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**Organic hydroperoxide-induced lipid peroxidation (LPO) and signal
transduction pathways in human keratinocytes**

Dissertation

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1. Introduction

Research on lipid peroxidation has intensified over the past years as a result of increased awareness that these processes may play a role in a variety of disorders such as atherosclerosis, neurodegeneration, cancer, diabetes, and UV-induced skin cancer. Especially increased incidence of skin cancers and skin damage due to (over-) exposure of skin to UVR (ultraviolet radiation) have stimulated efforts in understanding the molecular mechanisms involved.

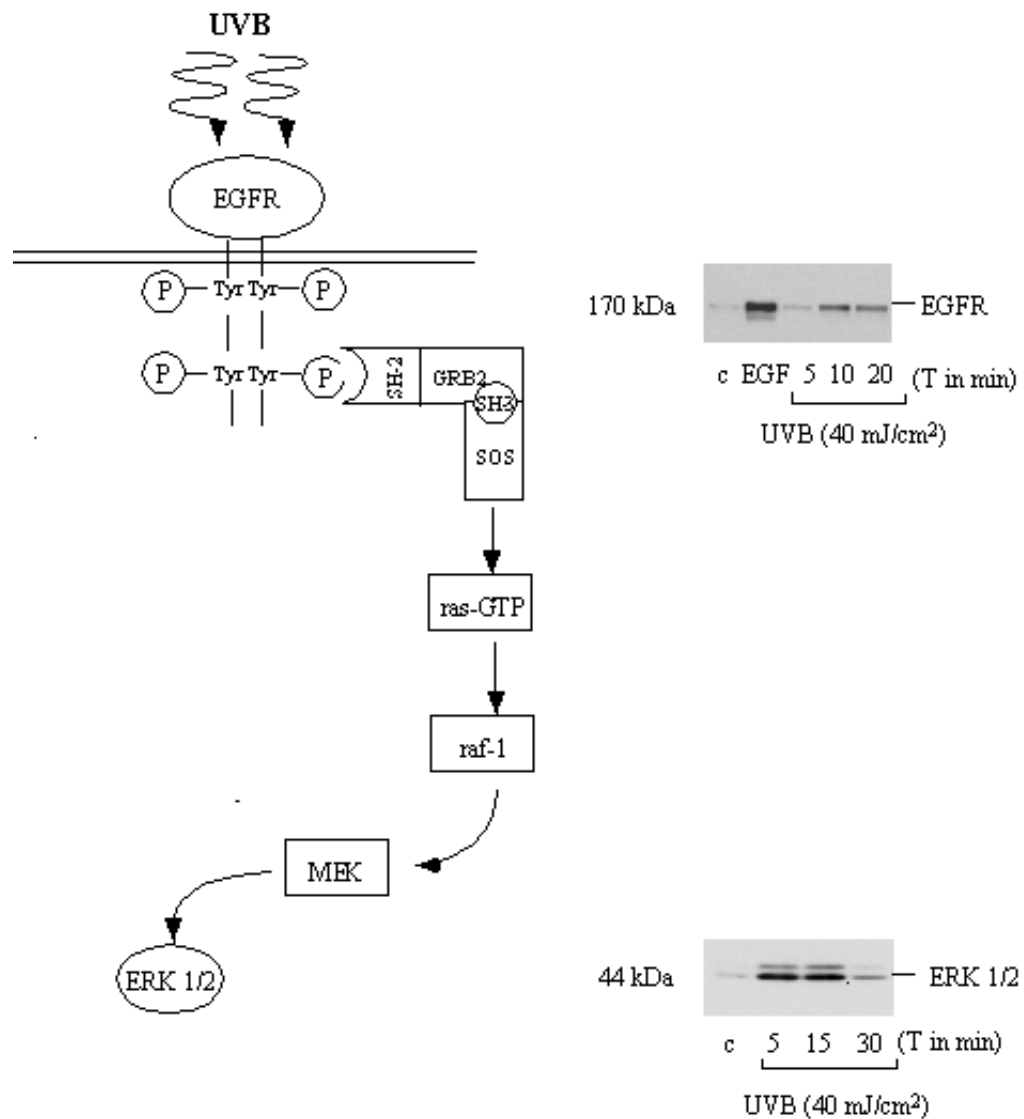
UVR has been identified as a potent activator of mitogen-activated protein kinases (MAPK) signaling cascades, as well as EGFR (epidermal growth factor receptor) phosphorylation (Knebel, 1996). These are cell signaling cascades that play important roles in promulgating events that contribute to cutaneous carcinogenesis. Responses to UVR are mediated by reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. Cumene hydroperoxide (CumOOH) is an organic hydroperoxide that has often been used as a model compound to initiate radical formation (van den Berg, 1992), lipid peroxidation (Weiss, 1986) and as a skin tumor promoter (Patamalai, 1994). As such, it was the aim of this study to further delineate pathways initiated by lipid peroxidation that are involved in stress signaling responses and in tumor promotion in human keratinocytes.

1.1. Signal transduction in human keratinocytes

1.1.1. EGF receptor and signal transduction pathways

EGF (epidermal growth factor), a 53-amino-acid polypeptide, plays an important role in cell proliferation and differentiation in many cell types. Growth factors, as well as other

peptide hormones are unable to directly cross the plasma membrane of their target cell. They require a cell surface receptor to bind growth factor ligand in order to function, such as the EGF receptor.



Scheme 1.1 depicts signal transduction pathways of EGFR activated by UVB. UVB irradiation leads to EGFR phosphorylation and subsequent ERK 1/2 stimulation.

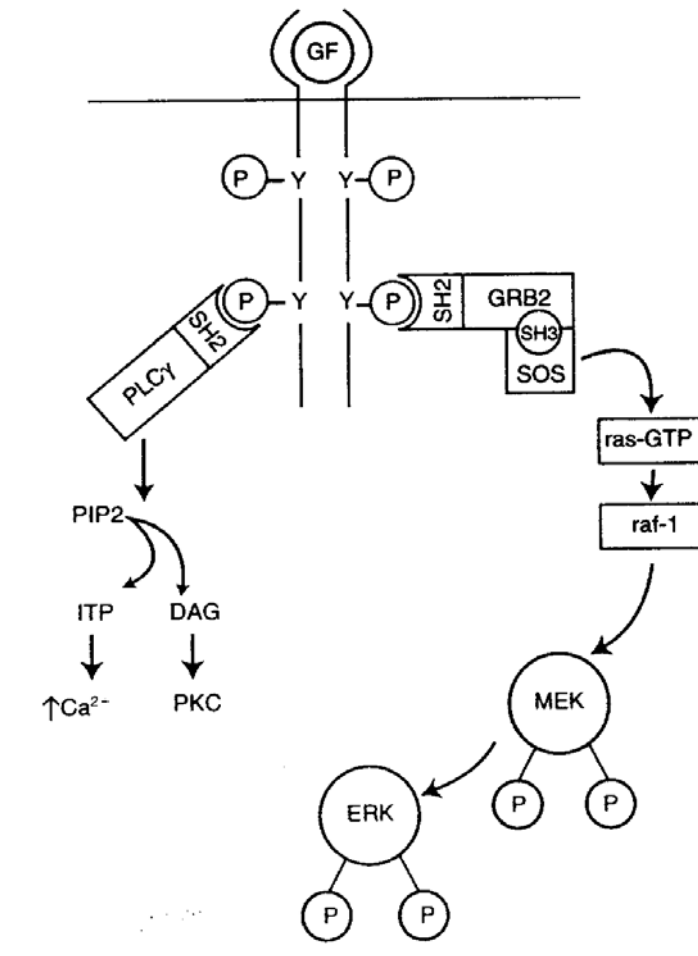
The EGF receptor belongs to a family of transmembrane cell surface receptors that bind ligand at the cell surface and are directly linked to intracellular enzymes on the cytoplasmic portion of the receptor. These receptors contrast with the G protein-coupled receptors and other receptors such as the steroid hormone receptors. EGF receptor belongs to the largest of the family of receptor protein-tyrosine kinases, which phosphorylate their substrate proteins on tyrosine residues, as first described by Dr. Stanley Cohen (Carpenter, G and S. Cohen, 1990).

The structure of the EGF receptor includes an N-terminal extracellular ligand-binding domain that is cysteine rich, a single transmembrane α -helix domain, and a C-terminal cytosolic domain that contains an ATP-binding site and has protein tyrosine kinase activity. EGF receptor is a monomer, i.e. a receptor in the resting state. However, there are two receptor binding sites that can induce conformational changes that promote receptor dimerization. Dimerization of EGF receptor induces autophosphorylation of the cytosolic domains due to cross-phosphorylation of the dimerized polypeptide chains. This creates *de novo* binding sites, within the activated receptor protein-tyrosine kinase, that serve as a docking mechanism for proteins that transmit intracellular downstream signals.

Binding of specific adaptor proteins to EGF receptor is mediated by specific protein domains within these substrate molecules termed SH2 domains that consist of approximately 100 amino acids. They mediate binding of proteins to specific phosphotyrosine-containing peptides. The designation, SH2 stems from the prototypic domain identified in the Rous sarcoma virus oncogene homologue, src and is called src homology (SH) 2. This binding is specific to pTyr and determined by the residues immediately carboxyl to pTyr (pTyr-X-X-X) (Cohen, 1995). The resulting association of SH2-containing proteins with activated RTKs (receptor protein-tyrosine kinases) may have several effects: stimulation of enzymatic

activity, promotion of phosphorylation, association with other proteins, and localization of the SH2-containing protein closer to the membrane. One form of phospholipase C, PLC- γ , contains

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Scheme 1.1.1. Cytoplasmic signaling of a tyrosine kinase receptor. Binding of growth factor (GF) results in phosphorylation of the intracellular components on tyrosine (Y). SH-2 mediated association of enzymes (for example phospholipase C γ occurs). Another molecule that binds on activated receptors via SH-2 linkage is the growth factor receptor binding protein 2 (GRB-2). SH-3 linkage then mediates the docking of son-of-sevenless (SOS) to GRB-2. From: "The Basic Science of Oncology", McGraw-Hill Company, (editors: Tannock, IF, and Hill, RP), New York, 1998.

SH2 domains which mediate the association of PLC- γ with the activated RTK. This leads to localization of PLC to the plasma membrane as well as its tyrosine phosphorylation, which

stimulates its catalytic activity: PIP₂ (phosphatidylinositol 4,5-bisphosphate) is hydrolyzed and yields DAG (diacylglycerol) and IP₃ (inositol trisphosphate). DAG mediates activation of protein kinase C family, IP₃ signals the release of Ca²⁺ from intracellular stores.

PKC targets the MAP kinase pathway and also the transcription factor NF-κB.

1.1.2. MAP kinase pathway --ERK

The mitogen-activated protein (MAP) kinase superfamily may be divided into at least three groups: ERK, SAPK/JNK, and p38 kinase. Central parts of the MAP kinase pathways are activated as a response to growth factors and other signaling molecules. Especially in higher eukaryotes, MAP kinases are regulators of cell growth and differentiation. One major family of MAP kinases is the extracellular signal-regulated kinase (ERK) family. Either protein-tyrosine kinase receptors or G protein-coupled receptors can activate ERK. As noted above, PKC also targets MAP kinase pathways, which may constitute the mechanism of cell stimulation by phorbol ester tumor promoters.

Activation of ERK is mediated by RAS, a small GTP-binding protein. RAS (from rat sarcoma virus, oncogenic proteins of tumor viruses that cause sarcoma in rats) functions as a monomer, rather than to associate with β or γ subunits, its function being dependent upon guanine nucleotide exchange factors, that stimulate the release of bound GDP and its exchange for GTP. (Activity of this complex is terminated by GTP hydrolysis through GTPase-activating proteins. Unregulated proliferation of cancer cells has been associated with inhibition of GTP hydrolysis.) Autophosphorylated RTKs result in binding of the guanine nucleotide exchange factor SOS via Grb2, a SH2 containing protein. This interaction translocates SOS to the plasma membrane, where it can stimulate guanine nucleotide exchange and thus activate RAS (Cohen, 1995). RAS-GTP binds to the N-terminus of RAF,

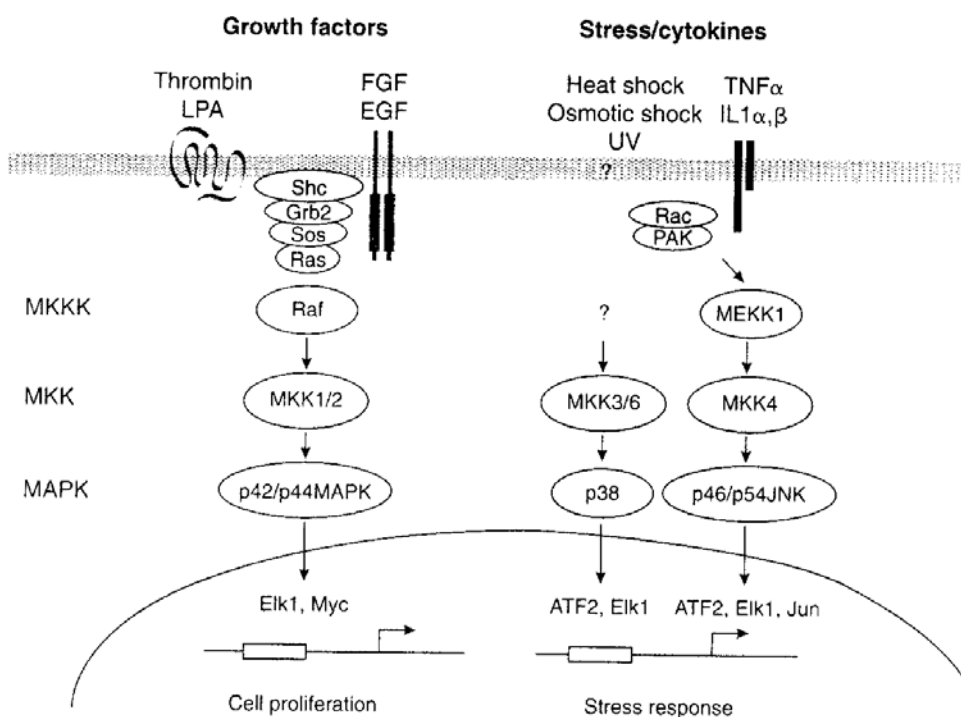
bringing RAF to the membrane and resulting in RAF autophosphorylation and activation. RAF is considered a MAPKKK, or MAP-kinase-kinase-kinase. RAF then activates and serine phosphorylates the cytoplasmic MEK1/MEK2 MAPKKs, dual specificity phosphokinases. Dual specific threonine/tyrosine phosphorylation activate ERK 1/2, MAPK (Hunter, 1995). ERK 1/2 phosphorylate a variety of target proteins, like PLA₂, the catalyst in formation of arachidonic acid. A fraction of the activated ERK 1/2 population translocates into the nucleus and phosphorylates transcription factors such as TCF/Elk 1 and 2. Elk 1 binds to SRE (serum response element)/SRF (serum response factor) and initiates thus early gene induction (Hill, 1995)

1.1.3. MAP kinase pathways --JNK and p38

Additional recently identified sets of the MAPK family are JNK and p38. They are termed stress-activated protein kinases (SAPK). Diverse external stimuli, such as UV irradiation, mitogens and cytokines (TNF α and IL-1) are activators of the JNK and p38 subgroup of MAP kinases. JNK is activated by the MAP kinase kinases MKK 4 and MKK7; this activation is mediated by dual phosphorylation within the Thr-Pro-Tyr motif. p38, another subgroup of the MAP kinases that is associated with the stress-activated protein kinases (SAPK) and is the mammalian homologue of the *Sacharomyces cerevisiae* HOG 1 gene, is only activated by MKK 4, not by MKK 7 (Davis, 1999). JNK activation leads to phosphorylation of specific sites of the transcription factor c-jun at the amino terminal trans-activation domain, enhancing its activity. C-jun is an important component of transcriptional activator AP-1; several other transcription factors are phosphorylated, most of them contribute to activation of AP-1. p38 does not phosphorylate the c-jun activation domain and must therefore be regarded as different in substrate specificity and function.

