloproteinases (MMPs), respectively (17-22). AP-1 and MMPs play critical roles in development of skin cancer and photoaging (23,24), indicating the importance of EGFR tyrosine phosphorylation in the pathophysiology of UV-induced human skin damage. In addition, EGFR activation protects against UV-induced apoptosis, through activation of PI-3-kinase/AKT pathway (16).

Biological effects of UV irradiation occur as a consequence of absorption of electromagnetic energy by certain molecules within all cells. The excess energy is dissipated either by chemical modification of the absorbing molecule, or transfer of energy to an acceptor molecule. Molecular oxygen, which is in high concentrations in eukaryotic cells, can readily accept energy from UV-absorbing molecules (25,26). This photochemical activation of molecular oxygen generates reactive oxygen species (ROS), which can oxidize cellular constituents including proteins lipids, and nucleic acids (27-30). ROS are the major chemical initiators of UV irradiation-induced responses in human cells (21,31-34). While at high concentrations ROS can be cytotoxic, at low concentrations ROS appear to serve a physiological function as mediators of cellular response (35,36). Recent studies have shown that activation of cell surface growth factors and cytokine receptors by UV results in the formation of ROS, which play a critical role in mediating downstream signaling pathways in cultured cells (37-39) Peus *et al.* have reported that H_2O_2 generation precedes EGFR phosphorylation and ERK activation following UVB irradiation of human keratinocytes (20).

All members of the protein tyrosine phosphatase (PTP) family contain an active site cysteine residue, which is required for phosphohydrolase activity. This active site cysteine is highly susceptible to oxidation, particularly by hydrogen peroxide (H_2O_2) (40). The pKa of the cysteine within the active site, is relatively low (5.5) at physiological pH, which promotes formation of the reactive thiolate form. The thiolate reacts readily with H_2O_2 to form a stable sulfenic acid (40), or sulphenyl-amide species (41), which renders the phosphatase catalytically inactive. Recent evidence indicates that reversible oxidative inactivation of PTP activities occurs as a consequence of ROS generated in response to growth factor and cytokine receptor activation, and regulates tyrosine phosphorylation-dependent signal transduction pathways (18,38,42-44).

Oxidative inhibition of PTP activity by ROS has been proposed as a mechanism for activation of EGFR by UV irradiation (45). Critical testing of this proposed mechanism has been hindered by lack of knowledge regarding phosphatases that directly regulate EGFR at the cell surface. We have recently identified receptor type protein tyrosine phosphatase-kappa (RPTP-κ) as a regulator of EGFR tyrosine phosphorylation, in human keratinocytes (6). RPTP-κ directly dephosphorylates EGFR *in vitro*, and functions in cells to maintain low levels of EGFR tyrosine phosphorylation in the absence of exogenous ligand. RPTP-κ counteracts EGFR intrinsic tyrosine kinase activity by preferentially dephosphorylating EGFR tyrosine residues 1068 and 1173 (6). We report here that activation of EGFR by UV irradiation is mediated by oxidative inhibition of RPTP-κ activity.

EXPERIMENTAL PROCEDURES

Materials

Adult human primary keratinocytes were purchased from Cascade Biologics Inc., (Portland, OR). Chinese hamster ovary (CHO) cells were obtained from ATCC. EGFR and Phospho-EGFR (pY1068) antibodies used for Western analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA), respectively. EGFR antibody for immunofluorescence was from Neomarkers (Fremont, CA). Neutralizing EGFR antibody LA1 which blocks ligand-binding, was obtained from Upstate Biotechnologies (Waltham, MA). RPTP-κ antibody was generated and affinity purified from rabbits immunized with a peptide derived from the intracellular domain of human RPTP-κ, as described (6). Phosphotyrosine peptide derived from EGFR (Biotin-KGSTAENAE(pY)LRV-amide) was synthesized by New England Peptide, Inc. (Gardner, MA). PD169540 is a generous gift from Dr. David Fry (Pfizer Inc.). Oligonucleotide probes used for *in situ* hybridization were synthesized by http://www.GeneDetect.com (Bradenton, FL). Purified, full length active human EGFR was obtained from BioMol (Plymouth Meeting, PA). Intracellular region of RPTP-κ was cloned into pGEX-6-P, and expressed as a HIS-tagged GST fusion protein in BL21. Expressed RPTP-κ was purified by nickel chelate and glutathione affinity chromatography to a purity of greater than 90%, as judged by SDS PAGE.

Cell culture

Subcultures of adult human primary keratinocytes were expanded in modified MCDB153 media (EpiLife, Cascade Biologics, Inc.) at 37°C under 5% CO_2. CHO cells were cultured in Ham's F12 medium with 1.5 g/ml sodium bicarbonate, supplemented with 10% FBS under 5% CO_2 at 37°C.

UV source and irradiation

Sub-confluent cells in a thin layer of Tris-buffered saline were irradiated using a Daavlin lamp apparatus containing six FS24T12 UVB-HO bulbs. A Kodacel TA401/407 filter was used to eliminate wavelengths below 290 nm (UVC) resulting in a UV spectrum consisting of 48% UVB, 31% UVAII and 21% UVA1. The irradiation intensity was monitored with an IL1400A phototherapy radiometer and a SED240/UVB/W photodetector (International Light, Newbury, MA). Human subjects were phototested to determine the dose of UV irradiation that caused the skin to become slightly pink. (minimal erythema dose). Subjects were exposed to twice this dose for studies. All procedures involving human subjects were approved by the University of Michigan Institutional Review Board and all subjects provided written informed consent.

