Redox Cycling of Phenol Induces Oxidative Stress in Human Epidermal Keratinocytes

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A variety of phenolic compounds are utilized for industrial production of phenol-formaldehyde resins, paints, lacquers, cosmetics, and pharmaceuticals. Skin exposure to industrial phenolics is known to cause skin rash, dermal inflammation, contact dermatitis, leucoderma, and cancer promotion. The biochemical mechanisms of cytotoxicity of phenolic compounds are not well understood. We hypothesized that enzymatic one-electron oxidation of phenolic compounds resulting in the generation of phenoxyl radicals may be an important contributor to the cytotoxic effects. Phenoxyl radicals are readily reduced by thiols, ascorbate, and other intracellular reductants (e.g., NADH, NADPH) regenerating the parent phenolic compound. Hence, phenolic compounds may undergo enzymatically driven redox-cycling thus causing oxidative stress. To test the hypothesis, we analyzed endogenous thiols, lipid peroxidation, and total antioxidant reserves in normal human keratinocytes exposed to phenol. Using a newly developed *cis*-parinaric acid-based procedure to assay site-specific oxidative stress in membrane phospholipids, we found that phenol at subtoxic concentrations (50 μM) caused oxidation of phosphatidylcholine and phosphatidylethanolamine (but not of phosphatidylserine) in keratinocytes. Phenol did not induce peroxidation of phospholipids in liposomes prepared from keratinocyte lipids labeled by *cis*-parinaric acid. Measurements with ThioGlo-1 showed that phenol depleted glutathione but did not produce thiyl radicals as evidenced by our high-performance liquid chromatography measurements of GS·-5,5-dimethyl1pyrroline N-oxide nitrone. Additionally, phenol caused a significant decrease of protein SH groups. Luminol-enhanced chemiluminescence assay demonstrated a significant decrease in total antioxidant reserves of keratinocytes exposed to phenol. Incubation of ascorbate-preloaded keratinocytes with phenol produced an electron paramagnetic resonance-detectable signal of ascorbate radicals, suggesting that redox-cycling of one-electron oxidation products of phenol, its phenoxyl radicals, is involved in the oxidative effects. As no cytotoxicity was observed in keratinocytes exposed to 50 μM or 500 μM phenol, we conclude that phenol at subtoxic concentrations causes significant oxidative stress. ***Key words: antioxidant reserve/cytotoxicity/glutathione/keratinocytes/oxidative stress/phenol/phospholipids. J Invest Dermatol 114:354–364, 2000***

The skin is continuously exposed to physical (e.g., solar irradiation) and chemical insults of environmental and occupational origin. Among the latter, a variety of phenolic compounds are routinely used in the chemical industry. A large group of synthetic polymers of phenolic origin are utilized commercially as bonding powders and cast articles, laminating materials, thermal and acoustic insulation, adhesives, etc. Phenol, xylene, *p*-*tert*-butylphenol, *p*-phenylphenol, and bisphenol are commonly used for production of phenol-formaldehyde resins, lacquers, and paints. Phenol, thymol, cresol, *p*-*tert*-butylphenol, butyl-hydroxyanisole, resorcinol, eugenol, and isoeugenol are widely utilized for the production of pharmaceuticals as well as toiletry, cosmetic and food flavoring goods.

It has been estimated that over half a million people in the United States are exposed to phenol at work (WHO, 1994, 1995; ATSDR, 1997). Skin contact with phenols during manufacturing is considered the major route of exposure in the workplace (ACGIH, 1991). Phenolic compounds are known to cause skin rashes, burns and ulceration, dermal inflammation and necrosis, irritant and allergic contact dermatitis, eczematous ''black-spot'' dermatitis, leukoderma, and cancer promotion (Ames *et al*, 1975; Bracher *et al*, 1990). In many cases the mechanisms underlying these toxic effects are unknown.