C-jun concentration is low in non-stimulated cells, with levels even lower in serum-deprived, quiescent cells. C-jun is encoded by the c-jun proto-oncogene, a sequence-specific transcription factor. Termed an immediate early gene, it is characterized with a rapid increase in c-jun transcription, increased c-jun mRNA and finally in an increase of c-jun protein. Post-translationally, phosphorylation of c-jun at serine 73 and to a lesser extent at serine 63 of the N-terminal activation domain occurs. JNK is the only MAPK that phosphorylates serines 63 and 73 at these positions (Minden, 1997).

Other JNK substrates that contribute to AP-1 activity are:



Scheme 1.1.3. Illustrates the differences between growth factor and stress-activated MAPK in mammalian cells. From: *Essays in Biochemistry*, Vol. 32, pp 1-14, 1997.

ATF-2, a transcription factor that dimerizes with c-Jun and then stimulates the expression of the c-jun gene. C-fos, an immediate response gene that is induced by serum response element (SRE). Although c-fos induction may also be mediated by ERK in response to growth

factors, it is JNK that is responsible for c-fos induction in response to cellular stress and cytokines. C-fos and c-jun form a heterodimer that is more stable than c-jun/c-jun homodimer, and thus exerts its effects longer on AP-1. Elk-1, a ternary complex factor (TCF), and serum response factor (SRF) can bind simultaneously to the SRE and induce gene transcription of genes like c-fos. Like c-jun, Elk-1 activity is regulated by phosphorylation. Although p38 does not phosphorylate c-jun, it may still contribute to AP-1 activity and to c-fos induction (Sugden, 1998).

There are more than 50 low molecular GTPases that control GTP-binding proteins. On the basis of sequence homology, these are divided into five classes: Ras, Rab, Arf, Ran, and Rho. As outlined above, Ras family members play an important role in cell growth and differentiation. Although also stimulated by growth factors, the Rho pathway is primarily activated in response to cytokines and environmental stress (e.g., ultraviolet irradiation). The Rho family includes Cdc42/G25K, Rac 1, Rac 2, Rho A, Rho B, Rho C. The JNK/SAPK (for Jun N-terminal kinases or Stress-activated protein kinases) and also the P38 pathways are activated by RhoA, Rac 1 and Cdc42 (Lopez, 1998), however, RhoA activates JNK only poorly (Coso, 1995). Chronic activation of these factors induces malignant transformation (Khosravifar, 1994).

For activation of the cascade to occur, Rac and Cdc, activated by GTP, bind to PAK65, a MAPKKKK. PAK 65 then activates MEKK1, which phosphorylates SEK1/MKK4. SAPK/JNK is activated by phosphorylation of Tyr 185 and then through autophosphorylation of Thr 183 (Verheij, 1998). This is the adequate stimulus for phosphorylation/activation of transcription factors such as TCF/Elk1, ATF-2, and c-Jun (phosphorylation sites are Ser 63/73. The p38 pathway is different: MEKK1 phosphorylates MKK3, 6 and then p38 MAP kinase is phosphorylated. It does not phosphorylate c-Jun, but

transcription factors TCF/Elk1, ATF-2 and Max/Myc, as well as CHOP, also known as growth arrest and DNA damage-inducible gene 153 (GADD 153) (Wang, 1996).

Given its activation by cellular stress, it has been proposed that JNK activation is involved in programmed cell death (apoptosis). Apoptosis seems to be an event that is mediated by SAPK/JNK with pro-apoptotic stimuli and on the other hand counterbalanced by MAPK/ERK with anti-apoptotic stimuli (Verheij, 1998). JNK functions, however, vary depending upon cell type and cell condition. In B cells, for example, JNK suppresses apoptosis. On the other hand, mechanisms guiding apoptosis may be different in each cell type (Minden, 1997).

1.2. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are widely found in biological systems; aerobic cells produce them. They include both, oxygen-free radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($HO\cdot$), perhydroxyl radical ($HO_2\cdot$), peroxy radical ($ROO\cdot$), and alkoxy radical ($RO\cdot$), and non-radical species such as singlet oxygen (1O_2) and hydrogen peroxide (H_2O_2). Sources of ROS are numerous: the electron transport chain, phagocytosis, oxidant enzymes, autooxidation, drugs, tumors, sunlight and ionizing radiation. Superoxide anion is transformed by superoxide dismutase (SOD) into H_2O_2 . TPA, one of the classical tumor promoters has been classified as a peroxisome proliferator. Peroxisomes oxidize activated long- and medium-chain fatty acids and by removal of two carbons can generate one molecule of H_2O_2 (Lillehaug, 1986). ROS are thought to be involved in activation of phospholipase A_2 , the activation of protein kinases and the inhibition of protein tyrosine phosphatases (Goldmann, 1997). Recently, our laboratory has shown that UVB-induced H_2O_2 is an important mediator in EGF-receptor phosphorylation (Peus, 1998), as well as in activation of

ERK 1/2 . The primary lines of defense against H_2O_2 include catalase ($2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$) and Glutathione with Glutathioneperoxidase ($\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow 2 \text{GSSG} + 2 \text{H}_2\text{O}$) (Florence, 1995). The latter one is also capable of disposing of lipid hydroperoxides ($\text{LOOH} + 2 \text{GSH} \rightarrow 2\text{GSSG} + \text{LOH} + \text{H}_2\text{O}$). Components of the secondary defense system are ascorbic acid, alpha-tocopherol (vitamin E), vitamin A, and others. Usually, detoxification of ROS occurs readily by the cell's antioxidant system. Oxidative stress is then a condition, where this balance is distorted. Persisting oxidative stress has a profound impact on the biological system of the cell, such as alterations in signal transduction, gene expression, and cell death. For example, exposure of cells to H_2O_2 stimulates increased expression of jun-B, jun-D, c-fos, and fos-B (Crawford, 1988). PKC seems to be an important mediator in stimulation of these early-response genes (Rao, 1993). Effects of antioxidants seem to be rather specific, as target genes are induced with different kinetics. This specificity could be a result from a combination of oxidant and chemical properties of a substance, that may lead to a complementation and promotion of oxidative stress.

Recently, our laboratory was able to demonstrate that UVB activates ERK 1/2 and p38 signaling pathways via ROS. It was shown that H_2O_2 stimulates the formation of an SHC-Grb2-SOS complex (Rao, 1996), that activates Ras-GDP by converting it to its activated GTP-bound form. As mentioned before, activated Ras stimulates Raf-1 translocation to the plasma membrane, where Raf-1 is activated by phosphorylation. In turn, activated Raf-1 phosphorylates serines on the active sites of MKK/MEK, thus regulating down-stream responses.

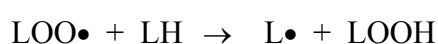
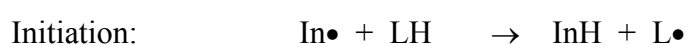
ROS that target phosphatases also have a profound effect on signal transduction processes, as normal cellular functions rely on the reversibility of kinase actions. H_2O_2 is a potent inhibitor of phosphatases (PTPs) (Mahadev, 2001) It was found that cyclin D1 expression is regulated

by ERK 1/2 activation and could be inhibited by p38 activation. Cyclin D1 expression is the earliest cell cycle related event to occur during G0/G1 to S-phase transition (Lavoit, 1996).

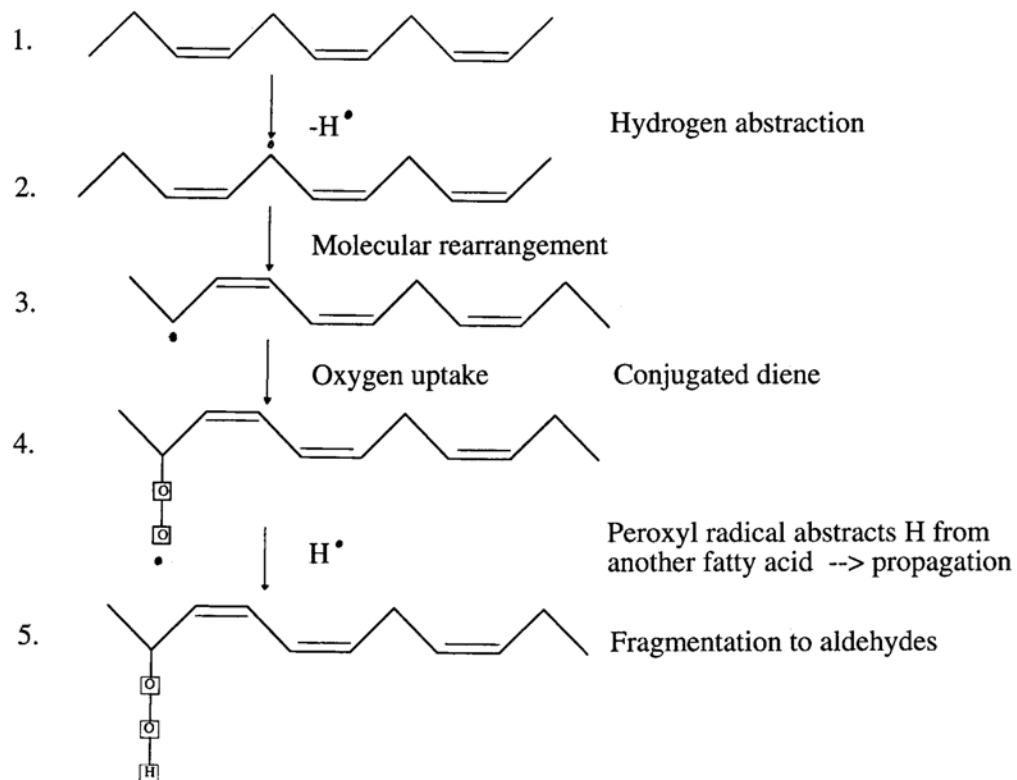
ROS and oxidative damage have been implicated in the induction of apoptosis (Tamarit, 1998). Glutathione depletion may play a major role in this process. In a human lymphoblastoid cell line, low doses of H₂O₂ induced apoptosis via production of OH• radicals and alteration of the oxidant and anti-oxidant pathway (Toledano, 1997).

1.3 Lipid peroxidation (LPO)

Lipid peroxidation is one feature of cellular damage brought about by free radical attack and oxidation of unsaturated lipids. Polyunsaturated fatty acids make up essential building blocks of the cell's structural elements, including the cell membrane, the endoplasmatic reticulum, and mitochondria. Lipid peroxidation disrupts the proper cell structure and can have dire consequences for cellular function. The peroxidation process involves three defined processes: initiation, propagation and termination (Yu, 1994). Initiation occurs, for example, when an initiator radical (In•) abstracts a hydrogen atom from L-H and forms thus a lipid radical, L•. Propagation involves the addition of molecular oxygen to the lipid radical, obtaining a peroxy radical, LOO•. Propagation is complete, when LOO• abstracts a hydrogen atom from LH to generate another radical L•. 2 LOO• can form LOOOOL and thus terminate the sequence (Porter, 1995): In this case, a dimer is formed at the expense of a polyunsaturated fatty acid cross linkage, resulting in alteration of membrane properties.

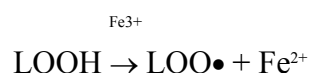


Termination: $2 \text{ LOO}\bullet \rightarrow \text{LOOOOL}$

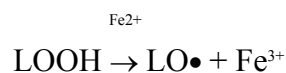


Scheme 1.3.1 illustrates the steps involved in ROS-induced Lipid peroxidation.

Lipid hydroperoxide (LOOH) are more stable than its precursors and if not disposed of by the glutathione system, they might be converted to alkoxyl ($\text{LO}\bullet$) or peroxy ($\text{LOO}\bullet$) radicals. A prerequisite is the presence of iron (Yu, 1994):

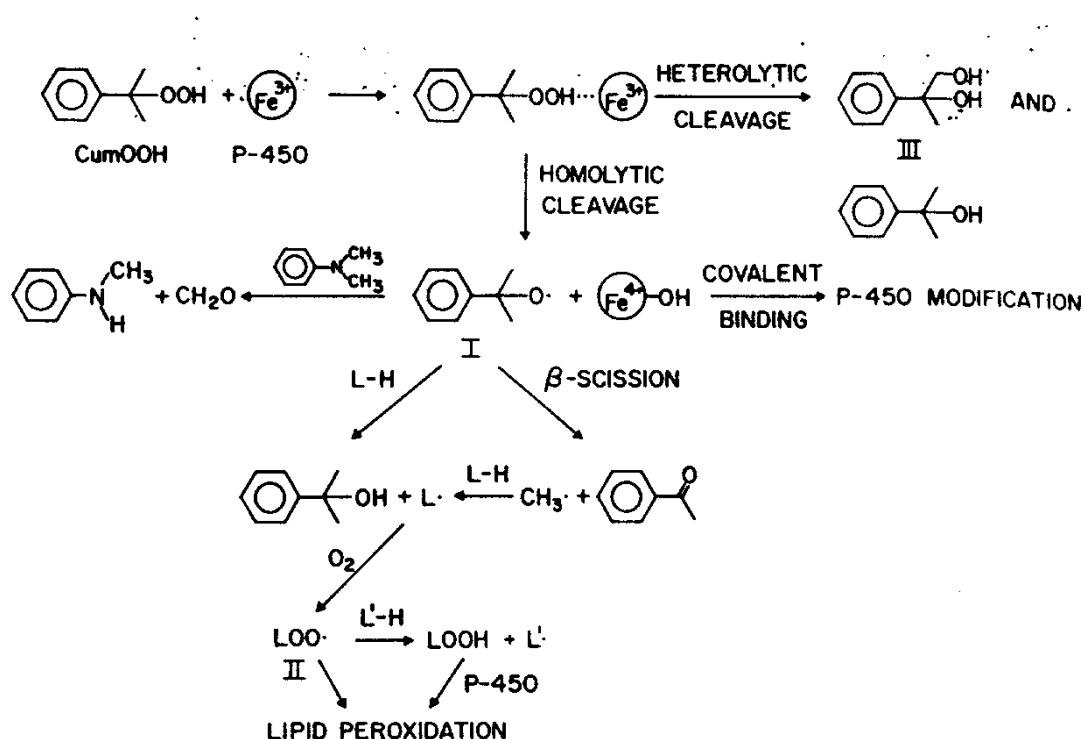


or



The importance of LPO is significant, given the direct impact on membrane integrity, the production of second messengers or the depletion of GSH, which predisposes cells to oxidative stress. Lipid peroxidation products have important influences on cell signaling pathways, as it is thought that they increase cellular calcium content (Sweetman, 1995).

The mode of action of CumOOH-mediated lipid peroxidation is illustrated in the following scheme.



Scheme 1.3.2. Depicts the pathway to lipid peroxidation for CumOOH and microsomal P-450. From: Weiss, R.H.; Estabrook, R.W. 1986. *Archives of Biochemistry and Biophysics*. 251,1:348-360

There are numerous ways of assessing LPO. Most common is the spectrophotometric assay of malondialdehyde (MDA) following its reaction with thiobarbituric acid. Sensitivity and specificity of this method can be enhanced by high performance liquid chromatography with fluorimetric detection of the MDA-TBA adduct (Young, 1991). Biochemical assay kits (LPO Assay Kit, Calbiochem, La Jolla, Ca) measure MDA and 4-Hydroxyalkenals, such as 4-Hydroxynonenals (4-HNE). 4-HNE, the aldehydic end product of lipid peroxidation, has been found to cause EGFR phosphorylation and subsequent MAPkinase activation in human epidermoid carcinoma A 431 cells, ultimately resulting in inhibition of cellular growth (Liu, 1999). It has also been implicated in the direct interaction with JNK isoforms in ITO cells of the liver (Parola, 1998), followed by increased levels of c-jun. Interestingly, this activation occurred independently from JNK phosphorylation. 4-HNE could conceivably interact directly with JNK isoforms.