Transient transfection of CHO cells

Mammalian expression vectors harboring EGFR (pRK5 EGF) or RPTP-κ pShuttle RPTP-κ) coding sequences were transiently transfected by Lipofectamine 2000 method into CHO cells according to manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA).

siRNA silencing of endogenous RPTP-κ in primary human keratinocytes

A 21mer RNA sequence (5' AAG GTT TGC CGC TTC CTT CAG 3') derived from RPTP-κ coding sequence was designed using Oligoengine's software (Seattle, WA). Homology search was performed on this RNA sequence using Blast (http://www.ncbi.nlm.nih.gov/BLAST/) to ensure it was not presented in any other known sequence in the database. Double-stranded siRNA was synthesized by Qiagen-Xeragon Inc. (Valencia, CA). The synthetic siRNA was transfected into primary human keratinocytes using Human

Keratinocytes Nucleofector kit and device from Amaxa Biosystems (Cologne, Germany) according to manufacturer's protocol.

RPTP-κ immunoprecipitation and protein tyrosine phosphatase assay

Keratinocytes whole cell lysates were made in TGH buffer (50 mM Hepes, pH 7.2, 20 mM NaCl, 10% glycerol and 1% Triton X-100), supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 1 mM PMSF, and were pre-cleared with normal rabbit IgG before incubation with RPTP-κ antibody for three hours at 4°C. For some assays, as indicated in the text, 10mM iodoacetic acid was added to TGH buffer to irreversibly inhibit non-oxidized protein tyrosine phosphatase activity (18). Protein A-conjugated agarose beads were then added, and further incubated at 4°C for two hours, followed by extensive washing. Washed immunoprecipitates were analyzed by Western blot, or assayed for protein tyrosine phosphatase activity. For some assays, as indicated in the text, 10mM DTT was added to the assay buffer to reduce oxidized RPTP-κ (18). For measurement of protein phosphatase activity, tyrosine-phosphorylated peptide derived from EGFR was added to a final concentration of 0.5 mM in 50 μl PTP assay buffer (50 mM Tris, pH7.6, 100 mM NaCl, 100 μg/ml BSA). Reactions were terminated by addition of 100 μl of BIOMOL Green Reagent (BIOMOL, Plymouth Meeting, PA) and absorbance measured at 620 nm.

Western blot analysis of UV irradiation-induced oxidation of RPTP-κ in human primary keratinocytes

Human primary keratinocytes were mock irradiated or UV irradiated (90 mJ/cm^2). Five minutes post UV irradiation, cells were lysed in the presence of 100 mM Iodacetice acid, and RPTP-κ was immunoprecipiated as dscribed above. The immunoprecipitate was reduced by addition of 10 mM dithiothreitol in TGH buffer, containing protease inhibitors, for 30 minutes at 4°C. The immunoprecipitate was washed three times, and then irreversibly oxidized by incubation with 2 mM pervanadate at 4°C for one hour. Oxidized RPTP-κ was analyzed by Western blot probed with oxPTP antibody (A gift from Dr. Arne Ostman, Cancer Center Karolinska, Stockholm, Sweden) as described (47).

Western analysis detection and quantitation

Western blots were developed and quantified using a chemifluorescent substrate (ECF Western Blot Reagents, Amersham Biosciences, Arlington Heights, IL). Detection of chemifluorescense was performed using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Sample loads, antibody concentration, and incubator times were adjusted to yield fluorescent signals within the linear range of detection. Fluorescent intensity of protein bands were quantified by ImageQuant software, which is an integral application of the STORM.

Detection of UV irradiation-induced DNA fragmentation in human primary kerationcytes

Human primary keratinocytes were infected with either empty or RPTP-κ adenovirus. Cells were mock or UV-irradiated two days post infection. Six hours post UV irradiation, cells were lysed, and DNA fragmentation was measured by Cell Death Detection ELISA according to manufacturer's instructions (Roche Applied Science, Penzberg, Germany)

In situ hybridization

Hybridization buffer (4x SSC, 20% dextran sulfate, 50% formamide, 0.25mg/ml salmon sperm DNA, 0.25mg/ml yeast tRNA, 0.1M DTT, 0.5X Denhardt's solution) with three fluorescine-conjugated sense or antisense DNA oligonucleotide probes, corresponding to nucleotides 1549-1596, 3440-3487, and 4290-4337 in the human RPTP-κ mRNA sequence

(genebank accession number NM_002844), at 37°C overnight. Sections were washed in 2X PBS with 0.01% Tween 20, then 1X PBS, 0.01% Tween 20. Washed slides were incubated with protein block (Biogenex, San Ramon, CA), biotin-labeled anti-fluorescence antibody, followed by horse radish peroxidase-strepaviden. Hybridized probes were visualized by addition of AEC as substrate.