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Abbreviations: AAPH, azo-initiator, 2,2′-azobis(2-aminodinopropane)-dihydrochloride; AMVN, 2,2-azobis(2,4-dimethyl-valeronitrile); BHT, butylated hydroxytoluene; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; EPR, electron paramagnetic resonance; hAS, human serum albumin; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PnA, *cis*-parinaric acid; PS, phosphatidylserine.

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Enzymic oxidation of phenols is an effective metabolic pathway leading to their bioactivation. In particular, one-electron oxidation of phenolic compounds to their respective free radical intermediates, phenoxyl radicals, may trigger cyto- and genotoxic effects (Hess *et al*, 1991; Corbett *et al*, 1992; Chen and Eastmond, 1995; Thompson *et al*, 1995; BogadiSare *et al*, 1997; Hiramoto *et al*, 1998; Paolini *et al*, 1998; Tuo *et al*, 1998). For example, myeloperoxidase-catalyzed generation of phenoxyl radicals has been demonstrated to produce oxidative stress and cause oxidative modification of lipids, proteins and DNA in cells of myelogenous lineage (Stoyanovsky *et al*, 1996). A number of enzymes eminently expressed in skin cells, such as prostaglandin synthase, lipoxygenase, tyrosinase, and isoforms of CYP450 (Bickers, 1980; Pence and Naylor, 1990; Punnonen *et al*, 1991; Schmidt and Chung, 1992; Shindo *et al*, 1994; Scholz *et al*, 1994; Applegate *et al*, 1998), may create a redox environment favoring one-electron oxidation of phenolic compounds. This suggests that enzymatic generation of phenoxyl radicals from phenolic compounds may initiate their redox cycling by intracellular reductants such as thiols producing new reactive intermediates such as thiyl radicals. The latter yield superoxide in the course of their reactions with glutathione (GSH) and other thiols, and (in the presence of transition metal complexes) hydroxyl radicals, thus causing severe oxidative stress.

As keratinocytes are predominant cells in the skin epidermis, we undertook this study to determine whether phenol can induce oxidative stress in normal human epidermal keratinocyte cell cultures. We found phenol-induced changes in total antioxidant reserves, as well as in GSH content and peroxidation of specific classes of membrane phospholipids. Assays of these markers of radical-driven oxidation were complemented with evaluations of cell viability and apoptosis.

MATERIALS AND METHODS

Reagents *Cis*-parinaric acid (PnA) (Z-9, E-11, E-13, Z-15-octadecatetraenoic acid) was purchased from Molecular Probes (Eugene, OR). The purity of each lot of PnA purchased was determined by ultraviolet spectrophotometry using the molar extinction ε_{304nm} in ethanol, 80×10^3 M per cm. Fatty acid-free human serum albumin (hSA), phenol, polyoxyethylenesorbitan monolaurate (Tween 20), sodium molybdate, malachite green base, butylated hydroxytoluene (BHT), Hank's balanced salt solution, luminol, and GSH were purchased from Sigma (St Louis, MO). HCl, acetone, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Methanol, chloroform, hexane (HPLC grade), 2-propanol (HPLC grade), water (HPLC grade), and sodium nitrite were purchased from Aldrich Chemical (Milwaukee, WI). Silica G plates (5×5 cm) were purchased from Whatman (Clifton, NJ). Medium 154 was purchased from Cascade Biologics (Portland, OR). Phospholipid standards were purchased from Avanti Polar Lipids (Alabaster, AL). ThioGlo-1 was obtained from Covalent (Wobum, MA). The azo-initiator, 2,2′-azobis(2-aminodinopropane)-dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethyl-valeronitrile) (AMVN) were purchased from Wako Chemicals USA (Richmond, VA).