Finally, Uchida (Uchida, 1999) linked expression of c-jun after treatment of rat liver epithelial cells with 4-HNE to depletion of intracellular glutathione (GSH) and the induction of intracellular peroxides.

Yet another commercially available kit (Cayman Chemical Company, Ann Arbor, MI) measures hydroperoxides directly.

In our experiments, none of these methods were sufficient for the measurement of appreciable amounts of LPO, therefore, we used cis-parinaric acid in conjunction with flow cytometry (Hedley, 1992). It could be argued that the non-dye methods described above did not bring sufficient results because of the fact that cultured keratinocytes unsupplemented with unsaturated fatty acids have only very small amounts of polyunsaturated fatty acids in their membranes (Ponec, 1988). Therefore, the products of lipid peroxidation would be harder to detect.

2. Materials and Methods

2.1. Methods

2.1.1. Culture of human keratinocytes.

Human keratinocytes were isolated from neonatal foreskin specimens obtained within 1-2 h after routine circumcision of newborn male infants. Subcutaneous tissue was removed; skin was cut into 4-5 mm pieces and digested with 0.25 % trypsin/EDTA overnight. The epidermis was then separated from the dermis, human keratinocytes were isolated, primary cultures were initiated by seeding approx. $5 \times 10^3/\text{cm}^2$ into 75 cm^2 tissue culture flasks and incubated at 37°C in humidified atmosphere of 5 % CO_2 and 95% air. Stocks were maintained in a replicative state with complete, serum free MCDB 153 medium + growth factors, they were re-fed every other day (Wille JJ, 1984). At a density of approx. $8 \times 10^3/\text{cm}^2$ (subconfluence) cells were then trypsinized for 20 min, trypsin was deactivated by 2% dFBS in Solution A, and the number of cells was determined with a hemacytometer. Cells were then spun down at 1000 rpm for 10 min at 4°C , resuspended in medium and then passed into secondary culture at different densities. For differentiation, keratinocytes were grown to confluence with MCDB 153 medium + growth factors. Where indicated, cells were grown without growth factors for 48 h after confluence, as to minimize stimulation of the EGF receptor.

The keratinocyte cell line HaCat was cultured in DMEM (Mediatech, Herndon, VA) containing 10% dFBS (Life Technologies, Grand Island, NY). Bcl-2 transfected cells (vector: pcDNA1/Neo + bcl-2cDNA) received media supplemented with 400 $\mu\text{g}/\text{ml}$ of geneticin. The cell line termed Neo expresses very low levels of Bcl-2 protein, similar to parental HaCat cells, and was thus used as a control

2.1.2. Detection of intracellular H₂O₂ by flow cytometric analysis

Keratinocytes were loaded with 5 μ M dihydrorhodamine DHR 123 (obtained from Molecular Probes, Eugene, OR) for 45 min before treatment with CumOOH. Intracellular production of H₂O₂ oxidizes reduced DHR and irreversibly converts it to the red fluorescent compound rhodamine (Royal JA, 1993). Fluorescence intensity of 10,000 cells per sample was measured using a Becton-Dickenson FACS Star flow cytometer with an argon-ion laser, excitation/emission wavelengths were set at 488 and 580 nm longpass respectively, control mean fluorescence was set at 100.

2.1.3. Lipid peroxidation assay

Primary human keratinocytes were loaded with 10 μ M cis-parinaric acid (cPA) (Molecular Probes, Eugene, OR) in complete medium for one hour (Hedly and Chow, 1992). Cells were then washed and treated with CumOOH. After trypsinization and fixation in paraformaldehyde, 10,000 cells were analyzed by flow cytometry immediately, using a Becton-Dickenson FACS Star Plus with an argon ion laser with excitation/emission wavelength set at 334-364 and 424 nm respectively.

For laser scanning confocal microscopy, cells were treated in the same manner as above. Keratinocytes were cultured on coverslips and images were obtained with an inverted laser scanning confocal microscope 1SM410 (Zeiss, Germany), which is equipped with argon-krypton laser, emitting 488 nm for excitation, deflected by LP510 dichroic filter and an Em filter, LP 515. Pinhole size was set at 1 μ m. Fluorescent intensities were converted into pseudocolors using the program Analyze® (Mayo Foundation, Rochester, MN)

2.1.4. Immunoprecipitation and tyrosine phosphorylation assay

Cell lysates were obtained by scraping treated, confluent cultures grown in -GF for 48 h into 1 ml of Frackelton buffer with protease inhibitors, centrifugation for 15 min at 12,000g. After adding monoclonal EGFR antibody (gift of Peter Dempsey, Vanderbilt) and Protein G-Plus Agarose beads (Calbiochem, La Jolla, CA) to the supernatant, samples were incubated and rotated for 3 hours at 4° C. Release of protein from beads into 4x sample buffer was obtained by heating samples for 5 min @ 100° C and centrifugation for 5 min @ 5000 rpm. After 10% SDS-PAGE, transfer to an Immobilon-PVDF-transfer membrane (Millipore Corporation, Bedford, MA), blocking with 2 % bovine serum albumin in TBS/Tween for one hour, anti-phosphotyrosine (PY 20) coupled to peroxidase (Transduction Laboratories, Lexington, KY) was added 1:20. Bands were finally visualized using the ECL detection system (Amersham, Arlington Heights, IL). Total EGFR protein was detected with EGFR-15E11 SB1 (A10) hybridoma antibody, courtesy of Dr. N. Maihle, Mayo Clinic, Rochester, MN and secondary ab 1: 4000 goat anti-mouse Ig (H+L)-HRP, (Cat # 1010-05, Southern Biotechnology Associates, Birmingham, AL) after stripping the membrane with guanidine solution.

2.1.5. Western blot assay

Cells were lysed using a modified lysis/sample buffer, heated to 100° C for 5 min, and then subjected to 10 % SDS-PAGE. After transfer and blocking in 2% BSA/TBS/Tween solution, polyclonal MAPK antibody (Promega, Madison, WI) or polyclonal phospho-p 38 MAPK (Thr 180/Tyr 182) (New England Biolabs, Beverly, MA) were added. After addition of appropriate secondary antibody (rabbit Ig HRP, DACO, Denmark), bands were visualized

with the ECL detection system as above. Total p38 protein was detected using p38-MAPK AB, 1:1000 in 2% TBS/Tween (New England Biolabs, Beverly, MA, # 9212).

2.1.6. JNK assay

JNK activity was determined by incubating lysates from keratinocytes challenged with CumOOH with GST-c-Jun (1-223) GSH-agarose beads for several hours (Hibi, 1993): Cell lysates were extracted in a lysis buffer (WCE buffer) with protease inhibitors. After rotation for 30 min at 4° C and centrifugation (15 min at 12,000 g), supernatants were mixed with the GSH-agarose beads and incubated for several hours or overnight at 4° C. After aspiration of the supernatant and extensive washing with HBB buffer, an in-vitro kinase assay with ³²P-gamma ATP at 0.25mCi/4500Ci/mMol (cat. # 35001.X, ICN, Costa Mesa, CA) in KB buffer for 20 min was performed, followed by 12% SDS-PAGE, gel drying and autoradiography (Kodak, New Haven, CT)

2.1.7. Extracellular hydrogen peroxide assay

Measurement of extracellular levels of H₂O₂ following treatment with CumOOH was performed using an Amplex™ assay (Molecular Probes, Eugene, OR), a derivative of dehydrophenoxazine, which -in the presence of horseradish peroxidase (Sigma, P6104)- becomes highly fluorescent upon oxidation by H₂O₂. Fluorescence was quantified using a CytoFluor II® microplate fluorometer (Perseptive Biosystems, Framingham, MA), with excitation/emission wavelength filters set at 590/645 nm respectively. A standard curve was produced for each experiment. Control fluorescent values of CumOOH alone and no cells or cells alone were monitored over time and subtracted from the fluorescent intensity of the experimental samples. Using the standard curve, these values were converted into μM.

Measurements were performed in 24-well plates, with 50 μ M Amplex™ and 1 U/ml horseradish peroxidase (Mohanty et al, 1997)

2.1.8. Electroporation

At 90% confluency, cells were harvested by trypsin treatment and resuspended in growth factor-free complete medium at a density of 10^7 cells/ml. 400 μ l of the cell suspension were placed in an electroporation chamber in the presence or absence of catalase (30,000 units/ml) (Sigma, St. Louis, MO). Electroporation was then performed by subjecting cells to a field strength of 375 ± 25 V/cm for 20 ms (Bae et al, 1997), using a Bio Rad Gene Pulser (Bio Rad, Richmond, CA). Cells were then transferred to growth factor supplemented complete media and placed in the incubator for 12 h and then washed twice in Solution A. Medium without growth factors was added for 2 days.

2.1.9. GSH detection assay

Cells were grown to confluency in 96-well plates. After treatment, cells were lysed by adding 50 μ l of 2% trichloroacetic acid (TCA) and incubated on a rocker (37° C) for 45 minutes. After the incubation, 50 μ l of 200 mM Trizma Base (pH 9.3) were added. A 1:75 dilution of GSH-S-transferase in KRH buffer and a 1:150 dilution of monochlorobimane in acetonitrile (10 mM) was added in 100 μ l of media without growth factors and incubated on a rocker (37° C) for 15 minutes. Plates were then evaluated in a CytoFluor II™ plate reader, with excitation wavelength set at 360 nm and emission wavelength set at 460 nm.

2.2. Materials

2.2.1. Solutions for cell culture

2.2.1.1. 153 Basal media (MCDB 153)

The water used was purified by a Millipore Filtering system.

Amount/4 liters

Glucose	4.32 g
NaCl	30.39 g
Hepes	26.40 g
Na Acetate•3 H ₂ O	2.00 g
L-glutamine	3.50 g
Na-pyruvate	0.22 g
Cysteine	0.16 g

Stock #	ml/4 Liters
1	40
2	40
3	80
4a	2
4b	4
K2	4
6c	4
Phenol red	4
8	40
9	40
10	40
L	40

pH to 7.4 using 10 N NaOH, then 4.704 g of NaHCO₃ is added.

QS to 4 liters. Filter sterilized with .2 µ filters. Stable at 4°C for up to 2 weeks.

Standard Media = -GF = MCDB 153 with the standard non-protein factors

Added. These are ethanolamine, phosphorylethanolamine and hydrocortisone. Stable for 2 weeks.

Complete Media = +GF = MCDB 153 has the complete requirements for growth. It is the -GF media with EGF, insulin, and BPE added to it. Stable for approximately 1 week.

2.2.1.2 Preparation of media with or without growth factors (+GF or -GF)

MDCB 153 medium was poured into a bottle-top 0.2 μ filter attached to a sterile bottle. Ethanolamine, phosphorylethanolamine, hydrocortisone, Pen/Strep, and 6 high amino acids were added into MCDB153. After filtration, the filter was removed and EGF, insulin, and BPE were added sterilely.

-GF Media:

	<u>Final Conc</u>
Ethanolamine	10^{-4} M
Phosphorylethanolamine	10^{-4} M
Hydrocortisone	5×10^{-7} M
Penicillin/Strep	see antibiotics
6 high amino acids	see high amino acids

+GF Media:

EGF	10 ng/ml
Insulin	5 μ g/ml
BPE	≈ 28 μ g/ml

Ethanolamine (2-aminoethanol): a 16.4 M solution. Made up as 10^{-1} M stock in solution A, stored in freezer at -20°C .

Phosphorylethanolamine (O-phosphoryl-ethanolamine): 141.1 g/mole, made up as 10^{-1} M stock in solution A and stored at -20°C .

Epidermal Growth Factor = EGF: Made up as 10 μ g/ml stock in Solution A and stored in freezer at -20°C .

Insulin: Made up as 5 mg/ml stock in 0.01 M HCl and stored at -20°C .

Hydrocortisone: Made up as 5×10^{-4} M stock in 95% EtOH and stored in freezer at -20°C .

2.2.1.3. Media for HaCat cells

Serum-free:

13.36 g/l Cellgro DMEM (with glucose and L-glutamine; without Na Bicarbonate) 3.7

g/l NaHCO₃.

Made in 900 ml H₂O. Do not pH. Filter sterilized .2 μ.

With serum:

10% FBS (.2 μ filter sterilized) are added. 1:1000 P/S are added.

2.2.1.4. Preparation of MDCB 153

Stock 1

Component	Final Medium moles/l
Arginine•HCl	1x10 ⁻³
Histidine•HCl•H ₂ O	8x10 ⁻⁵
Isoleucine allofree	1.5x10 ⁻⁵
Leucine	5x10 ⁻⁴
Lysine•HCl	1x10 ⁻⁴
Methionine	3x10 ⁻⁵
Phenylalanine	3x10 ⁻⁵
Threonine	1x10 ⁻⁴
Tryptophan	1.5x10 ⁻⁵
Tyrosine	1.5x10 ⁻⁵
Valine	3x10 ⁻⁴
Choline chloride	1x10 ⁻⁴
Serine	6x10 ⁻⁴

Stock 1 is storable at 4°C for up to two months or frozen at -20°C for longer periods.

Stock 2

Component	Final Medium moles/l
Biotin	6x10 ⁻⁸
Ca pantothenate	1x10 ⁻⁶
Niacinamide	3x10 ⁻⁷
Pyridoxine•HCl	3x10 ⁻⁷
Thiamine•HCl	1x10 ⁻⁶

KCl 1.5×10^{-3}

Stock 2 is storable at 4°C up to two months, or frozen at -20°C for longer periods.

Stock 3

Component	Final Medium
	moles/l
Folic acid	1.8×10^{-6}
Na ₂ HPO ₄ •7 H ₂ O	2×10^{-3}

Stock 3 is storable at 4°C up to two months, or frozen at -20°C for longer periods.

Stock 4a, 4b, K2 (Individually prepared)

Component	Final Medium
	moles/l
K2 FeSO ₄ •7 H ₂ O	5×10^{-6}
4b MgCl ₂ •6 H ₂ O	6×10^{-4}
4a CaCl ₂ •2 H ₂ O	1×10^{-4}

K2 - for 100 ml: 0.139 g FeSO₄•7 H₂O in 2.0 ml of .5 M Hcl were dissolved. The solution was then diluted to 100 ml with d H₂O (i.e. 10 mM HCl) and filter sterilized.

4b - for 100 ml: 12.2 g MgCl₂•6 H₂O were dissolved in 100 ml d H₂O and filter sterilized.

4a - for 100 ml: 2.9406 g of 2000x (2×10^{-1} M stock, 100 μM Ca⁺⁺ in final medium) were dissolved in 100 ml d H₂O and filter sterilized.

4a, 4b, and K2 are storable at room temperature indefinitely.

Stock 5

Component	Final Medium
	moles/l
Phenol red	3.3×10^{-6}

Stock 5 was frozen in -20°C.

Stock 6c

Component	Final Medium moles/l
Riboflavin (1000x)	1×10^{-7}

Stock was frozen at -20°C and protected from light.

Stock 8

Component	Final Medium moles/l
Asparagine	1×10^{-4}
Proline	3×10^{-4}
Putrescine	1×10^{-6}
Vitamin B ₁₂	3×10^{-7}

Stock 8 is storable at 4°C for up to two months, or frozen -20°C for longer periods.

Stock 9

Component	Final Medium moles/l
Alanine	1×10^{-4}
Aspartic acid	3×10^{-5}
Glutamic acid	1×10^{-4}
Glycine	1×10^{-4}
Phenol red	3.3×10^{-6}

Stock 9 is storable at 4°C for up to two months, or frozen at -20°C for longer periods.

Stock 10

Component	Final Medium moles/l
Adenine	1.8×10^{-4}
Myo-inositol	1×10^{-4}
Lipoic acid	1×10^{-6}
Thymidine	3×10^{-6}
CuSO ₄ •5 H ₂ O	1×10^{-8}

Stock 10 is storable at 4°C for up to two months, or frozen at -20°C for longer periods.