Immunohistology and immunofluorescence

Human full thickness skin samples were embedded in OCT and frozen in liquid nitrogen. Frozen sections (7μm) were cut with a cryostat (LEICA CM3050). Sections were air dried for 10 minutes, fixed with 2% paraformaldehyde for 20 minutes at room temperature, and washed for 20 minutes. Slides were loaded on an automated immunostainer (Biogenex i6000). For immunoperoxidase staining, slides were incubated with peroxide block (10 minutes), protein block (20 minutes), rabbit affinity-purified anti-RPTP-κ (30 minutes), Multilink-biotin conjugate (10 minutes), streptaviden-conjugated horse radish peroxidase (10 minutes), AEC substrate (3 minutes), and Hemotoxylin (20 seconds). For double immunofluorescence, peroxide block was omitted, and following incubation with RPTP-κ antibody, biotin-conjugated anti-rabbit antibody (Vector Laboratories, Burlingame, CA), and streptavidin-conjugated AlexaFluor 594 (Invitrogen-Molecular Probes, San Diego, CA) were each added for 10 minutes. Slides were washed with distilled water, EGFR antibody (Ab-10) was added overnight at 4°C, and anti-mouse IgG_1-conjugated FITC (Caltag, Burlingame, CA) was added for 10 minutes. Stained slides were washed with distilled water, and covered with Supermount. For negative control, staining was performed using RPTP-κ antibody plus peptide used to raise the antibody, or pre-immune serum, instead of primary antibody. Staining was observed under a Zeiss microscope (Axioskop 2) and images were obtained with digital camera (SPOT2, Diagnostic Inc. Sterling Heights, MI). All reagents, except as noted, were from Biogenex.

RESULTS AND DISCUSSION

To investigate the role of RPTP-κ in UV irradiation regulation of EGFR tyrosine phosphorylation, we initially examined the effects of ROS on purified RPTP-κ activity and EGFR tyrosine phosphorylation *in vitro*. Addition of hydrogen peroxide (H_2O_2) caused dose-dependent inhibition of RPTP-κ activity, with 80% loss of activity observed at 100μM (Fig. 1A). In the presence of ATP/Mg^{++}, purified EGFR was phosphorylated by its intrinsic tyrosine kinase activity. In contrast to RPTP-κ, H_2O_2 had no effect on tyrosine phosphorylation of purified EGFR, *in vitro* (Fig. 1B). Incubation of purified RPTP-κ and EGFR together resulted in a low level of steady state EGFR tyrosine phosphorylation, representing the balance between the rates of tyrosine kinase and tyrosine phosphatase activities. In the presence of H_2O_2, which inhibits RPTP-κ, EGFR tyrosine phosphorylation increased to the level observed in the absence of RPTP-κ (Fig. 1C). These data provide proof of concept for RPTP-κ-dependent regulation of EGFR tyrosine phosphorylation by ROS, in a cell-free system.

To further investigate UV irradiation regulation of EGFR tyrosine phosphorylation by RPTP-κ, we used a model mammalian cell system. Chinese hamster ovary (CHO) cells do not express either EGFR or RPTP-κ. We have previously used this system to screen PTPs for EGFR phosphatase activity (6). Transient transfection of CHO cells with EGFR expression vector resulted in high level of constitutive (i.e. in the absence of ligand) EGFR tyrosine phosphorylation (Fig. 2). This constitutive EGFR tyrosine phosphorylation was abolished by specific EGFR tyrosine kinase inhibitor PD169540, indicating tyrosine phosphorylation was due to intrinsic tyrosine kinase activity (data not shown. Exposure of EGFR-expressing CHO cells to UV irradiation did not further increase EGFR tyrosine phosphorylation. However, co-expression of EGFR with RPTP-κ substantially reduced

EGFR tyrosine phosphorylation (Fig. 2). UV irradiation of CHO cells expressing both EGFR and RPTP-κ increased the level of EGFR tyrosine phosphorylation to the level observed in the absence of RPTP-κ (Fig. 2). These data demonstrate that RPTP-κ is required for UV irradiation induction of EGFR tyrosine phosphorylation, in the CHO cell model system.

We next investigated the role of RPTP-κ in UV irradiation regulation of EGFR tyrosine phosphorylation in human keratinocytes. In addition to expressing both EGFR and RPTP-κ, keratinocytes express several EGFR ligands, including transforming growth factor-alpha, amphiregulin, HB-EGF, betacellulin, and epiregulin. To examine potential involvement of ligand-binding in UV irradiation induction of EGFR tyrosine phosphorylation, we used a neutralizing monoclonal antibody, which blocks ligand binding to EGFR. While this antibody reduced EGF-induced EGFR tyrosine phosphorylation to near basal levels, it had no significant effect on UV irradiation induction of EGFR tyrosine phosphorylation (Fig. 3A). We conclude that ligand-binding has little, if any, role in activation of EGFR by UV irradiation. This conclusion is consistent with data demonstrating that the EGFR ligand-binding domain is not required for UV irradiation induction of EGFR tyrosine phosphorylation in NIH 3T3 cells (45).

We next examined whether UV irradiation inhibited RPTP-κ activity in human keratinocytes. For these studies, keratinocytes were mock-exposed or exposed to UV irradiation (50mJ/cm^2), and harvested in lysis buffer five minutes post UV irradiation. RPTP-κ was immunoprecipitated, and its activity measured by dephosphorylation of a phosphotyrosine-containing synthetic peptide substrate, derived from the amino acid sequence of the EGFR (amino acids 1164-1176). UV irradiation reduced RPTP-κ activity in human keratinocytes more than 60%, compared to mock-irradiated cells (Fig 3B). It has recently been reported that UV irradiation can reduce protein levels of PTP 1B and LAR, in certain cell types, though activation of proteolytic cleavage (46). However, no reduction of RPTP-κ protein level in human keratinocytes was observed within 90 minutes following UV irradiation (data not shown).