Normal human epidermal keratinocyte cell culture and treatment with phenol and AMVN Normal human epidermal keratinocytes from adults were obtained from Clonetics (San Diego, CA). Cells were plated at a density of 2.5×10^3 cells per cm^2 in 75 ml tissue culture flasks (Greiner Laboratories, GmbH, Germany). Normal human epidermal keratinocytes were grown in KGM-2 medium (Clonetics) at 37°C in a tissue culture incubator (5% CO_2) until confluent monolayers were obtained. Normal human epidermal keratinocytes between three and four passage were used for the assays. Repeated counts of normal human epidermal keratinocytes using a hemacytometer revealed a concentration of $1–2 \times 10^6$ cells per flask at the time of harvest. To induce oxidative stress in normal human epidermal keratinocytes, cells were incubated with 50 μM or 500 μM phenol or 500 μM AMVN in KGM-2 medium for 2 h at 37°C. After incubation, cells were scraped and homogenized in phosphate-buffered saline.

Chemiluminescence measurements of total anti-oxidant reserve in normal human epidermal keratinocytes A water soluble azo-initiator, 2,2′-azobis(2-amonodinopropane)-dihydrochloride (AAPH) was used to produce peroxyl radicals at a constant rate (Niki, 1990). Oxidation of luminol by AAPH-derived peroxyl radicals was assayed by monitoring chemiluminescence. A delay in the chemiluminescence response, which is caused by interaction of endogenous antioxidants with AAPH-derived peroxyl radicals, was observed upon addition of normal human epidermal keratinocyte homogenates. Based on the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants can be determined. The incubation medium contained 50 mM phosphate buffer (pH 7.4 at 37°C), AAPH (50 mM), and luminol (400 μM). The protein content in cells was measured by Bradford method using BioRad assay kit (BioRad, Richmond, CA). The concentration of protein in samples was 0.2 mg per ml. The reaction was started by addition of AAPH. Luminescent analyzer 633 (Coral Biomedical, San Diego, CA) was employed for determinations.

Fluorescence assay of GSH and protein sulfhydryls GSH and total protein sulfhydryl concentration in normal human epidermal keratinocyte was determined using ThioGlo-1, a maleimide reagent, which produces a highly fluorescent product upon its reaction with SH groups (Langmuir *et al*, 1996). GSH content was estimated by an immediate fluorescence response registered upon addition of Thio Glo-1 to the cell homogenate. Protein sulfhydryls were determined as an additional increase in fluorescence response after addition of sodium dodecyl sulfate (4.0 mM) to the same cell homogenate. A standard curve was established by the addition of GSH (0.04–4.0 μM) to 100 mM phosphate buffer, pH 7.4 containing 10 μM ThioGlo-1 (dimethyl sulfoxide solution). Shimadzu RF-5301PC spectrofluorophotometer (Kyoto, Japan) was employed for the assay of fluorescence using excitation at 388 nm and emission at 500 nm. The data obtained were exported and treated using RF-5301PC Personal Fluorescence Software (Shimadzu).

Electron paramagnetic resonance (EPR) measurement of GS-5,5dimethyl-1pyrroline N-oxide nitroxide (GS-DMPO) stock solutions were cleaned with activated charcoal prior to use as previously described (Stoyanovsky *et al*, 1996). Detection of thiyl radicals as DMPO-nitroxide was performed in a model system (containing myeloperoxidase 2.0 U per ml, GSH 4.0 mM, phenol 400 μM, H_2O_2 200 μM, and DMPO 100 mM) as well as in normal human epidermal keratinocytes and HL60 cells (10^6 cells per ml exposed to 100 mM DMPO, 400 μM phenol, 200 μM H_2O_2 (without addition of exogenous GSH). EPR measurements were performed on a JEOL-RE1X spectrometer (Kyoto, Japan) at 25°C in gas permeable Teflon tubing (0.8 mm internal diameter, 1.3 μm thickness, Alpha Wire, Elizabeth, NJ). The tubing was filled with 60 μl of a sample immediately after the addition of reagents, folded into quarters, and placed in an open EPR quartz tube in such a way that all the sample was within the effective microwave irradiation area. Spectra were recorded at 3352 G center field, 20 mW power, 0.79 G modulation, and 50 G per min scan time. Under these conditions, GS-DMPO nitroxide has a four-line EPR spectrum with hyperfine splitting constants ($a^N = 15.4$ G and $a^H = 16.2$ G) (Schreiber *et al*, 1989).