Stock L (Trace Elements):

Component	Final Medium
	moles/l
CuSO ₄ •5 H ₂ O	1.1x10 ⁻⁸
H ₂ SeO ₃	3x10 ⁻⁸
MnSO ₄ •5 H ₂ O	1x10 ⁻⁹
Na ₂ SiO ₃ •9 H ₂ O	5x10 ⁻⁷
(NH ₄) ₆ MO ₇ O ₂₄ •4 H ₂ O	1x10 ⁻⁹
NH ₄ VO ₃	5x10 ⁻⁹
NiCl ₂ •6 H ₂ O	5x10 ⁻¹⁰
SnCl ₂ •2 H ₂ O	5x10 ⁻¹⁰
ZnSO ₄ •7 H ₂ O	5x10 ⁻⁷

Stock for high amino acids:

Component	Final Medium
	moles/l
L-histidine•HCl• H ₂ O	8x10 ⁻⁵
L-isoleucine	1.5x10 ⁻⁵
L-methionine	3x10 ⁻⁵
L-phenylalanine	3x10 ⁻⁵
L-tryptophan	1.5x10 ⁻⁵
L-tyrosine	2.5x10 ⁻⁵

2.2.1.5. Preparation of BPE (bovine pituitary extract)

Fresh, trimmed, mixed-sex beef pituitaries were obtained from Pel-Freeze, P.O. Box 68, Rogers, AR 72756, USA

The preparation of a saline extract of whole bovine pituitaries was followed as described earlier (Tsao, 1982). Protein content was verified by Bio-Rad protein assay method (Bradford method) average 12-14 mg/ml. Samples were filter sterilized (0.2 µm), aliquoted and then frozen at -20°C .

2.2.1.6. Solution A

	<u>Final</u>
Glucose	10.0 mM
KCl	3.0 mM
NaCl	130.0 mM
Na ₂ HPO ₄ anhydrous	1 mM
Phenol red	0.0033 mM
Hepes	30.0 mM

Hepes was dissolved and pH was brought to 7.4 with 1 or 10 M NaOH. The other components were dissolved and the pH was re-checked. Storage at 4°C. When making the 1x from the 10x stock, the pH was re-checked. Solution was filter-sterilized. (Shipley, 1981)

2.2.1.7. 0.025% trypsin with 0.01% EDTA for passing cultures

0.08 g EDTA (disodium) was dissolved in 800 ml Solution A (room temperature). The pH was adjusted to 7.4 with 1 M NaOH. Before adding trypsin, this solution was covered with parafilm and chilled overnight in the cold room. 0.2 g trypsin was dissolved in the EDTA solution. The solution was then filtered through a 0.2 µ filter into 500 ml bottles on ice and stored at -20°C. Used for routine passing of cell cultures.

2.2.1.8. Antibiotics/antimycotics

Antibiotics (culture prophylaxis)

Penicillin-G - Precautionary: was used at 50 U/ml.

Streptomycin sulfate - Precautionary: used at 50 µg/ml.

P/S is mixed together in d H₂O, filtered, and used at 1 ml/l

Gentamycin sulfate: was used between 10 and 200 µg/ml (tissue culture). A stock was made up as 5 mg/ml and used at 1:100.

Antimycotics

Fungizone = Amphotericin B, was used at 0.25 µg/ml.

A stock was made up at 25 µg/ml and used at 1:100.

2.2.2 Solutions for experiments

2.2.2.1. Modified Frackelton buffer (lysis buffer for immunoprecipitations)

	<u>Final conc.</u>
Tris pH7.4	20 mM
Na ₄ P ₂ O ₇ (Na Pyrophosphate FW 446.1)	30 mM
NaCl	40 mM
NaF	50 mM
EDTA	5 mM
Triton-X 100	0.5%
Glycerol	5%
H ₂ O	

Day of use the following was added:

Na-O-vanadate	0.1 mM*
Aprotinin	12TIU/ml
Leupeptin	10 µg/ml
PMSF EtOH	1.0 mM
Pepstatin EtOH	5 µg/ml

*Na-O-vanadate was prepared freshly the day of use.

2.2.2.2. Sample buffer for immunoprecipitation

	<u>Final conc.</u>
Tris HCl pH 8.0	40 mM
Glycerol	40 %
2-mercaptoethanol	2 %
SDS	4 %

EDTA	4 mM
Bromophenol blue	0.01 %
H ₂ O	

Day of use the following inhibitors were added:

Na-O-vanadate	0.1 mM
Aprotinin	0.12 TIU/ml
Leupeptin	0.002 mM
PMSF	1.0 mM
Pepstatin	5 µg/ml

2.2.2.3. Sample buffer for MAPK assay

	<u>Final conc.</u>
Tris HCl pH 6.8	60 mM
Glycerol	20%
2- mercaptoethanol	2%
SDS	2%
EDTA	2 mM
Na-O-vanadate	50 µM
NaF	50 mM
Nitrophenyl-phosphate	20 mM

Brought to 5 ml with H₂O.

2.2.2.4. Sample buffers for JNK-assay

WCE Lysis-buffer	<u>Final conc.</u>
HEPES pH 7.6	5 M
MgCl ₂	1 M
EDTA	0.5 M
Triton X 100	10 %
H ₂ O	
Beta-glycerophosphate	1M
Na-vanadate	0.1 M
PMSF (34.8 mg/ml 95% EtOH)	1.0 mM
Leupeptin	0.01 mM
Aprotinin	0.12 TIU/ml

B incubation buffer	<u>Final conc.</u>
HEPES pH 7.6	1 M
MgCl ₂	1 M
EDTA	0.5 M
Triton X 100	10 %
H ₂ O	
Beta-glycerophosphate	1M
Na-vanadate	0.1 M
PMSF (34.8 mg/ml 95% EtOH)	1.0 mM
Leupeptin	0.01 mM
Aprotinin	0.12 TIU/ml

HBB Washing buffer	<u>Final conc.</u>
HEPES pH 7.6	1 M
NaCl	5 M
MgCl ₂	1 M
EDTA	0.5 M
Triton X 100	10 %
H ₂ O	

KB buffer for suspension of radioactive ATP	<u>Final conc.</u>
HEPES pH 7.6	1 M
MgCl ₂	1 M
H ₂ O	
Beta-glycerophosphate	1M
Na-vanadate	0.1 M
ATP	1 mM
DTT	1 M

2.2.2.5. Solutions for running gels

Lower Gel Buffer, pH 8.8	
Tris Base (1.5M)	181.7 g
SDS (0.4%)	4.0 g
Millipore H ₂ O	750 ml
Conc HCl to pH 8.8	
Millipore H ₂ O qs to 1.0 l	
Refrigerate	

Upper Gel Buffer, pH 6.8

Tris Base (1.5M)	30.0 g
SDS (0.4%)	4.0 g
Millipore H ₂ O	350 ml
Conc HCl to pH 6.8	
Millipore H ₂ O qs to 500 ml	
Refrigerate	

Transfer Buffer, 10x Stock

Tris Base	60.6 g
Glycine	288.0 g
Millipore H ₂ O qs to 2.0 l	

Was refrigerated and diluted 1:10 before using.

SDS Running Buffer, 10x Stock

Tris Base	60.6 g
Glycine	288.0 g
SDS	20.0 g
Millipore H ₂ O qs to 2.0 l	

Stored at room temperature and diluted 1:10 before using.

TBS Tris Buffered Saline pH 7.4

Tris Base FW 121.1	50 mM	6.05 g
Tris HCl FW 157.6	50 mM	39.40 g
NaCl	150 mM	52.59 g
pH with conc. HCl to 7.4, qs to 6 l		

Wash Buffer

TBS
0.1% Tween 20
Refrigerated

Blocking Buffer

2% BSA (Fraction V) in Wash Buffer

Ammonium Persulfate

0.1 g/ml was made up prior to usage, stable for two weeks

10 % SDS-PAGE

Millipore H ₂ O	26.5 ml
Lower Gel Buffer	16 ml
30% Acrylamide	21 ml
10%(NH ₄) ₂ S ₂ O ₈	100 µl
TEMED	50 µl

12.5 % SDS-PAGE

Millipore H ₂ O	21.3 ml
Lower Gel Buffer	16 ml
30% Acrylamide	26.7 ml
10%(NH ₄) ₂ S ₂ O ₈	100 µl

TEMED	50 µl
Stacking gel	
Millipore H ₂ O	8.85 ml
Upper Gel Buffer	3.75 ml
30% Acrylamide	2.4 ml
10%(NH ₄) ₂ S ₂ O ₈	45 µl
TEMED	15 µl

2.2.2.6. KRH buffer for GSH-detection assay

	<u>Final conc.</u>
HEPES	25 mM
MgSO ₄ •7 H ₂ O	1 mM
NaCl	120 mM
KCl	5 mM
KH ₂ PO ₄	1 mM
CaCl ₂ (pH 7.4)	1 mM

2.3. List of Chemicals

AA6-P (ascorbic acid 6-palmitate)	Sigma, St. Louis, MO
Acrylamide	National Diagnostic, Atlanta, GA
Adenine	Sigma, St. Louis, MO
Alanine	Sigma, St. Louis, MO
Ammonium persulfate	Serva, Westbury, NY
Amphotericin B	Squibb, NJ
Ampicillin	Sigma, St. Louis, MO
Amplex	Molecular Probes, Eugene, OR
Anti-phosphotyrosine	AB
conjugated	HRPO Transduction Laboratories, Lexington, KY
Aprotinin	Sigma, St. Louis, MO
Arginine•HCl	Sigma, St. Louis, MO
Ascorbic acid 6-palmitate (AA6-P)	Sigma, St. Louis, MO
Asparagine	Sigma, St. Louis, MO
Aspartic acid	Sigma, St. Louis, MO
ATP	Sigma, St. Louis, MO
ATP, ³² P	ICN Biomedicals, Costa Mesa, CA
Beta-glycerophosphate	Sigma, St. Louis, MO
Biotin	Sigma, St. Louis, MO
BHA (butylated hydroxyanisole)	Sigma, St. Louis, MO
Bovine saerum albumin	Sigma, St. Louis, MO
Bovine pituitary extract (BPE)	Pel-Freeze, Rogers, AR
Bromophenol blue	IBI, New Haven, CT
Butylated hydroxyanisole (BHA)	Sigma, St. Louis, MO

Catalase (from bovine liver)	Sigma, St. Louis, MO
c-PA (cis-parinaric acid)	Molecular Probes, Eugene, OR
Ca-pantothenate	Sigma, St. Louis, MO
Ca(Cl ₂)•2 H ₂ O	Fisher Scientific, Pittsburgh, PA
Cholinechloride	Sigma, St. Louis, MO
CumOOH	Sigma, St. Louis, MO
Cu(SO ₄)•5 H ₂ O	Sigma, St. Louis, MO
Cysteine	Sigma, St. Louis, MO
DFBS (dialyzed fetal calf serum)	Life Technology, Grand Island, NY
DHR 123 (dihydrorhodamine 123)	Molecular Probes, Eugene, OR
Dihydrorhodamine	Molecular Probes, Eugene, OR
DMEM	Mediatech, Hirndon, VA
DMSO	Sigma, St. Louis, MO
DTT (dithioreitol)	Sigma, St. Louis, MO
ECL detection reagents	Amersham Life Sciences, England, UK
EDTA	Sigma, St. Louis, MO
EGF	Upstate Biotechnology, Lake Placid, NY
EGFR AB 15E11 SB1(A10) mouse	Gift of Dr. N. Maible, Mayo Clinic, MN
EGFR AB 528	Gift of Dr. P. Dempsey, Vanderbilt U., TN
Ethanolamine	Sigma, St. Louis, MO
FeSO ₄ •7 H ₂ O	Fisher Scientific, Pittsburgh, PA
Folic acid	Sigma, St. Louis, MO
Geneticin	Life Technologies, Grand Island, NY
Gentamycin	Sigma, St. Louis, MO
Glucose	Sigma, St. Louis, MO
Glutamic acid	Sigma, St. Louis, MO
Glycerol	Sigma, St. Louis, MO
Glycine	Sigma, St. Louis, MO
Goat-anti-mouse Ig Ab,HRP-conjugated	Daco, Carpinteria, CA
GSH-s-transferase	Sigma, St. Louis, MO
H ₂ SeO ₃	Johnson Matthey Chemicals
Hepes	US Biochemical Corporation, Cleveland, OH
Histidine•HCl•H ₂ O	Sigma, St. Louis, MO
Hydrocortisone	Sigma, St. Louis, MO
Insulin	Sigma, St. Louis, MO
Isoleucine	Sigma, St. Louis, MO
KCl	Sigma, St. Louis, MO
L-glutamine	Sigma, St. Louis, MO
L-histidine	Sigma, St. Louis, MO
L-isoleucine	Sigma, St. Louis, MO
L-phenylalanine	Sigma, St. Louis, MO
L-tryptophane	Sigma, St. Louis, MO
L-tyrosine	Sigma, St. Louis, MO
Leucine	Sigma, St. Louis, MO
Leupeptin	Sigma, St. Louis, MO
Lipoic acid	Sigma, St. Louis, MO
Lysine•HCl	Sigma, St. Louis, MO
MAPK, anti-active AB, rabbit IgG	Promega, Madison, WI

Mercaptoethanol	Sigma, St. Louis, MO
Methionine	Sigma, St. Louis, MO
MgCl ₂ •6 H ₂ O	Fisher Scientific, Pittsburgh, PA
MnSO ₄ •5 H ₂ O	Sigma, St. Louis, MO
Molybdic acid ((NH ₄) ₆ MO ₇ O ₂₄ •4 H ₂ O)	Sigma, St. Louis, MO
Monochlorobimane	Sigma, St. Louis, MO
Mycostatin	Sigma, St. Louis, MO
Myo-inositol	Sigma, St. Louis, MO
N-acetylcysteine (NAC)	Sigma, St. Louis, MO
Na-acetate•3 H ₂ O	Sigma, St. Louis, MO
NAC (N-acetylcysteine)	Sigma, St. Louis, MO
Na-O-vanadate	Sigma, St. Louis, MO
Na-pyruvate	Sigma, St. Louis, MO
Na-vanadate	Sigma, St. Louis, MO
Na ₂ HPO ₄ anhydrous	Sigma, St. Louis, MO
Na ₂ HPO ₄ •7 H ₂ O	Sigma, St. Louis, MO
Na ₂ SiO ₃ •9 H ₂ O	Sigma, St. Louis, MO
Na ₄ P ₂ O ₇	Sigma, St. Louis, MO
NaCl	Sigma, St. Louis, MO
NaF	Sigma, St. Louis, MO
NaHCO ₃	Sigma, St. Louis, MO
(NH ₄) ₂ S ₂ O ₈	Sigma, St. Louis, MO
(NH ₄) ₆ MO ₇ O ₂₄ •4H ₂ O	Sigma, St. Louis, MO
NH ₄ VO ₃	Sigma, St. Louis, MO
Niacinamide	Sigma, St. Louis, MO
NiCl ₂ •6 H ₂ O	Sigma, St. Louis, MO
Nitrophenylphosphate	Sigma, St. Louis, MO
P38 MAPK AB rabbit polyclonal	New England Biolabs, Beverly, MA
P38-phospho-specific MAPK AB	New England Biolabs, Beverly, MA
PD 098	Gift of Dr. D. Fry, Parke Davis, Ann Arbor, MI
PD 153	Gift of Dr. D. Fry, Parke Davis, Ann Arbor, MI
PDTC (pyrrolidinedithiocarbamate)	Sigma, St. Louis, MO
Penicillin	Sigma, St. Louis, MO
Pepstatin	Sigma, St. Louis, MO
Phenol red	Sigma, St. Louis, MO
Phenylalanine	Sigma, St. Louis, MO
Phosphoryl-ethanolamine	Sigma, St. Louis, MO
PMSF	Sigma, St. Louis, MO
Proline	Sigma, St. Louis, MO
Putrescine	Sigma, St. Louis, MO
Pyridoxine HCl	Sigma, St. Louis, MO
Pyrrolidinedithiocarbamate	Sigma, St. Louis, MO
Riboflavin	Sigma, St. Louis, MO
SDS	Sigma, St. Louis, MO
Serine	Sigma, St. Louis, MO
SnCl ₂ •6 H ₂ O	Johnson Matthey Chemicals, Ltd.
Streptomycin	Sigma, St. Louis, MO
TCA (trichloroacetic acid)	EM, Gibbstown, NJ