The data above indicate UV irradiation inhibits RPTP-κ activity, in human keratinocytes. To determine whether inhibition results from oxidation, iodoacetic acid was included in the lysis buffer that was used to harvest cells following mock or UV irradiation. Iodoacetate forms a stable adduct with non-oxidized, but not with oxidized, cysteine thiols. Therefore non-oxidized RPTP-κ is irreversibly inhibited by iodoacetate, whereas oxidized RPTP-κ is not (18). The activity of oxidized, but not acetylated, RPTP-κ can be restored by reduction with dithiothreitol (DTT) (18). Immunoprecipitates from mock-irradiated keratinocytes, prepared in the presence of iodoacetate, and treated with DTT, contained four-times less RPTP-κ activity, compared to immunoprecipitates from UV-irradiated cells (Fig. 3C). These data indicate that UV irradiation caused oxidation of RPTP-κ, which protected it against acetylation, in human keratinocytes.

To confirm that UV irradiation leads to oxidation of RPTP-κ in human keratinocytes, we utilized an antibody that specifically recognizes the oxidized active site of protein tyrosine phosphatases (47). RPTP-κ was immunoprecipitated from keratinocytes following mock exposure or exposure to UV irradiation. Immunoprecipatated RPTP-κ was analyzed for active site oxidation by Western analysis. The level of oxidized RPTP-κ was increased three-fold in UV-irradiated, compared to non-irradiated keratinocytes (Fig 3D).

We observed that expression of exogenous RPTP-κ conferred UV irradiation induction of EGFR tyrosine phosphorylation, in CHO cells (Fig. 2). Keratinocytes, however, express endogenous RPTP-κ. Therefore, we utilized siRNA-mediated knockdown to examine the

role of RPTP-κ in UV irradiation regulation of EGFR tyrosine phosphorylation. Transient transfection of RPTP-κ siRNA caused 80% and 70% reduction of RPTP-κ mRNA and protein, respectively (6). Knockdown of RPTP-κ had no effect on gene expression levels of other related RPTPs expressed in keratinocytes (RPTP-μ, β, δ, or ζ) (6). UV irradiation induced EGFR tyrosine phosphorylation nearly five-fold in keratinocytes transfected with scrambled control siRNA (Fig. 4), similar to that observed in non-transfected keratinocytes (Fig. 3). Knockdown of RPTP-κ increased EGFR tyrosine phosphorylation in non-irradiated keratinocytes nearly four-fold. Exposure to UV irradiation further increased EGFR tyrosine phosphorylation only 20% (Fig. 4). These data indicate that normal levels of RPTP-κ function to maintain low basal EGFR tyrosine phosphorylation. In the presence of reduced levels of RPTP-κ, basal EGFR tyrosine phosphorylation is increased, and therefore can only be marginally further increased by UV irradiation. In the presence of normal levels of RPTP-κ, basal EGFR tyrosine phosphorylation is low, and oxidative inhibition of RPTP-κ by UV irradiation alters the EGFR tyrosine kinase/phosphatase balance to elevate EGFR tyrosine phosphorylation.

UV irradiation can damage skin cells, and with sufficient damage, induce apoptosis (48). In human keratinocytes, EGFR protects against UV-induced apoptosis, primarily through activation of the PI-3-kinase/ATK pathway (16). Therefore, we examined whether overexpression of RPTP-κ could modulate UV irradiation-induced DNA fragmentation, a marker of apoptosis, in human keratinocytes. At a dose of 50mJ/cm^2, UV irradiation did not cause significant DNA fragmentation, compared to mock irradiation, in keratinocytes infected with control vector (Fig. 5). In contrast, this dose of UV irradiation causes a significant increase of DNA fragmentation of keratinocytes overexpressing RPTP-κ (Fig 5). Higher doses of UV irradiation (70-90mJ/cm^2) caused increased DNA fragmentation in both control and RPTP-κ overexpressing cells. However, increased expression of RPTP-κ caused increased levels of DNA fragmentation, at all doses of UV irradiation.

Finally, we examined RPTP-κ expression and regulation by UV irradiation in human skin *in vivo*. Epidermis primarily consists of stratified layers of keratinocytes. The lowest layer of keratinocytes (basal keratinocytes) undergoes cell division. Daughter cells (suprabasal keratinocytes) migrate upward towards the surface, and, as they migrate, undergo a coordinated, complex program of maturation. Suprabasal keratinocytes normally do not proliferate. RPTP-κ mRNA was expressed predominantly in suprabasal keratinocytes (Fig. 6A). A similar pattern of expression was observed for RPTP-κ protein (Fig. 6B). In contrast, EGFR protein, the substrate for RPTP-κ, was expressed throughout the epidermis, in both basal and suprasbasal keratinocytes (Fig. 6C). Erk MAP kinase is a major EGFR effector in many cells, including human keratinocytes. UV irradiation activates Erk1/2 in human keratinocytes in skin *in vivo*, and this activation is dependent on EGFR (49). Interestingly, the localization of activated Erk closely coincides with that of RPTP-κ in UV irradiated human skin (5). This observation is consistent with inhibition of RPTP-κ leading to EGFR-dependent Erk activation of suprabasal keratinocytes in human skin *in vivo*. In addition, EGFR is a major mitogenic pathway in basal keratinocytes. Therefore, predominant expression of RPTP-κ in non-proliferating suprabasal keratinocytes is consistent with its role in limiting EGFR tyrosine phosphorylation. Our observation that overexpression of RPTP-κ in cultured basal keratinocytes completely inhibits proliferation provides support for this notion (6).