HPLC assay of GS-DMPO nitrone GS-DMPO nitrone was determined as described previously (Stoyanovsky *et al*, 1996). Briefly, normal human epidermal keratinocyte (1×10^5 per ml) were treated with phenol (50 μM or 500 μM) at 37°C for 1 h. DMPO (100 mM) was added 2 min before the addition of phenol. After normal human epidermal keratinocytes were washed twice with phosphate-buffered saline and harvested by trypsinization, pellets of cells were resuspended in 100 mM Na-phosphate buffer and disrupted by three sonic pulses using a 4710 series Ultrasonic Homogenizer (Cole-Parmer Instrument, Chicago, IL). Then homogenates were spun down at $10,000 \times g$ and supernatants were used for determinations. A Shimadzu LC-10A HPLC system (Kyoto, Japan) was employed with an LC-600 pump and an SPD-10AV ultraviolet detector (detection was by absorbance at 258 nm). C18 reverse phase column (Ultrasphere ODS, 5 μM particle size, 4.6×250 nm. Beckman) was used for determinations. Eluent was 100 mM Na-phosphate buffer, pH 7.4 flow rate of 1.0 ml per min. Under this condition, retention time for GS-DMPO nitrone was 8.1 min.

EPR measurement of ascorbate radicals EPR measurements for ascorbate radicals were performed on a JEOL-RE1X spectrometer (Kyoto, Japan) at 37°C in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness obtained from Alpha Wire (Elizabeth, NJ). The tube (approximately 8 cm in length) was filled with 75 μl of a mixed sample, folded into quarters and placed in an open 3.0 mm internal diameter EPR quartz tube in such a way that all of the sample was within the effective microwave irradiation area. The spectra of ascorbate radicals were recorded at 3352 G, center field; 20 mW, power; 0.79 G, field modulation; 20 s,

sweep time; 2.5 G, sweep width; 4000, receiver gain; and 0.1 s, time constant. Spectra were collected using EPRware software (Scientific Software Services, Bloomington, IL).

PnA-based assay of oxidative stress in normal human epidermal keratinocyte phospholipids PnA was incorporated into normal human epidermal keratinocytes by addition of its hSA complex (PnA-hSA) to medium 154 without additives. The complex PnA-hSA was prepared as described by Ritov *et al* (1996). PnA-hSA complex was added to the cells during their log phase of growth (2.5×10^5 cells) to give a final concentration of 5 μg PnA/10^6 cells and incubated in medium 154 for 2 h at 37°C in a CO_2 incubator to allow metabolic incorporation of PnA into phospholipids. At the end of the incubation period, the cells were washed with medium 154 with hSA (0.5 mg per ml) followed by another wash with medium 154 without hSA to remove the excess of unbound PnA.

Extraction of cell lipids Total lipids were extracted from normal human epidermal keratinocyte (2.5×10^5 cells) using Folch's procedure (Folch *et al*, 1957). The lipid extract was dried under nitrogen and dissolved in 0.2 ml of 2-propanol/hexane:water (4:3:0.16, by vol.).

HPLC analysis of cell lipids Lipid extracts were separated by normal phase HPLC using a 5 μm Supelcosil LC-Si column (4.6 × 250 mm) and an ammonium acetate gradient as described by Kagan *et al* (1998). The separations were performed using a Shimadzu HPLC system (model LC-600) (Kyoto, Japan) equipped with an in-line configuration of fluorescence (model RF-551) and ultraviolet-VIS (model SPD-10AV) detectors. The effluent was monitored at 205 nm to detect lipids. Fluorescence of PnA was measured at 420 nm emission after excitation at 324 nm. Ultraviolet and fluorescence data were processed and stored in digital form with Shimadzu EZChrom software. Lipid phosphorus was determined using a modification of the method for microdetection as described by Chalvardjian and Rubnicki (1970).