TEMED	Sigma, St. Louis, MO
Thiamine•HCl	Sigma, St. Louis, MO
Threonine	Sigma, St. Louis, MO
Thymidine	Sigma, St. Louis, MO
Tocopherolpyrophosphate	Sigma, St. Louis, MO
Trichloroacetic acid (TCA)	EM, Gibbstown, NJ
Trizma-Base	Sigma, St. Louis, MO
Trizma-HCl	Sigma, St. Louis, MO
Triton-X 100	Sigma, St. Louis, MO
Trolox	Aldrich Chemical, Milwaukee, WI
Tryptophane	Sigma, St. Louis, MO
Tyrosine	Sigma, St. Louis, MO
Valine	Sigma, St. Louis, MO
Vitamin B12	Sigma, St. Louis, MO
ZnSO ₄ •7 H ₂ O	Sigma, St. Louis, MO

2.4. Instruments

Cell Culture Labware	Falcon, Lincoln Park, NJ
	Corning, Corning, NY
	Becton Dickinson, Oxnard, CA
Centrifuge: J6M (Cell culture)	Beckmann, Palo Alto, CA
Centrifuge: TJ-6	Beckmann, Palo Alto, CA
Electrophoresis: SE 600 electrophoresis unit	Hoefer Scientific Instruments, San Francisco, CA
Film: Bio Max MR	Kodak, Rochester, NY
Gel Dryer: Speed Gel SG 200	Forma Scientific Inc., Marietta, OH
Incubator: 3158 water-jacketed incub.	Forma Scientific Inc., Marietta, OH
Microcentrifuge: 235 C	Fisher Scientific, Pittsburgh, PA
Microcentrifuge: 5417R	Eppendorf, Hamburg, Germany
Microplate Reader: CytoFluor II	Perseptive Biosystems, Framingham, MA
Power Supply: 1000 V Power Supply	Labconco, Kansas City, MO
Rotator:	ATR, Laurel, MD
Transfer unit: TE Series Transphor	Hoefer Scientific Instruments, San Francisco, CA

3. Results

3.1. Lipid Peroxidation

Lipid peroxidation shown as loss of c-PA fluorescence following CumOOH treatment (300 μ M at 0, 15, 30, 60, and 90 minutes) reached a maximum value after 60 minutes (Fig. 1) (The 90-minute time point was disregarded due to an increased amount of dead cells and debris over time.). This correlates with JNK activation, which also showed a maximum after 60 min. LPO was also visualized using laser scanning confocal microscopy. Loss of c-PA fluorescence after 30 minutes indicates increased LPO (Fig. 17).

3.2. Measurement of intracellular H_2O_2

Figure 2 shows the CumOOH dose-dependent increase in DHR fluorescence. Maximum values of DHR fluorescence were measured following treatment with CumOOH (300 μ M) after 30 minutes (Fig. 3). This peak was sustained at time points 45 and 60 minutes, before it dropped off at 90 minutes. Standard deviations increased noticeably after the 30-minutes time-point, which might be caused by cellular leakage of H_2O_2 . DHR fluorescence could be effectively inhibited by pre-treating cells with ascorbic acid 6-palmitate, a fat-soluble anti-oxidant, for 30 minutes prior to CumOOH exposure (Fig. 4). Increase of DHR fluorescence following CumOOH treatment was also visualized with laser scanning confocal microscopy (Figure 16).

3.3. Measurement of extracellular H_2O_2

The increase of extracellular H_2O_2 was time and dose-dependent (Fig. 5 a) and b)). It was effectively inhibited by the peroxyl scavenger Trolox (20 μ g/ml with 1 hour pre-treatment). Cells pre-treated with Trolox and subjected to CumOOH stress elicited 25% less

extracellular H_2O_2 than the untreated cells (Fig. 6). Pretreating cells with 30mM of N-acetylcysteine (NAC) for 5 h also reduced the amount of extracellular H_2O_2 release following treatment with CumOOH (Fig. 8 b)). We also found that the amount of extracellular H_2O_2 produced after CumOOH treatment was dependent upon the growth stage of the culture. Cells that were confluent and rapidly dividing produced larger amounts of H_2O_2 than cells that were confluent and quiescent (unpublished observation).

3.4. EGFR phosphorylation

CumOOH induces EGFR phosphorylation in a dose- and time-dependent matter (Fig. 7) 200 μM CumOOH (20 minutes) evoked a strong EGFR phosphorylation. A timing-experiment revealed that for this effect to occur more than 15 minutes of exposure to CumOOH were needed. EGFR phosphorylation could be inhibited by pre-treating cells with 30 mM of the thiolic anti-oxidant N-acetylcysteine (NAC) for 5 h prior to exposure (Fig. 8 a)). This showed a correlation with the amount of extracellular H_2O_2 release (Fig. 8 b)). The same results were seen using tocopherolphosphate and Trolox, a vitamin E analog (unpublished observation). Furthermore, specifically using 500 nM of the EGFR-tyrosine kinase inhibitor PD 153035 could block EGFR phosphorylation after CumOOH exposure, when pre-treated for 30 minutes (Fig. 9).

Electroporated cells into which catalase (30,000 units/ml) had been introduced also showed a decreased susceptibility to EGFR phosphorylation when treated with 200 μM CumOOH. This effect was much less pronounced using 100 or 300 μM CumOOH. (Fig. 10)

3.5. MAP kinases --ERK 1/2

ERK 1/2 is maximally activated following 15-30 min of 200 μ M CumOOH exposure, activation decreases extending treatment time to 45 or 60 minutes (Fig. 11a)). CumOOH dosing (20 min) showed an early ERK 1/2 activation with 75 μ M, increasing to its maximum with 300 μ M (Fig. 11b)). The signal could be inhibited by pre-treatment with 30 mM NAC for 5 h (Fig. 8a)). 120 μ M (20 min) of CumOOH exposure showed little ERK 1/2 activation if pre-treated for 30 min with antioxidants BHA (Butylated Hydroxyanisole, 90 μ M), AA6P (Ascorbic-Acid-6-Palmitate, 30 μ M), and PDTC (pyrrolidinedithiocarbamate, 0.3, 1, 3 μ M) (Fig. 12 a and b)). The inhibition of the EGFR-tyrosine kinase with PD 153035 (500 nM, pre-treatment for 30 minutes) lead to no activation of ERK 1/2 following CumOOH exposure (120 μ M for 20 minutes) (Fig. 9 a)). Similarly, ERK 1/2 signals were strongly inhibited using the specific MEK 1 inhibitor PD 098059 (30 μ M, 30 minutes pre-treatment) (Fig. 9 b)).

3.6. MAP kinases --p38

This pathway is already activated after 5 minutes following treatment with 200 μ M CumOOH, showing a decline in phosphorylation after 15 minutes (Fig. 11a)). Between ERK 1/2 and p38 pathways, it is p38 that is activated more rapidly (Fig. 11a)). The dosing indicates little phosphorylation at 75 μ M CumOOH (20 minutes) but increased activation at subsequent higher doses (11b)). Strong inhibition of the signal (200 μ M CumOOH, 20 minutes) was achieved with 30 mM NAC after 5 hours pre-treatment (Fig. 8 a)).

3.7. MAP kinases --JNK

After 60 minutes of treatment with 300 μ M of CumOOH JNK was maximally activated (Fig. 1). The signal (200 μ M, 20') was inhibited when pre-treated with NAC 30

mM for 5 hours (Fig. 8a) or with tocopherolphosphate, but not with Trolox. In HaCat cells, into which the Bcl-2 gene had been incorporated, the signal (300 μ M 60') was markedly reduced when compared to parental HaCat cells and neomycin-resistant cells, subjected to the same regimen of CumOOH treatment (Fig. 13).

3.8. Glutathione assay

Glutathione depletion was assessed after treatment with CumOOH (200 μ M) for the times indicated (Fig. 15). Initial depletion of glutathione was very small, moreover, the amount of glutathione seemed to increase at 60 minutes and only thereafter did depletion occur, with 55% depletion measured after 260 minutes. A similar pattern was observed when measuring $O_2^{\cdot -}$ production with a lucigenin assay (unpublished data courtesy of Carsten Krautmacher, Fig. 14) Initial production was low (increase of 4.9 % over control) and increased markedly (after 180 and 240 minutes, 27.6% and 35.9% over control respectively).

4. Figures

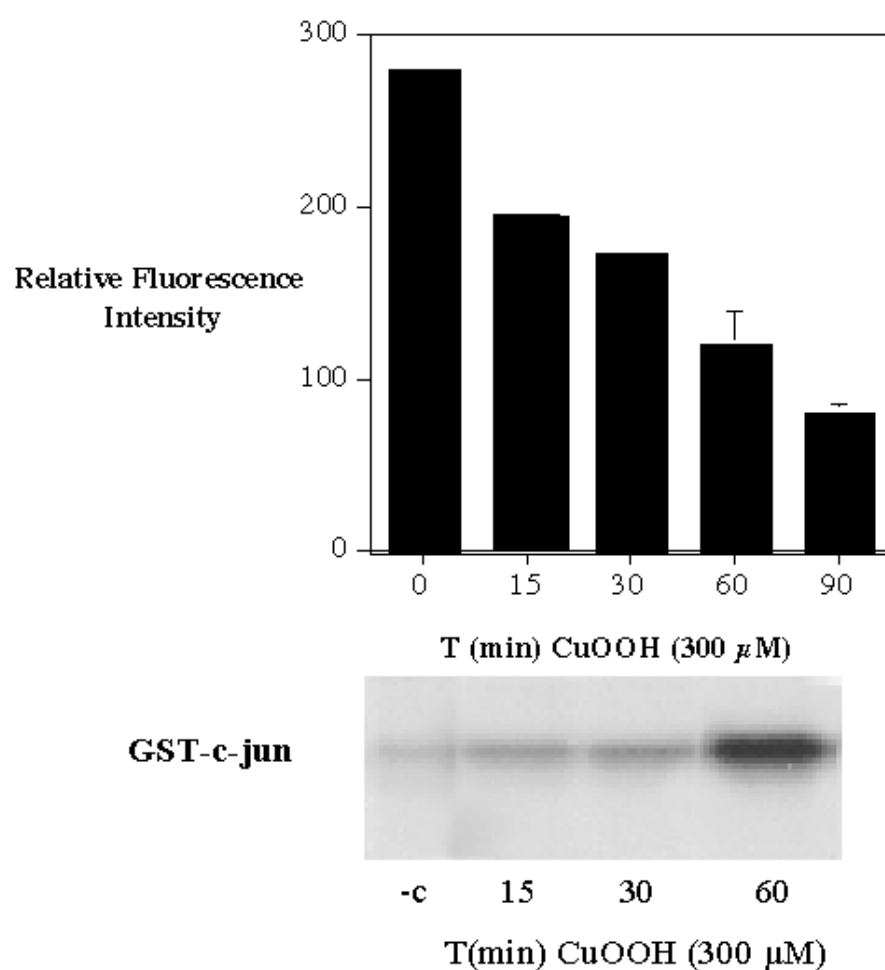


Figure 1. CumOOH induces lipid peroxidation (LPO). LPO correlates with JNK activity.

Lipid peroxidation was examined using flow cytometry and cis-parinaric acid (c-PA). Low levels of c-PA fluorescence reflect high lipid peroxidation. Maximum LPO was observed following treatment with 300 μ M CumOOH for 60 minutes. As membrane integrity is compromised after prolonged exposure, the 90-minute value should be disregarded. A similar pattern was observed in JNK activation of cells treated with the same amount of CumOOH for one hour.

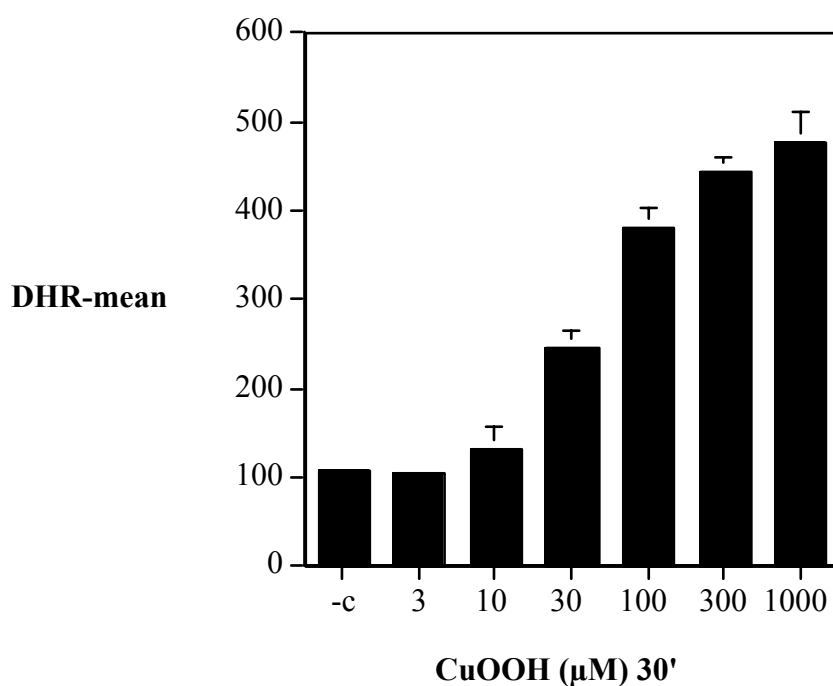


Figure 2. Dose-dependent increase of intracellular H_2O_2

Using the dye dihydrorhodamine (DHR) in conjunction with flow cytometric analysis, intracellular levels of H_2O_2 were examined. In the presence of H_2O_2 , DHR is irreversibly oxidized into the red fluorescent compound rhodamine. Increased levels of DHR fluorescence indicate high levels of H_2O_2 . Samples were treated with the doses of CumOOH indicated for 30 minutes. Immediately after harvesting the cells, flow cytometry was performed.

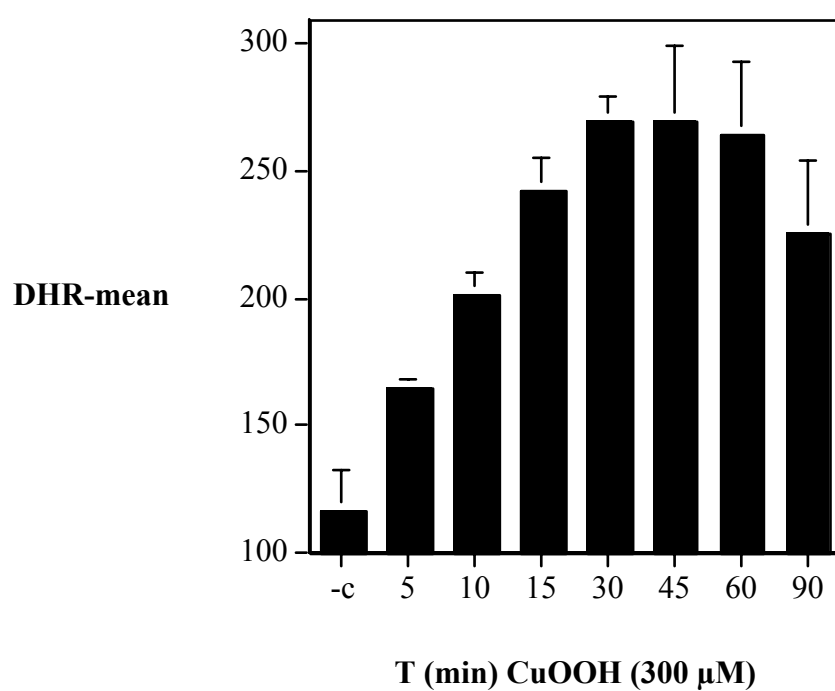


Figure 3. Intracellular H_2O_2 increases in a time-dependent manner.