We have previously shown that exposure of human skin *in vivo* to UV irradiation increases EGFR tyrosine phosphorylation (50). Increased tyrosine phosphorylation was maximal (5-fold) 30 minutes after exposure. To determine the effect of UV irradiation on RPTP-κ, sun-protected buttock skin of adult subjects was exposed to UV irradiation, and skin samples were obtained 30 minutes post exposure. Skin was homogenized, RPTP-κ was

immunoprecipitated, and its protein level and enzymatic activity were measured. UV irradiation had no effect on RPTP-κ protein level. In contrast, RPTP-κ activity was inhibited more than 60% (Fig 6D). These results are similar to those obtained in cultured keratinocytes, and provide support for RPTP-κ as a critical regulator of EGFR tyrosine phosphorylation, in UV-irradiated skin *in vivo*.

Activation of signal transduction cascades and concomitant alterations in gene expression that occur in skin cells in response to exposure to UV irradiation are largely dependent on increased EGFR tyrosine phosphorylation. In human skin, EGFR-dependent responses are critical elements in the pathophysiology of UV irradiation-induced cancer and aging. Currently, with the exception of sunscreens, there are no effective measures for prevention of these serious solar UV irradiation-induced skin conditions. Our data demonstrate that oxidative inhibition of RPTP-κ is a central mechanism by which UV irradiation activates EGFR in human skin. Anti-oxidants, as topical preparations or dietary supplements, have gained popular attention with claims for a multiplicity of health benefits. However, these claims have been difficult to substantiate. One reason for this difficulty is lack of specific molecular targets for assessment of anti-oxidant effect. Our findings identify RPTP-κ as a key molecular target for anti-oxidant action for prevention of the primary manifestations of solar UV-induced skin damage.

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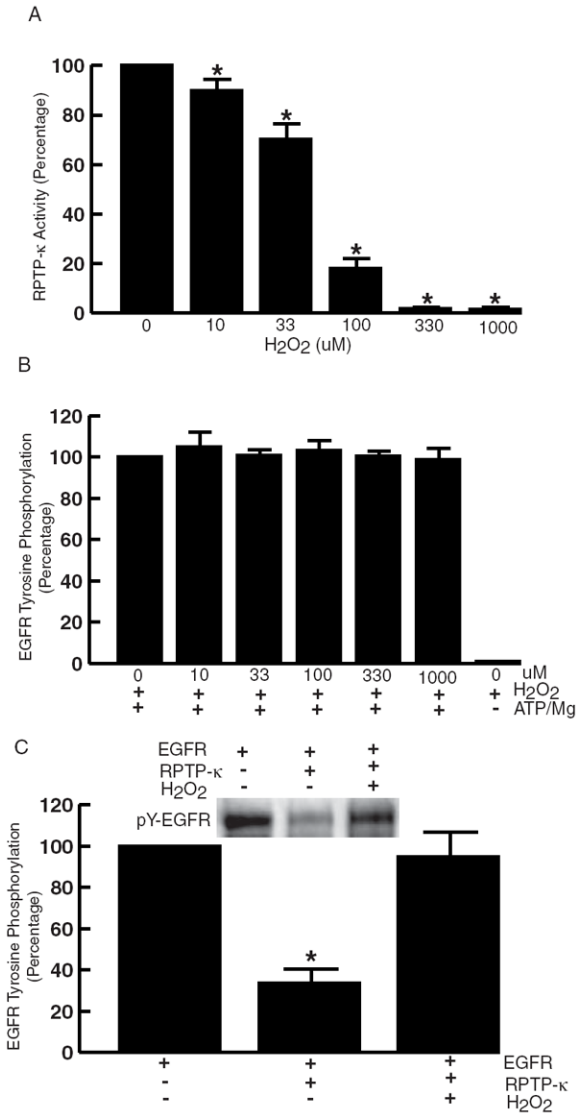

Figure 1.

Oxidative inhibition of RPTP-κ activity enhances EGFR tyrosine phosphorylation *in vitro*. A. Purified RPTP-κ GST-fusion protein was incubated with the indicated concentrations of H_2O_2 at room temperature for 30 minutes. RPTP-κ activity was measured using phospho-EGFR peptide as substrate. * $p<0.05$ vs Control (0). B. Purified EGFR, supplemented with EGF and ATP/Mg^{++}, was incubated with the indicated concentrations of H_2O_2 at room temperature for 30 minutes. Samples were subjected to Western analysis for EGFR tyrosine phosphorylation. Levels of phosphorylated EGFR were quantified by chemifluorescent detection. C. Purified EGFR, supplemented with EGF and ATP/Mg^{++}, and purified RPTP-κ GST fusion protein were incubated together in the presence or absence H_2O_2 (100μM), at room temperature for 30 minutes. Tyrosine phosphorylation of EGFR was quantified by chemifluorescence, as described in B. Results are means±SEM for three independent experiments. * $p<0.05$ vs H_2O_2 treated.