Preparation of PnA-labeled liposomes Normal human epidermal keratinocytes were incubated in the presence of PnA as described above and lipids were extracted from the cells by the Folch procedure (Folch *et al*, 1957). Lipid extract was evaporated under N_2 and the film of lipids was resuspended in HEPES buffer (pH 7.4) to achieve the lipid concentration equivalent to that used in the cellular experiments. Lipososmes were performed by sonication of lipid suspension (four 15 s pulses on ice) using tip sonicator (Ultrasonic Homogenizer 4710 series, Cole-Parmer Instrument, Chicago, IL). Liposomes containing PnA-labeled phospholipids were incubated for 2 h in the presence or absence of phenol (50 μM) at 37°C in the dark. After incubation lipids were extracted again and resolved by HPLC as above.

High-performance thin-layer chromatography (HPTLC) assay of phospholipid composition of normal human epidermal keratinocytes Individual phospholipid classes in lipid extracts were separated by two-dimensional HPTLC on silica G plates (5 × 5 cm) as described earlier (Kagan *et al*, 1998). The phospholipid spots identified by iodine staining were scraped and transferred to tubes. Lipid phosphorus was determined by a submicro method as described by Bottcher *et al* (1961).

Electron microscopy This technique was performed using a standard protocol. Briefly, cells were grown and treated as described above. Keratinocytes were then washed with phosphate-buffered saline, and fixed in Karnovsky Fixative (2.5 glutaraldehyde + 3% paraformaldehyde) in 1 M sodium cacodylate, pH 7.4, and postfixed with osmium tetroxide. Cells were dehydrated in graded alcohol solutions and propylene oxide and embedded in LX-112 (Ledd, Burlington, UT). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEOL 1220, Jeol, Kyoto, Japan).

Viability and apoptosis Cell viability was determined by Trypan Blue exclusion test. Apoptosis was evaluated by measurement of the number of hypodiploid cells using flow cytometry (Darzynkiewicz *et al*, 1992). Washed cell suspensions were fixed with 70% (vol/vol) aqueous ethanol at 4°C for 16 h. Low-molecular weight DNA was removed by washing the cells with 1:24 (vol/vol) 200 mM phosphate-citrate buffer (Tuo *et al*, 1998). To avoid clumping, the suspension was aspirated using a glass syringe with a 25 gauge needle and added back into the tube. The resuspended cells were treated with 0.1 mg propidium iodide per ml and 0.1 mg RNAse A per ml for 0.5 h at 37°C and stored at 4°C for 16 h. DNA content was determined using a Becton Dickinson Immunocytometry System (San Jose, CA) equipped with a 488 nm argon laser by measuring forward and orthogonal light scatter and the peak and area of red fluorescence. The cell populations were quantified using the Becton Dickinson Lysis II software on a standard count of 10,000 events.