After keratinocytes were loaded with DHR 300 μ M of CumOOH was added for the times indicated. Peak fluorescence was detected by flow cytometric analysis after 30 minutes. Increased standard deviations after this time point reflect changes due to cellular leakage.

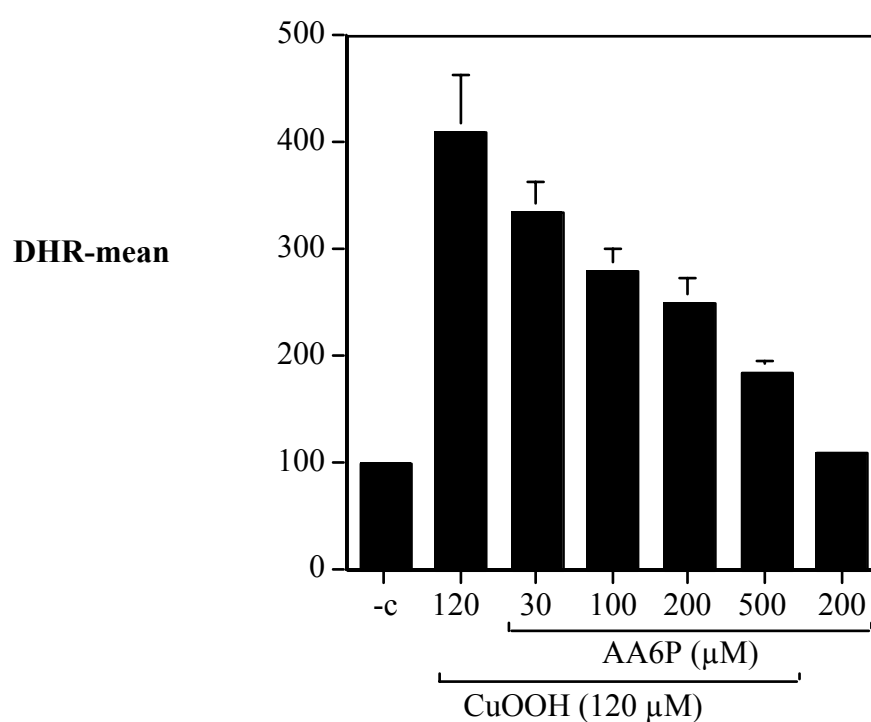


Figure 4. Inhibition of intracellular H_2O_2 by ascorbic acid 6-palmitate.

DHR fluorescence was strongly reduced when cells were pre-treated with the fat-soluble anti-oxidant ascorbic acid 6-palmitate (AA6P) for 30 minutes prior to stress with 120 μM CumOOH for 30 minutes.

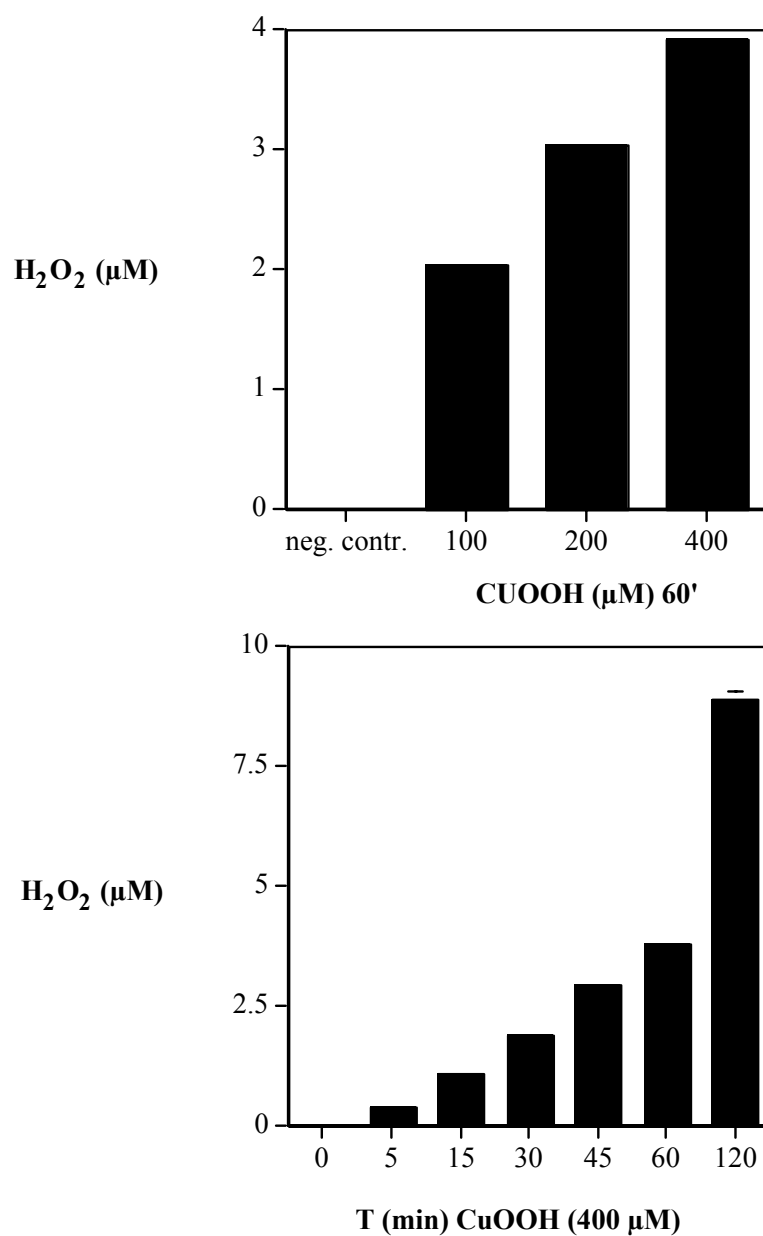


Figure 5. Extracellular concentration of H_2O_2 follows a dose- and time-dependent manner.

Extracellular H_2O_2 was detected using the AmplexTM assay. AmplexTM is a dehydrophenoxazine derivative, which becomes highly fluorescent when oxidized by H_2O_2 . Fluorescence was assessed by using a microplate reader. A standard curve was generated to convert fluorescent values into absolute values. After loading cells with the AmplexTM reagent, CumOOH was added as indicated.

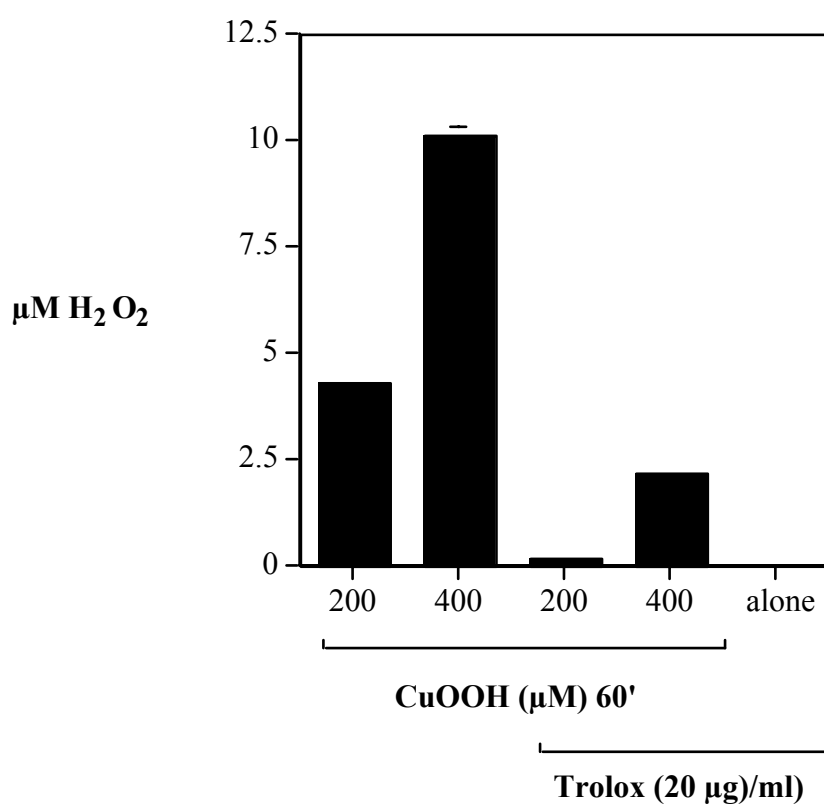


Figure 6. Peroxyl scavenger Trolox inhibits extracellular H_2O_2 .

Following exposure to 200 or 400 μM CumOOH for one hour, cells pretreated for one hour with 20 $\mu\text{g}/\text{ml}$ of Trolox showed decreased fluorescence in the AmplexTM assay. Pretreated cells showed a reduction of extracellular H_2O_2 by 95 % (200 μM CumOOH) or 78 % (400 μM CumOOH) when compared to cells that were not pretreated. Peroxyl radicals ($\text{LOO}\cdot$) are formed when lipid radicals react with oxygen.

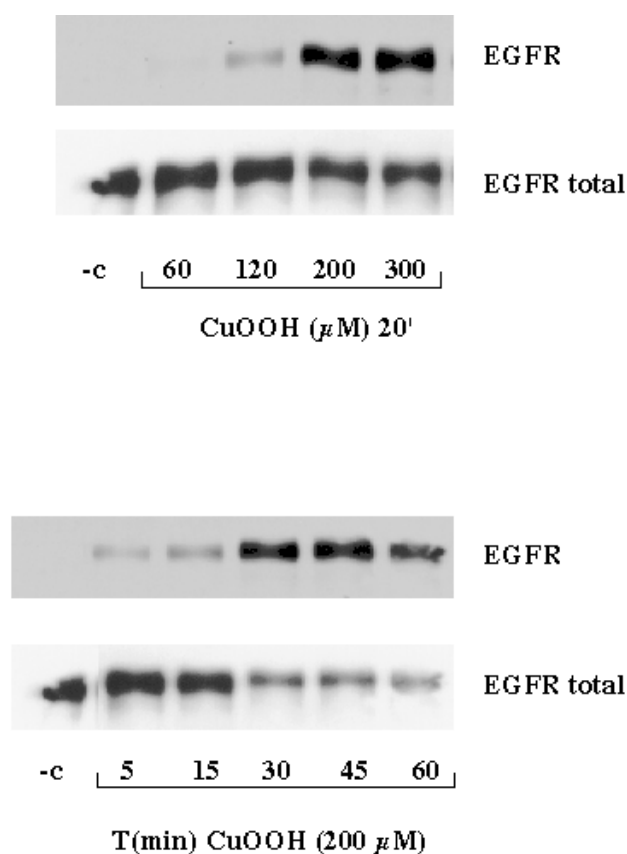


Figure 7. CumOOH induces EGFR phosphorylation in a dose- and time-dependent manner.

- a) CumOOH induces EGFR phosphorylation in a dose-dependent fashion, a strong signal is displayed with 200 μ M CumOOH (20 minutes).
- b) EGFR phosphorylation is also elicited in a time-dependent fashion starting 15-30 minutes after treatment with 200 μ M CumOOH.

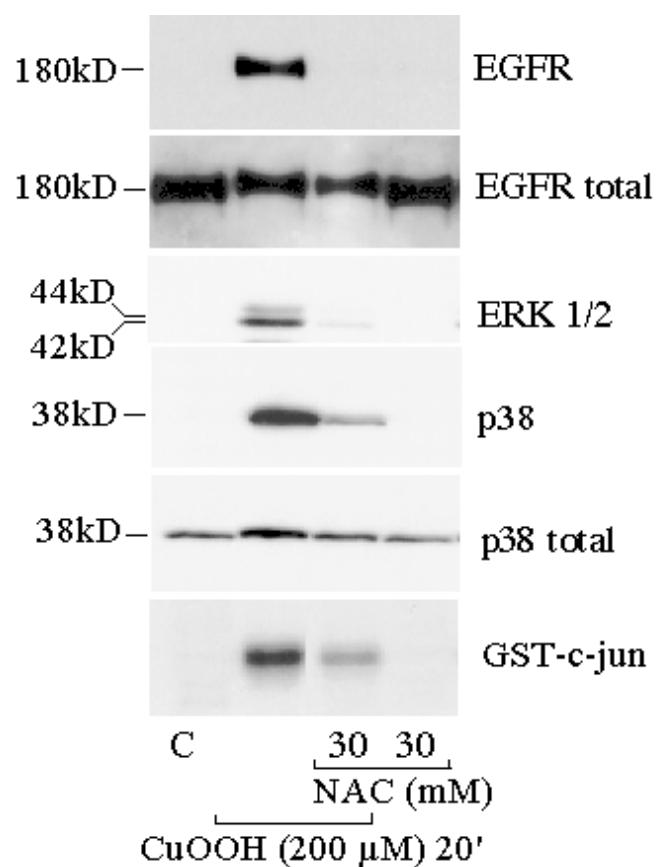
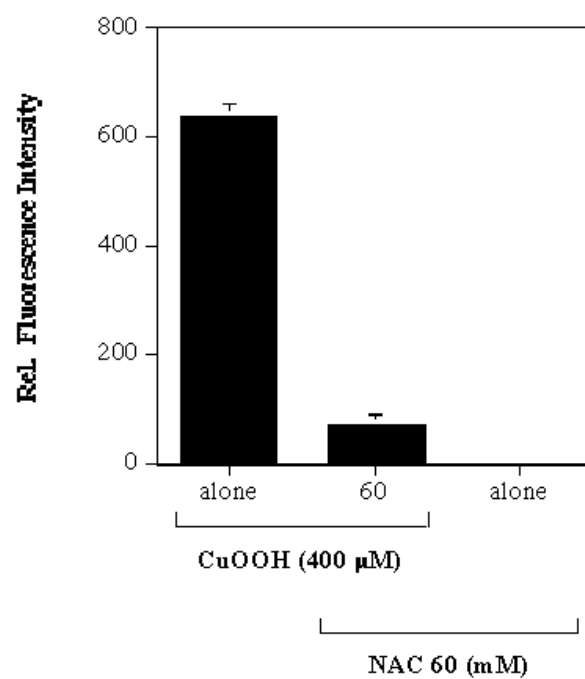


Figure 8 a). NAC, a thiol, inhibits EGFR phosphorylation and activation of ERK 1/2, P38, and JNK pathways.

Cells which were pretreated with 30 mM of the thiolic compound N-acetylcysteine (NAC) for 5 hours showed effective inhibition of CumOOH-induced (200 μM, 20 minutes) EGFR phosphorylation and activation of ERK 1/2, P38 and JNK when compared to control cells.

**Figure 8 b). Extracellular H₂O₂ is decreased by NAC**

Pretreatment of keratinocytes with 60 mM NAC for 5 hours effectively inhibited the effects of 400 μM CumOOH (60 minutes) in the Amplex™ assay. Pretreated cells showed 85 % less relative fluorescence intensity when compared to control cells.

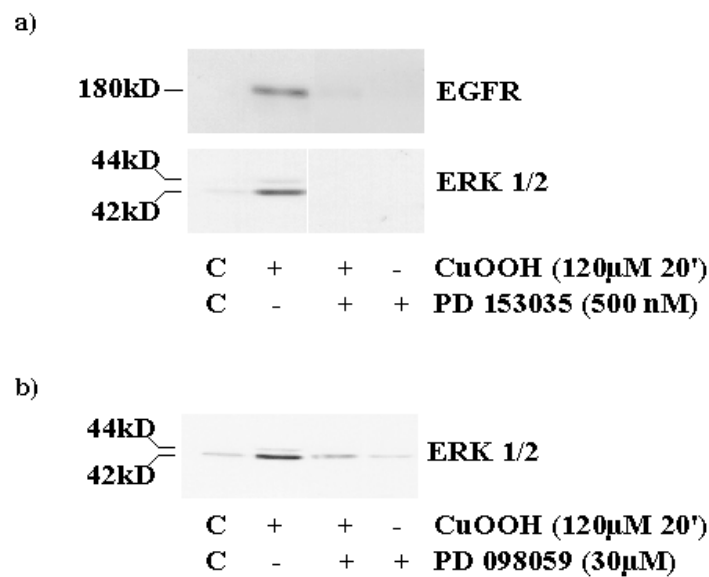


Figure 9. PD 153035 inhibits EGFR phosphorylation and ERK 1/2 activation, PD 098059 inhibits ERK 1/2.

a) PD 150053, a specific EGFR tyrosine kinase inhibitor (500 nM), was used to pretreat keratinocytes for 30 minutes before application of CumOOH (120 μM for 20 minutes). PD 153035 effectively inhibited EGFR phosphorylation and ERK 1/2 activation when compared to control cells.

b) 30 minutes pretreatment with 30 μM of PD 098059, a specific MEK 1 inhibitor, showed strong inhibition of ERK 1/2 activation after treatment with 120 μM CumOOH applied for 20 minutes.