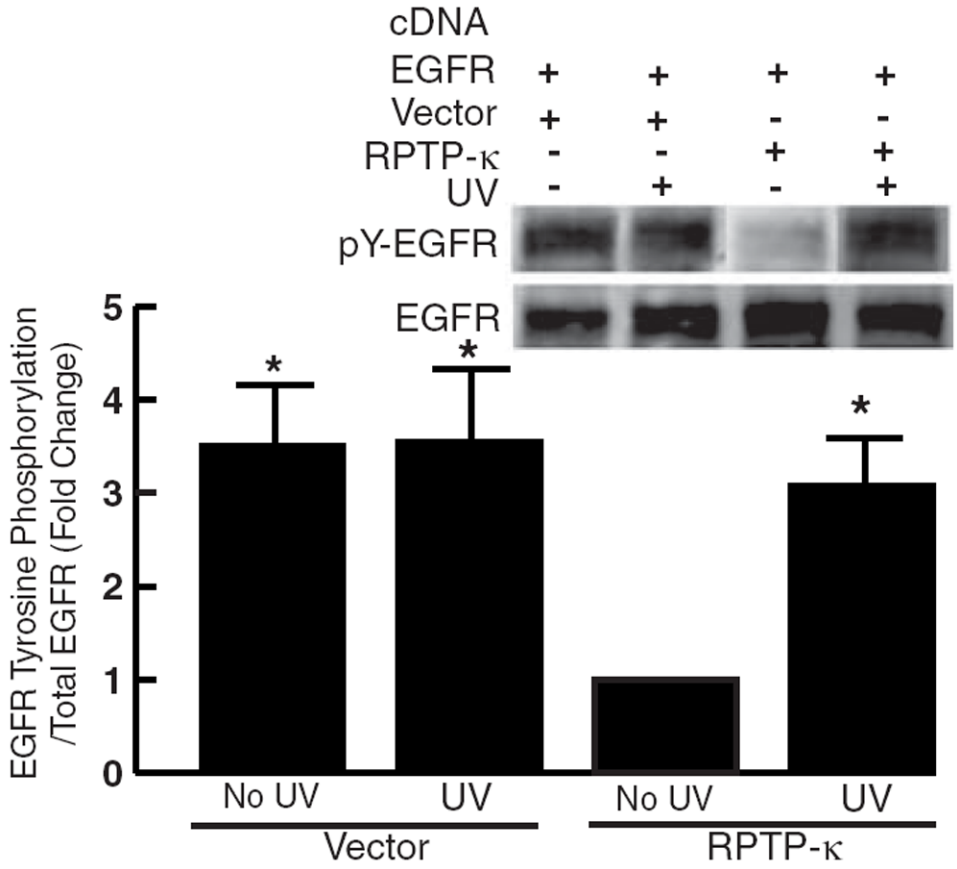

Figure 2.

RPTP-κ reduces constitutive EGFR tyrosine phosphorylation and confers UV-induction of EGFR tyrosine phosphorylation in CHO cells. A. CHO cells were transfected with pRK5 EGFR expression vector and empty or RPTP-κ vector. One day after transfection, cells were mock (No UV) or UV irradiated (50mJ/cm^2). Whole cell lysates were prepared 10 minutes post treatment, and subjected to Western analysis for total EGFR and tyrosine-phosphorylated EGFR. Levels of immunoreactive EGFR were quantified by chemifluorescent detection. Results are means±SEM of three independent experiments; *p<0.05. Inset shows a representative image of chemifluorescent immunoreactive bands.

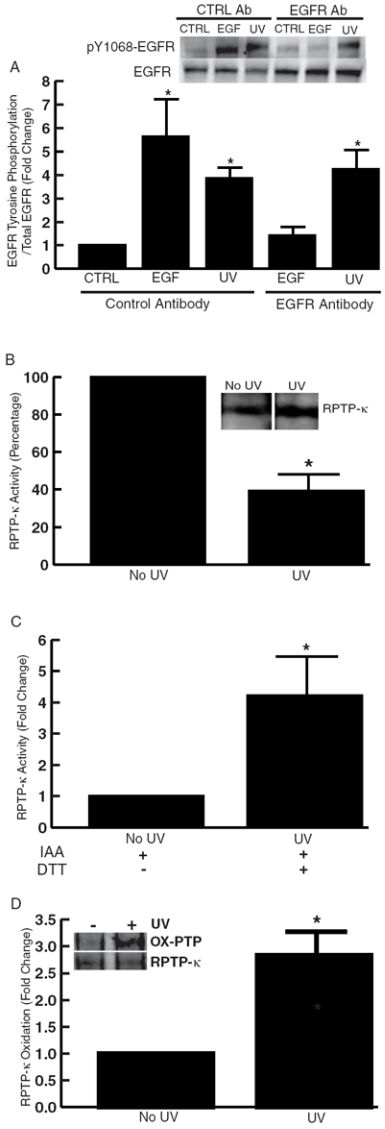

Figure 3.

UV-induced EGFR tyrosine phosphorylation is ligand-independent and mediated by oxidative inhibition of RPTP-κ, in primary human keratinocytes. A. Primary human keratinocytes were treated with control IgG_1 or EGFR antibody LA1 (1μg/ml), which blocks ligand-binding, as indicated. Cells were then treated with vehicle (CTRL), or EGF (10 ng/ml) for 10 minutes, or UV-irradiated (50 mJ/cm^2), and harvested 15 minutes post irradiation. Whole cell lysates were subjected to Western analysis for total EGFR and tyrosine-phosphorylated EGFR. Levels of immunoreactive EGFR were quantified by chemifluorescent detection. Results are means±SEM of three independent experiments; *$p<0.05$. Inset shows a representative image of chemifluorescent immunoreactive total and phospho-EGFR proteins. RPTP-κ activity is inhibited by UV irradiation-induced oxidation, and confers UV irradiation induction of EGFR tyrosine phosphorylation, in primary human keratinocytes. B. Primary human keratinocytes were mock (No UV) or UV-irradiated (50mJ/cm^2), and whole cell lysates were prepared five minutes post UV irradiation. Endogenous RPTP-κ was immunoprecipitated, and the immunoprecipitate was divided for determination of activity, using a tyrosine-phosphorylated EGFR peptide as substrate, and