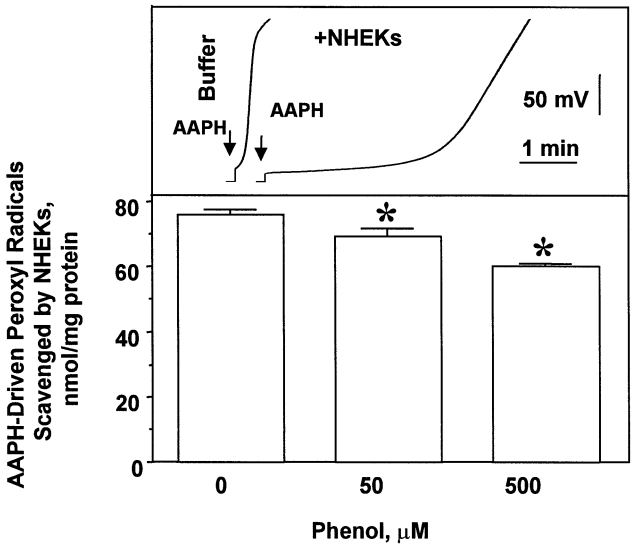

Figure 1. Effect of phenol on total antioxidant reserve of normal human epidermal keratinocytes. Normal human epidermal keratinocytes were incubated in medium 154 in the absence and in the presence of phenol (50 μM or 500 μM) for 2 h at 37°C. After incubation, cells were washed twice with Na-phosphate buffer, pH 7.4 and anti-oxidant reserve was estimated by chemiluminescence (oxidation) of luminol (400 μM) induced by AAPH (50 mM) in Na-phosphate buffer, pH 7.4 in the presence and in the absence of cell homogenates untreated (control) or treated with phenol (50 μM or 500 μM). All values are mean ± SEM, n = 10; *$p < 0.05$ *versus* control (untreated cells). Upper panel: Typical chemiluminescence responses in the absence and in the presence of cell homogenate (0.2 mg protein). *Arrows* indicate the addition of AAPH.

Statistical evaluation Data are expressed as mean ± SE values of at least three experiments. The data were analyzed by ANOVA, and the statistical significance of differences was set at $p < 0.05$.

RESULTS

Total anti-oxidant reserve of normal human epidermal keratinocytes Upon exposure to oxidative insult, the accumulation of biomarkers of oxidative stress occurs when anti-oxidant defenses are compromised. Therefore, we first investigated total antioxidant reserves of normal human epidermal keratinocytes exposed to phenol. Interaction of AAPH-derived peroxyl radicals with luminol in phosphate buffer (pH 7.4 at 37°C) resulted in a characteristic chemiluminescence response (**Fig 1**, upper panel). Addition of sonicated normal human epidermal keratinocytes to the oxidation system produced a lag period during which the chemiluminescence response was not observed. The duration of the lag period produced by normal human epidermal keratinocytes from control (nonexposed) cells was significantly greater than that observed in the presence of normal human epidermal keratinocytes treated with phenol (**Fig 1**). We found a 10% decrease in total antioxidant reserves of normal human epidermal keratinocytes exposed to 50 μM phenol, and a 21% decrease after exposure to 500 μM phenol ($p < 0.05$). Our calculations based on the rate constant for AAPH decomposition (Niki, 1990) and experimentally determined duration of lag periods, showed that antioxidants in nontreated normal human epidermal keratinocytes were able to scavenge 75.8 ± 1.7 nmol peroxyl radicals per mg protein. In exposed normal human epidermal keratinocytes, total antioxidant reserves were decreased such that 69.2 ± 2.6 and 60.2 ± 0.7 nmol peroxyl radicals per mg protein could be scavenged after incubation with 50 μM and 500 μM phenol, respectively.

GSH and protein sulfhydryls in normal human epidermal keratinocytes Addition of ThioGlo-1 to normal human epidermal keratinocyte homogenates produced an instantaneous increase in fluorescence due to the formation of the GSH ThioGlo-1 reaction product (**Fig 2*A***). The intensity of the response remained constant until sodium dodecyl sulfate was added to the incubation system to unfold protein SH groups at which point a slow increase of fluorescence was observed which leveled-off within 45–60 min. This latter fluorescence response was due to the interaction of protein SH groups with ThioGlo-1 (Kagan *et al*, 1999). As shown on **Fig 2(*B*)** the exposure to phenol produced a significant concentration-dependent drop in GSH concentration in normal human epidermal keratinocytes of 29% and 57% from the initial level after incubation in the presence of 50 μM and 500 μM phenol, respectively. Similarly, the content of protein sulfhydryls was significantly decreased in normal human epidermal keratinocytes incubated with phenol (**Fig 2*C***).