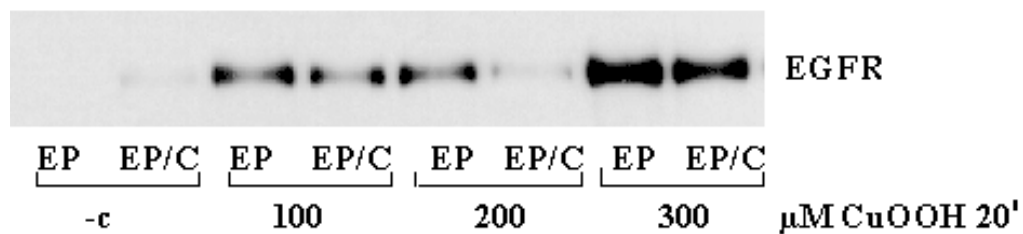


Figure 10. Catalase inhibits EGFR phosphorylation after CumOOH treatment.

Cells into which catalase (30,000 units/ml) had been incorporated by ways of electroporation showed decreased EGFR phosphorylation after CumOOH stress when compared to control cells, that were electroporated without catalase. This effect was more pronounced at 200 μM CumOOH, when strong inhibition of EGFR phosphorylation was observed, than in 100 or 300 μM CumOOH respectively. Following electroporation procedures, cells were grown in medium without growth factors for 2 days prior to the experiment.

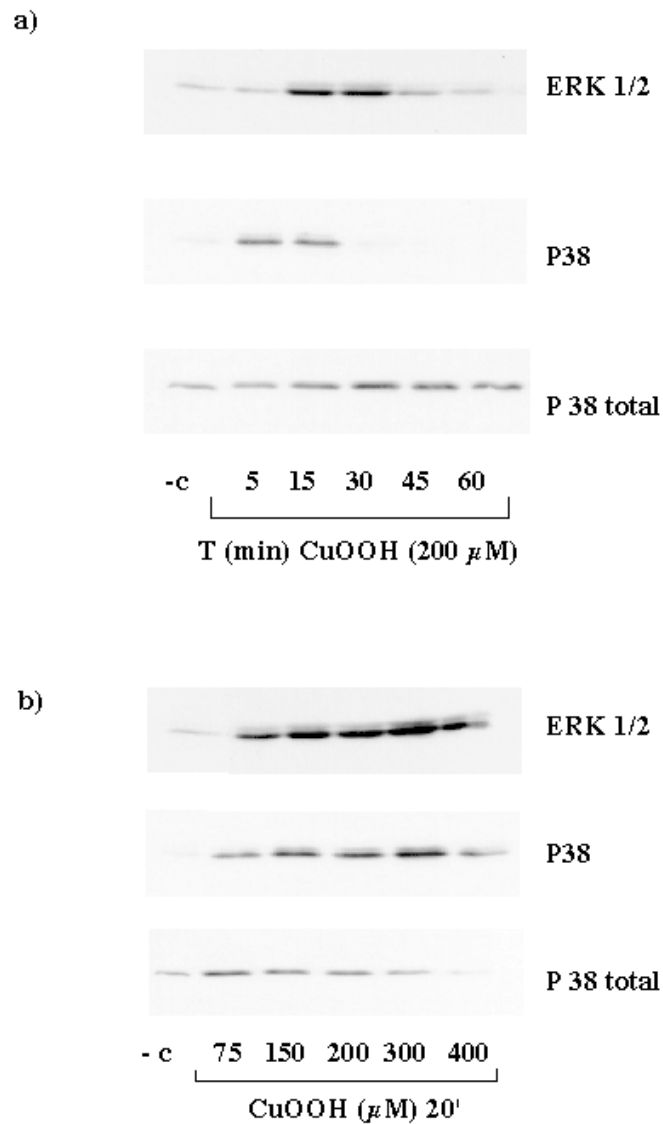


Figure 11. CumOOH activates ERK 1/2 and p38 in a time- and dose-dependent manner.

a) Following the addition of 200 μ M CumOOH, ERK 1/2 is maximally activated within 15-30 minutes, while p38 is activated maximally already after 5-15 minutes.

b) shows the dose-dependent activation of ERK 1/2 and p38. The treatment time with CumOOH was 20 minutes.

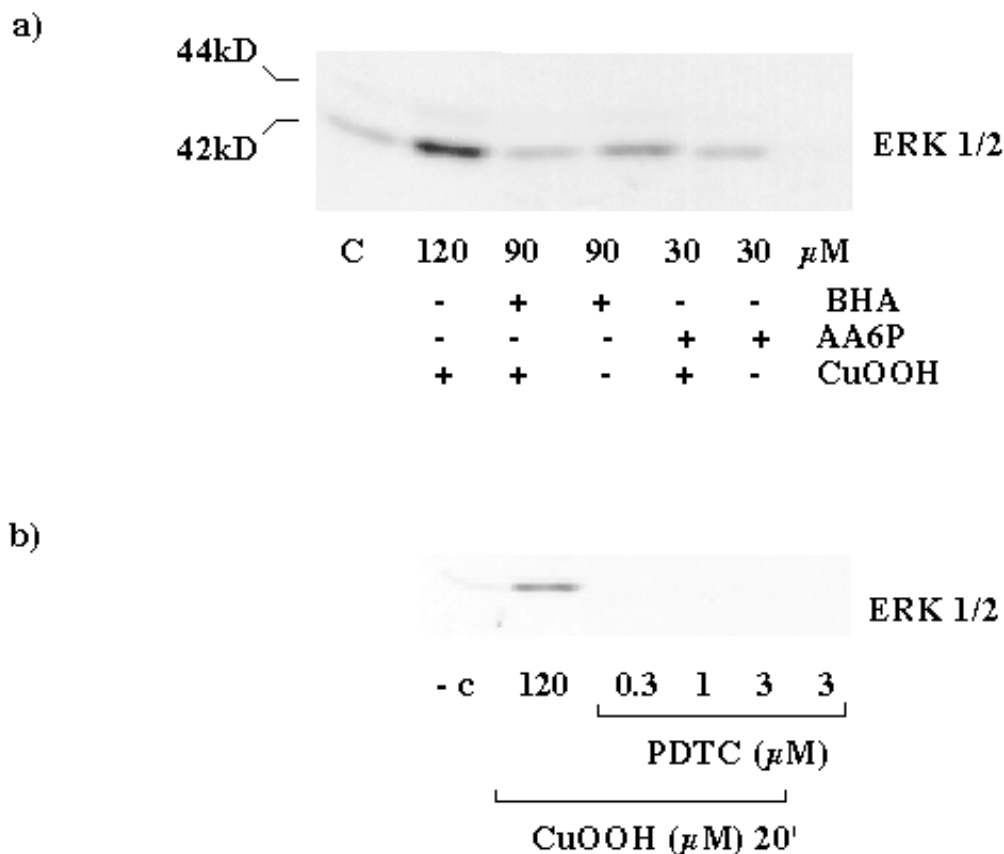


Figure 12. Antioxidants BHA, AA6P, and PDTC inhibit ERK 1/2 activation after treatment with CumOOH.

a) 90 μM of butylated hydroxyanisole (BHA) or 30 μM of ascorbic acid 6-palmitate inhibit ERK 1/2 activation in response to 120 μM CumOOH (20 minutes). Pretreatment time for the antioxidants was 30 minutes.

b) Low doses of pyrrolidinedithiocarbamate (0.3, 1, and 3 μM), a thiol, inhibit ERK1/2 activation after stressing keratinocytes with 120 μM CumOOH for 20 minutes. PDTC pretreatment time was 30 minutes.

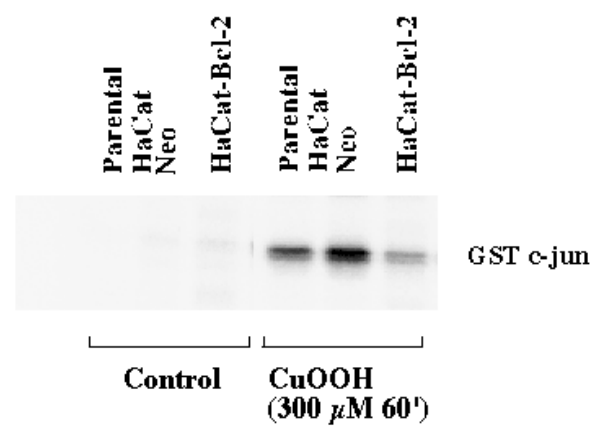


Figure 13. Bcl-2 overexpressing HaCat cells inhibit CumOOH induced JNK activation. HaCat cells overexpressing the Bcl-2 gene showed less JNK activation induced by CumOOH stress (300 μ M, 60 minutes) when compared to parental HaCat or control Neo cells.

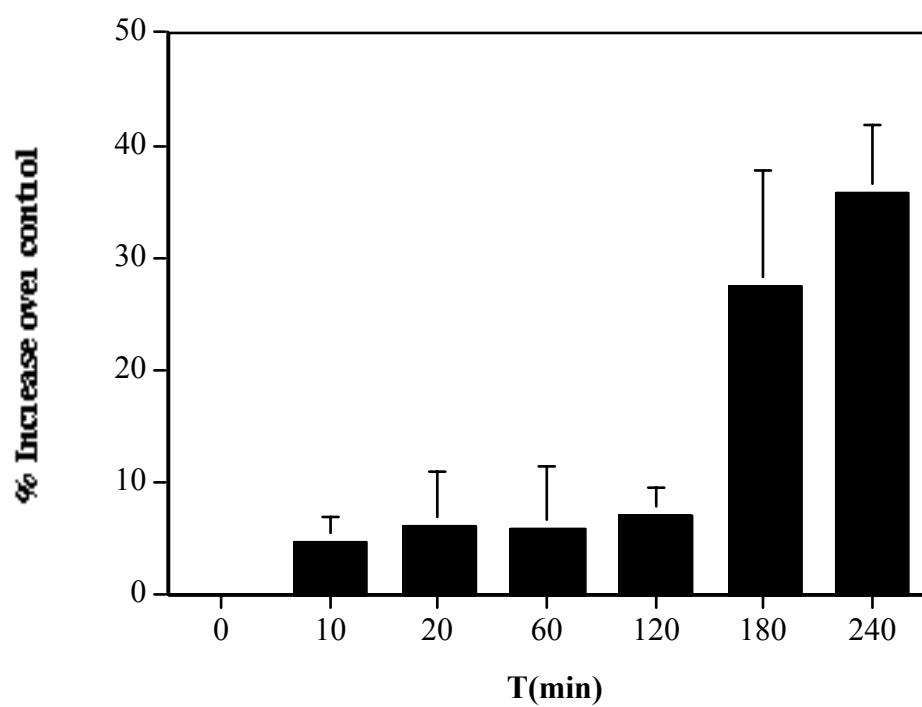


Figure 14. Superoxide anions appear late in CumOOH induced stress.

Superoxide anions were detected with a lucigenin assay after keratinocytes were stressed with 200 μ M CumOOH. Cells were confluent and grown in medium without growth factors two days prior to the experiment. Superoxide-mediated signaling seems to be of lesser importance in cells challenged with CumOOH given their late appearance. (Unpublished data from C. Krautmacher, Rochester, MN).

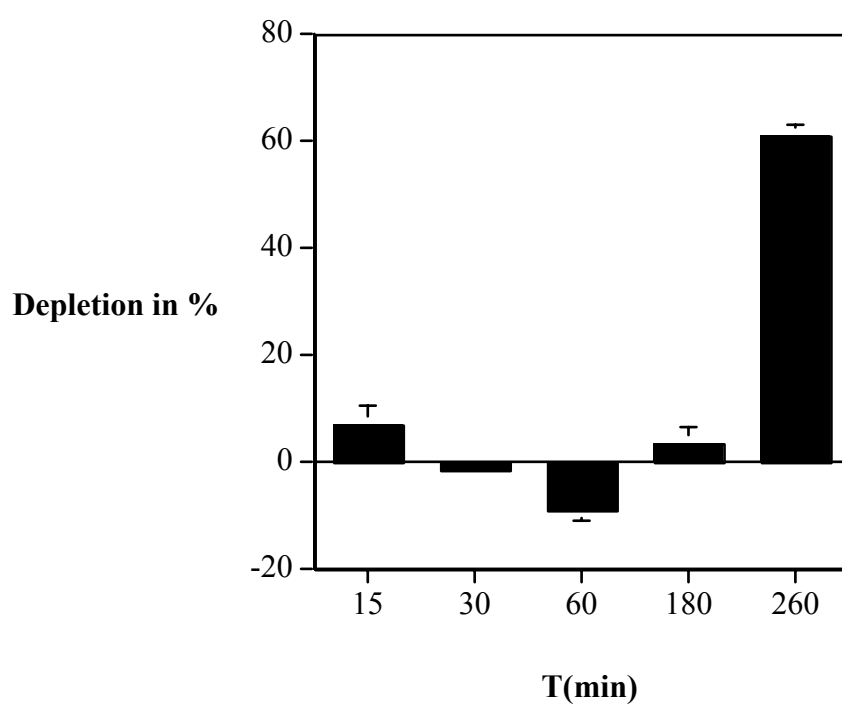


Figure 15. Glutathione depletion is a late-term event in CumOOH induced cellular stress.

Confluent keratinocytes in medium with growth factors showed an initial decrease of glutathione in response to 200 μ M CumOOH. At 15 minutes, glutathione was 7 % depleted. The glutathione pool was then replenished (enzyme induction) as seen at 60 minutes, and only after 260 minutes there was a marked depletion (more than 60 %) of glutathione, suggesting that this depletion is not the main target of CumOOH.

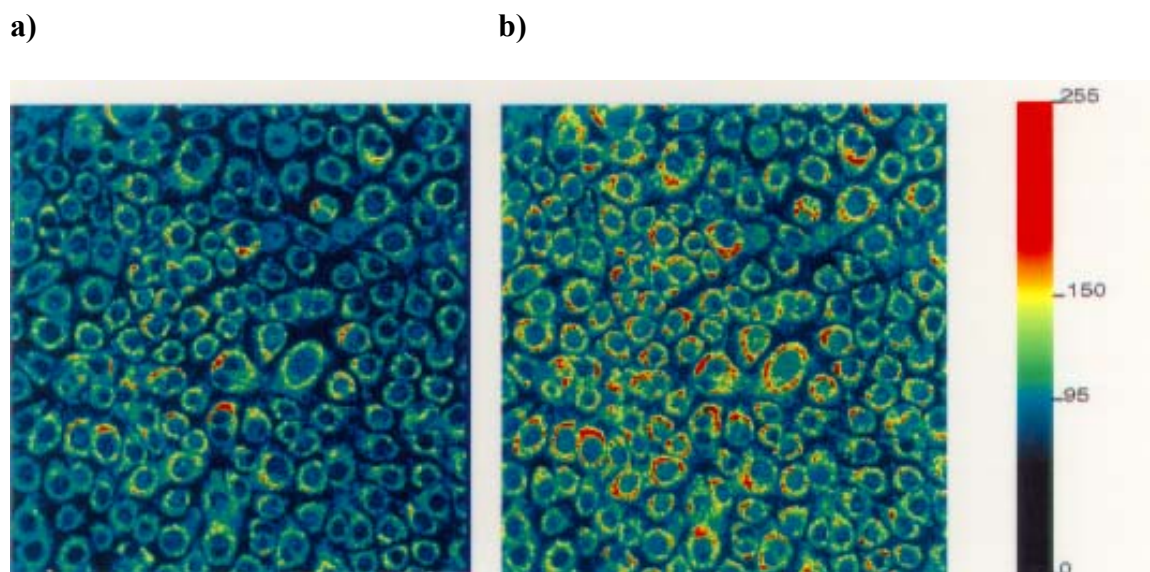


Figure 16. Laser scanning confocal microscopy: Increase of DHR fluorescence following treatment with CumOOH.