quantitation of RPTP-κ protein, by Western analysis. Levels of immunoreactive RPTP-κ protein were quantified by chemifluorescent detection. Results are means±SEM of three independent experiments; *p<0.05. Inset shows a representative image of chemifluorescent immunoreactive RPTP-κ protein. C. Primary human keratinocytes were mock or UV-irradiated (50mJ/cm^2), and whole cell lysates were prepared in buffer containing iodoacetic acid (IAA, 10mM) to irreversible inhibit non-oxidized protein tyrosine phosphatase activity, five minutes post UV irradiation. Endogenous RPTP-κ was immunoprecipitated, and assayed for activity in buffer containing dithiothreitol DTT to reduce oxidized RPTP-κ to restore enzymatic activity, using a tyrosine-phosphorylated EGFR peptide as substrate. Results are means±SEM of three independent experiments; *p<0.05. D. RPTP-κ was immunoprecipitated from mock or UV-irradiated human keratinocytes, and the Immunopreciptates were treated with DTT to reduce oxidized RPTP-κ, as described above in C. Reduced RPTP-κ was irreversibly oxidized by pervanadate, and oxidized (OX-PTP) and total RPTP-κ were detected by Western blot, using specific antibodies. Results are means±SEM of three independent experiments; *p<0.05. Inset shows a representative image of chemifluorescent immunoreactive RPTP-κ protein.

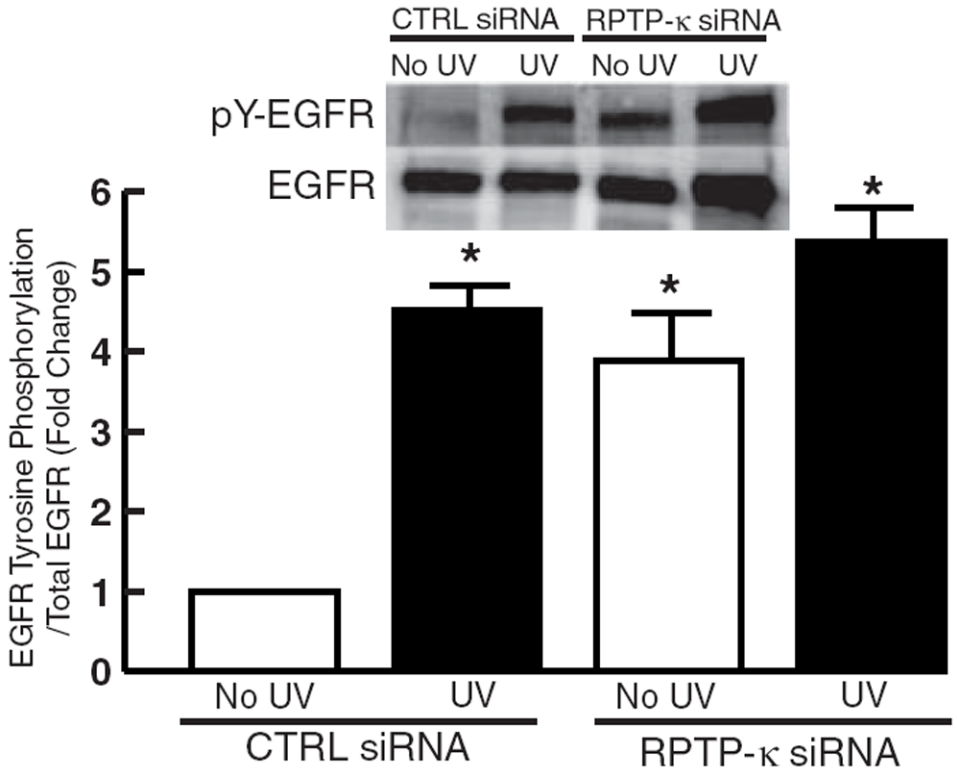

Figure 4.

Primary human keratinocytes were transfected with scrambled control (CTRL) or RPTP-κ siRNA using Amaxa Nucleofector. Two days after transfection, cells were UV irradiated (50mJ/cm^2). Whole cell lysates were prepared 15 minutes post UV irradiation, and subjected to Western analysis for total EGFR and tyrosine-phosphorylated EGFR. Levels of immunoreactive EGFR were quantified by chemifluorescent detection. Results are means ±SEM of three independent experiments; *$p<0.05$. Inset shows a representative image of chemifluorescent immunoreactive total and phospho-EGFR proteins.