Keratinocytes were loaded with DHR and then treated with CumOOH. a) After 5 minutes and b) after 30 minutes. The increase in fluorescence signifies an increase in intracellular H_2O_2 .

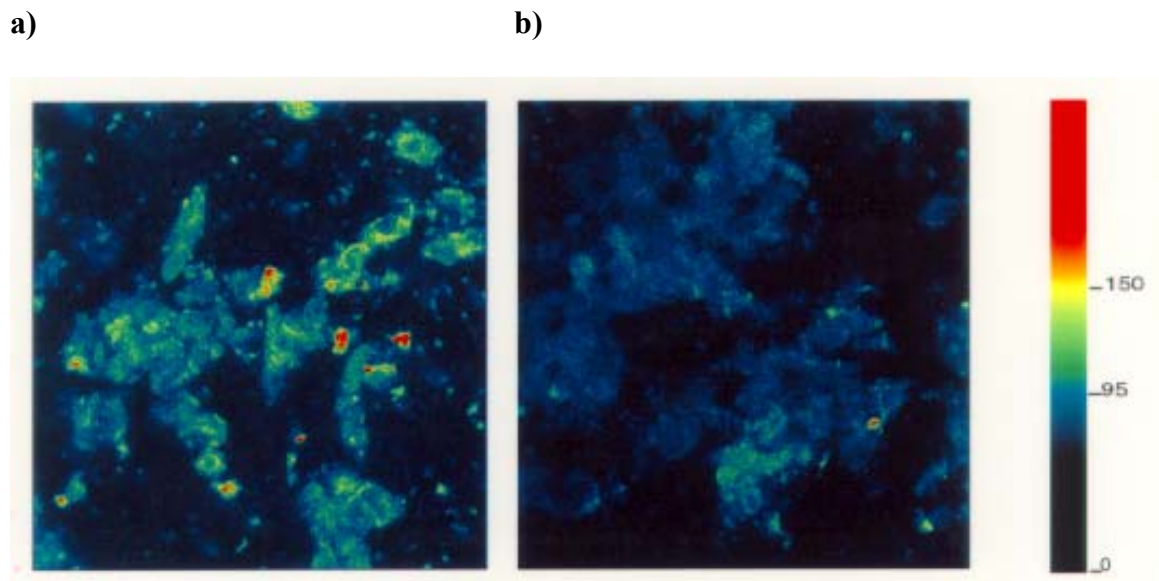


Figure 17. Laser scanning confocal microscopy: CumOOH induces lipid peroxidation (LPO).

Keratinocytes were loaded with 5 μM of cis-parinaric acid (c-PA) and then subjected to CumOOH stress (500 μM). a) before and b) after 30 minutes. Fluorescent values were obtained and then converted into pseudocolors. Loss of fluorescence indicates high levels of LPO.

5. Discussion

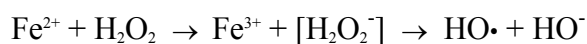
CumOOH is a well-known agent of lipid peroxidation and a promoter of skin tumors. Little is known about the effects of CumOOH on intracellular signaling pathways or about its mediators, which would be of particular interest in light of this substance's tumor promoting ability. Our findings demonstrate that, apart from causing lipid peroxidation, CumOOH also generates considerable amounts of H_2O_2 in human keratinocytes that act as endogenous mediators in CumOOH-initiated EGFR phosphorylation and subsequent cell signaling. This is consistent with previous findings from our laboratory, which indicated that H_2O_2 is an endogenous mediator of ligand-independent EGFR phosphorylation in response to UVB (Peus, 1998). EGFR phosphorylation was found to be a prerequisite for ERK 1/2 stimulation, as specific inhibition of EGFR phosphorylation with PD 153035 showed that ERK 1/2 stimulation did not occur. JNK activation was shown to be closely related to lipid peroxidation, both achieving maximal stimulation after one hour. Cells over-expressing the proto-oncogene Bcl-2 showed significant inhibition of JNK activity, a role potentially linked to the capacity of Bcl-2 to modulate cellular redox states or even its ability to modify the LPO process. The p38 pathway elicited the fastest responses to CumOOH-initiated oxidative stress.

To investigate the effects of CumOOH on the redox state of the cell, the generation of H_2O_2 was tested. This was achieved by measuring intracellular production as well as extracellular release of H_2O_2 following CumOOH exposure. H_2O_2 is produced by a variety of oxidases, and it can also be a product of the superoxide dismutase reaction that converts superoxide anions (O_2^-) into H_2O_2 . H_2O_2 is subsequently metabolized using catalase as a cellular defense system, which dismutates H_2O_2 into H_2O and O_2 . Another pathway employs

the use of glutathione with glutathione peroxidase as its catalyst. H_2O_2 must be viewed as a potentially destructive molecule, given its size and lack of charge, which allow it to freely cross biological membranes. Recently, this lab has shown that H_2O_2 is an important mediator in UVB-induced EGF-receptor phosphorylation (Peus, 1998). This action possibly results from the inhibition of protein phosphatases, that lead to enhanced tyrosine phosphorylation (Goldmann, 1997) by exerting stimulatory effects on protein tyrosine kinases. In a recent report, large alkyl-hydroperoxides alone had no effect on the catalytic activity of the phosphatases (Denu, 1998). Presumably, the bulkier alkyl-hydroperoxides are excluded from the active-site cleft. Activation of down-stream pathways include the formation of a SHC-Grb2-SOS complex, that activates RAS and finally ERK 1/2 (Rao, 1996). Activation of the ERK 1/2 pathway seems to depend on EGFR phosphorylation, as it could be inhibited through the specific tyrosine kinase inhibitor PD 153035. In a similar study with UVB-induced H_2O_2 , we demonstrated that the p38 pathway was not activated via EGFR phosphorylation. Similar patterns of stimulation of hydrogen peroxide generation were found in mouse skins that had been treated with tumor-promoting agents. Perchellet et al. (Perchellet, 1988) found that levels of hydroperoxides in mouse skin homogenates rose rapidly after incubation with 12-O-tetradecanoylphorbol-13-acetate (TPA).

The peroxy scavenger Trolox exhibited strong inhibition of H_2O_2 production in the Amplex[™] assay. Both, Trolox and tocopherolphosphate, another peroxy scavenger, inhibited EGFR-phosphorylation and the activation of ERK 1/2, p38 (data not shown). Peroxyl radicals are formed when lipid radicals react with oxygen. This indicates that H_2O_2 production is superceded by formation of the lipid radicals and that it may as such serve as a second messenger system. On the other hand, it may also contribute to lipid peroxidation by

the generation of hydroxyl radicals when hydrogen peroxide reacts with reducing agents (e^-) in what is called the modified Haber-Weiss or Fenton reaction:



Iron complexes play a central role in catalyzing this reaction (Brenneisen, 1998).

The thiol N-acetylcysteine (NAC) was a potent inhibitor of extracellular release of H_2O_2 , as well as an inhibitor of EGFR phosphorylation, ERK 1/2, P38 and JNK activation. Another thiol, PDTC, inhibited ERK 1/2. Given their thiol groups, substances like NAC and PDTC scavenge H_2O_2 , $\cdot\text{OH}$ (hydroxyl radical), and HOCl (hypochlorous acid). NAC can be deacetylated to cysteine and serve thus as a precursor in the synthesis of cellular glutathione. Given the limited depletion response of glutathione following CumOOH treatment, this mode seems to be of lesser importance. More likely, protection and reactivation of phosphatases, another important aspect of the thiolic function (Goldmann, 1997), could be the mechanism involved here.

In an effort to further distinguish the effects of H_2O_2 from other ROS, we attempted the use of a more specific scavenger for H_2O_2 . Earlier attempts to introduce high levels of catalase into keratinocytes via infection with an adenoviral vector did not produce convincing results. Peus et al. showed that by electroporation, high levels of catalase activity could be transferred into keratinocytes and that H_2O_2 levels were suppressed upon stimulation in keratinocytes (Peus et al, 1999). Electroporated cells into which catalase had been introduced showed decreased EGFR phosphorylation. CumOOH is a poor substrate for catalase (Anari, 1996), and thus, decreased phosphorylation probably stems from metabolizing H_2O_2 ,

implicating its role in EGFR phosphorylation; however, it does not seem to be the only mechanism in LPO-induced EGFR phosphorylation.

The increase of superoxide radicals ($O_2^{\cdot -}$) after the addition of CumOOH to keratinocytes has a rather slow onset: only after 180 min does a marked increase occur. This coincides with the depletion of glutathione after treatment with CumOOH. Initial levels of glutathione even show an increase after 60 minutes, as an expression of cellular regeneration or overproduction in response to a sublethal level of CumOOH. A similar result was observed by Vessey et al. (Vessey, 1992). Here, the critical dose for continuous decline in glutathione levels was estimated to be around 700 μ M CumOOH. Both of these effects might be attributable to late-term events in cellular damage; they do not seem to be the main pathways in CumOOH-mediated signaling. Arachidonic acid, in contrast, a product of phospholipase A_2 (PLA $_2$), does mediate cell signaling via superoxide anions. Nevertheless, the tumor promoting effect of CumOOH may very well be linked to its ability to produce large amounts of hydrogen peroxide and the decrease of cellular defense against peroxidative damage.

Interestingly, we found that the extracellular release of H_2O_2 following treatment with CumOOH was dependent upon the differentiation stage of keratinocyte cultures, where maximum release was obtained after one day after confluency (cells on +GF) and considerably lower levels before and after that day. Quiescent cells (-GF) showed no increase of fluorescence of the treated cells over negative control (data not shown). Conceivably, this is a result of the differentiation-induced change in the level of the antioxidant defense system of the cell (Vessey, 1995). It is unclear to what extent stimulation of EGFR plays a role in that process, since we measured high levels of hydrogen peroxide even in cells that were

confluent for several days. Guo et al. noted that levels of cytochrome P450 are higher in differentiated cells (Guo, 1991). Furthermore, levels of enzymes of the GSH pathway were increased with higher stages of differentiation, as was demonstrated in our own glutathione detection assay that was performed on keratinocytes that were quiescent. It has been speculated that the basal cell layer must invest all its energy in proliferation but can afford to expend very little energy on the antioxidant defense system (Vessey, 1995). Cell death assays with Alamar Blue™ showed higher cell mortality in cells that were rapidly dividing and +GF versus cells that were quiescent and –GF for two days, respectively. It was interesting to note that Northern Blot analysis revealed that c-jun was expressed much more strongly in cells that were –GF and quiescent versus cells that were +GF and dividing (unpublished data). It was noted that this was irrelevant for apoptosis to occur, as none of the conditions could demonstrate apoptosis in a caspase-3 assay, which shows that JNK functions are not only cell specific but also vary in a stimulus dependent manner (manuscript in preparation).

In summary, it was shown that CumOOH-induced lipid peroxidation and the generation of ROS in keratinocytes are early events that are involved in the activation of EGF receptor, ERK 1/2, p38, and JNK signal transduction pathways. Antioxidants and inhibitors of lipid peroxidation can suppress CumOOH-induced signal transduction via these pathways. H₂O₂ generation plays an important upstream event in the activation of these processes, and the tumor promoting effect of CumOOH may very well be linked to its ability to produce large amounts of hydrogen peroxide. These oxidant-induced pathologic alterations in signal transduction should be taken into consideration in further studies to identify mechanisms of tumorigenesis in the epidermis.

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Organic hydroperoxide-induced lipid peroxidation (LPO) and signal transduction pathways in human keratinocytes

Abstract

In den vergangenen Jahren beschäftigte sich ein zunehmender Anteil der molekularbiologischen Forschungsbemühungen mit dem Einfluß der Lipidperoxidation auf die Genese verschiedenener Erkrankungen. Auch im Bereich der Dermatologie hat die erhöhte Rate an Hauttumorerkrankungen zur Verstärkung der Bemühungen geführt, die molekularen Pathomechanismen zu erforschen und zu verstehen. Ultraviolette Strahlung (UVR, Ultraviolet Radiation) hat sich als ein potenter Aktivator von MAPK (Mitogen-Activated Protein Kinases) -induzierten Signaltransduktionskaskaden, als auch der Phosphorylierung von EGFR (Epidermal Growth Factor Receptor) erwiesen. Diese Zellsignalkaskaden spielen eine bedeutende Rolle in der Abfolge von Ereignissen, die zur Krebsentstehung in der Haut führen können. UVR vermittelt Teile dieses Ereignisses unter der Bildung von ROS (Reactive Oxygen Species) (z.B. H_2O_2). In dieser Studie wurde Cumene Hydroperoxide (CumOOH), ein organisches Hydroperoxid und ein bekannter Hauttumorpromoter, als Modellsubstanz verwendet, um ROS und Lipidperoxidation (LPO) zu initiieren und um den Einfluß auf die Zellsignalkaskaden in menschlichen Keratinozyten zu erhellen. Untersucht wurden der Einfluß auf den EGF Rezeptor, die MAP Kinasen ERK $\frac{1}{2}$ (Extracellular Regulated Kinases 1 and 2), JNK (C-Jun N-terminal Kinase) und p38.

Es konnte gezeigt werden, daß CumOOH im Keratinozytenmodell zur Lipidperoxidation führt. Ferner wurde H_2O_2 gebildet, welches sowohl intrazellulär als auch extrazellulär gemessen werden konnte. Es kam zur Phosphorylierung des EGFR in einem dosis- und zeitabhängigen Verhältnis. Ein ähnliches dosis- und zeitabhängiges Verhältnis zeigte sich auch bei der Aktivierung von ERK $\frac{1}{2}$ und p38. Wurde die EGFR Phosphorylierung durch den spezifischen Inhibitor PD 153035 inhibiert, so zeigte sich keinerlei Aktivierung des ERK $\frac{1}{2}$ Weges. Der p38 Weg wurde schon nach nur 5-minütigem Einfluß von CumOOH aktiviert. Es zeigte sich innerhalb der ersten 60 Minuten kein Abfall in der Glutathionkonzentration, vielmehr kam es zu einer reaktiven Steigerung. Das thiolische Antioxidans NAC (N-Acetylcysteine) und auch Trolox, ein Vitamin E Analogon, waren Inhibitoren der EGFR Phosphorylierung, sowie der Aktivierung von ERK $\frac{1}{2}$, p38. Die JNK Aktivierung folgte dem Verlauf der LPO und konnte durch NAC Vorbehandlung inhibiert werden. NAC und Trolox reduzierten beide extrazelluläres H_2O_2 . AA6P (Ascorbic Acid 6-Palmitate) war ein Inhibitor von intrazellulärem H_2O_2 . Keratinozyten, in die das Enzym Katalase durch Elektroporation eingefügt worden war, zeigten bei einer bestimmten Konzentration von CumOOH eine Reduktion der EGFR Phosphorylierung. Zusammenfassend konnte gezeigt werden, daß CumOOH frühzeitig Lipidperoxidation und die Produktion von ROS bewirkt. Beides sind wichtige Prozesse in der Initiierung der folgenden Zellsignalübertragung mit der Aktivierung von EGFR, ERK $\frac{1}{2}$, p38 und JNK. Verschiedene Antioxidantien konnten diese Aktivierung verhindern und wiesen somit einen protektiven Effekt auf.