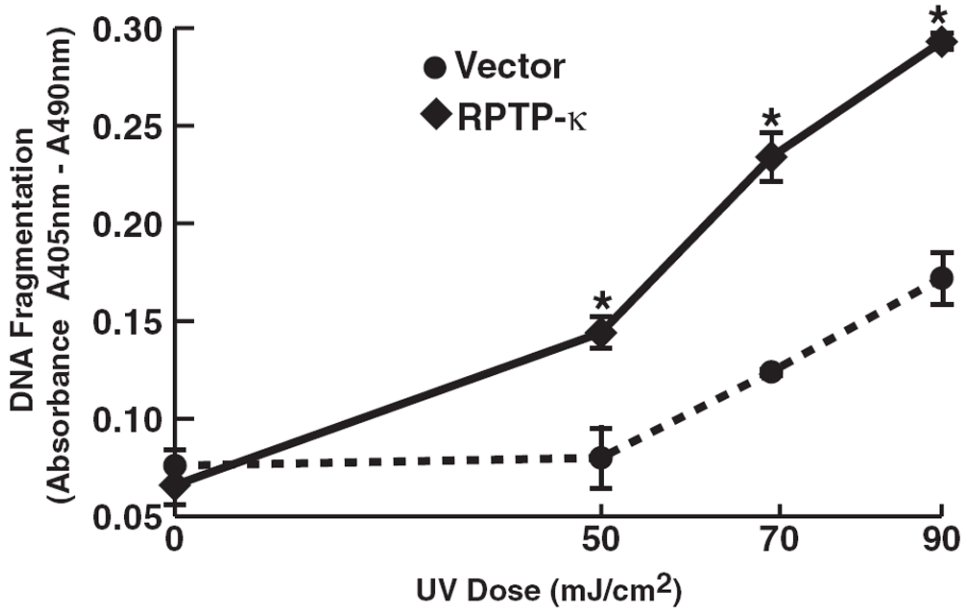

Figure 5.

RPTP-κ enhances UV irradiation-induced DNA fragmentation. Human primary keratinocytes were infected with either empty or RPTP-κ adenovirus. Cells were mock or UV-irradiated two days post infection. Six hours post UV irradiation, cells were lysed, and DNA fragmentation was measured by ELISA. Results are means±SEM of three independent experiments; *p<0.05 RPTP-κ vs. empty vector.

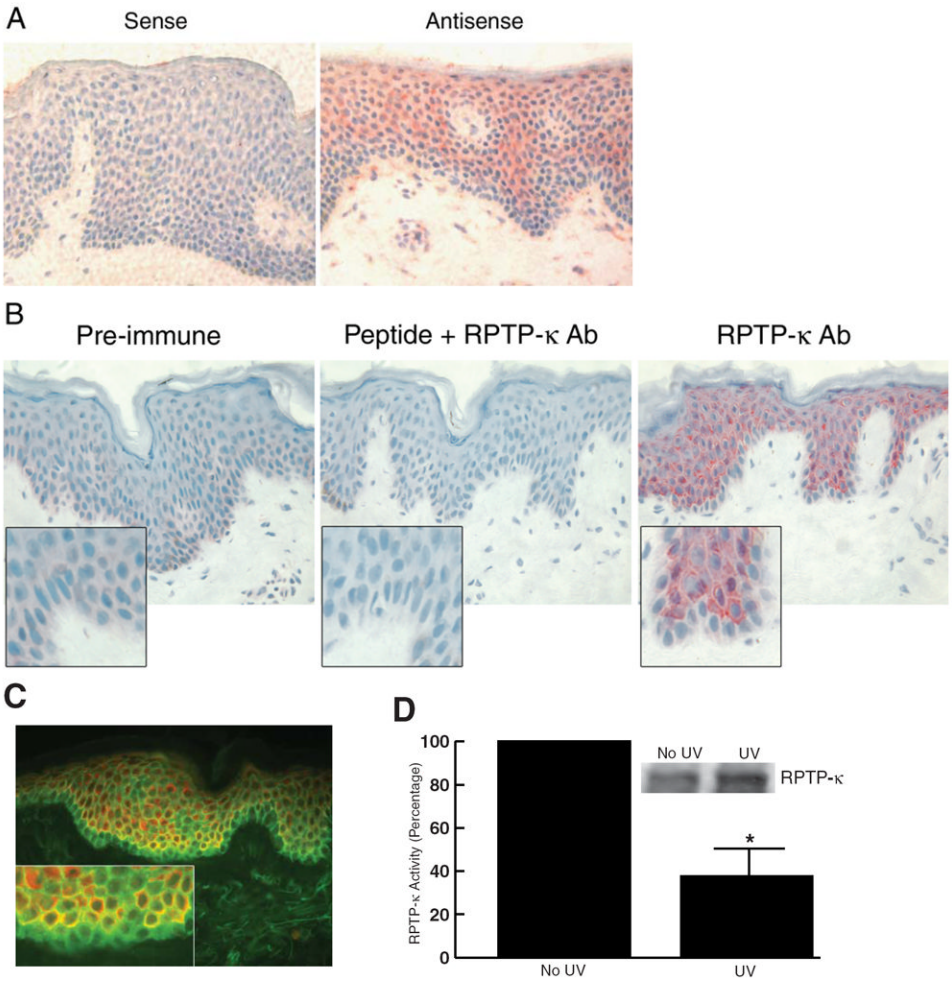

Figure 6.

Localization of RPTP-κ in human skin and inhibition of RPTP-κ activity by UV irradiation of *in vivo*. A RPTP-κ mRNA expression in human epidermis, detected by *in situ* hybridization. B. RPTP-κ protein expression in human epidermis, detected by immunohistochemistry. Pre-immune serum, and neutralization of RPTP-κ antibody (Ab) with immunogenic peptide were used as controls for specificity of staining. C. Co-localization of EGFR (green) and RPTP-κ (red) proteins in human epidermis, by double immunofluorescence staining. D. Sun-protected buttocks skin of human subjects were exposed to twice the minimal erythma dose of UV irradiation. Skin samples were obtained 30 minutes post irradiation. Endogenous RPTP-κ was immunoprecipitated, and the immunoprecipitate was divided for determination of activity, using a tyrosine-phosphorylated EGFR peptide as substrate, and quantitation of RPTP-κ protein, by Western analysis. Levels of immunoreactive RPTP-κ protein were quantified by chemifluorescent detection. Results are means±SEM of three independent experiments; *$p<0.05$. Inset shows a representative image of chemifluorescent immunoreactive RPTP-κ protein.