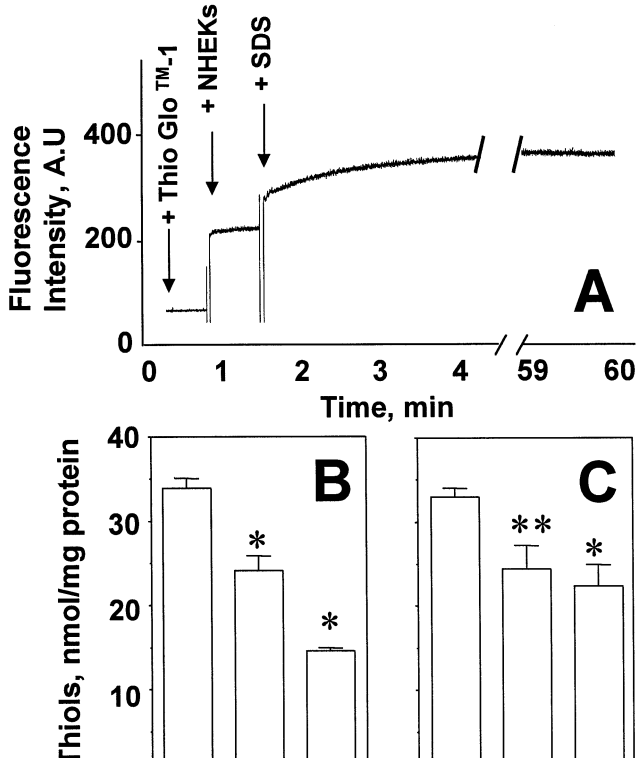

Figure 2. Effect of phenol on GSH and protein sulfhyryl content in normal human epidermal keratinocytes. Normal human epidermal keratinocytes were incubated in medium 154 in the absence and in the presence of phenol (50 μM or 500 μM) for 2 h at 37°C. After incubation cells were washed twice with Na-phosphate buffer, pH 7.4. Glutathione and protein thiols were determined using ThioGlo-1 (10 μM) as described in *Materials and Methods*. (*A*) Typical fluorescence response of ThioGlo-1 (10 μM) in Na-phosphate buffer, pH 7.4 in the absence and in the presence of normal human epidermal keratinocytes homogenate and sodium dodecyl sulfate. Arrows indicate addition of ThioGlo-1 (10 μM), normal human epidermal keratinocytes homogenate (30 μg protein) and sodium dodecyl sulfate (4 mM). (*B*) Effect of phenol (50 μM and 500 μM) on GSH content in normal human epidermal keratinocytes. (*C*) Effects of phenol (50 μM and 500 μM) on the content of protein thiols in normal human epidermal keratinocytes. *$p < 0.01$ *versus* control (untreated cells); **$p < 0.05$ *versus* control (untreated cells).

Assay of spin adducts of glutathionyl radicals with DMPO (GS-DMPO nitrone) in normal human epidermal keratinocytes Oxidation of thiols by phenoxyl radicals proceeds via a one-electron intermediate, the thiyl radical (GS·), whose formation may be monitored by spin-trapping with EPR detection (Schreiber *et al*, 1989) or HPLC detection (Stoyanovsky *et al*, 1996). As phenol caused a significant decrease of GSH content in normal human epidermal keratinocytes, we attempted to elucidate whether direct one-electron oxidation of GSH by phenoxyl radicals is accompanied by the formation of glutathionyl radicals. Whereas steady-state concentrations of glutathionyl radical adducts with a spin-trap, DMPO, have been previously registered by EPR in normal human epidermal keratinocytes, their detection has been only possible in the presence of very high, nonphysiologic concentrations of arachidonic acid (Schreiber *et al*, 1989). Our attempts to detect adducts of glutathionyl radicals with DMPO, DMPO-GS·, in normal human epidermal keratinocytes incubated in the presence of 50 μM or 500 μM phenol gave negative results (**Fig 3*e***), although a typical well-resolved EPR signal of DMPO-GS· was readily detectable in a model system containing myeloperoxidase/GSH/H_2O_2/phenol (**Fig 3*c***). Only a weak signal of DMPO-GS· adduct was observed when myeloperoxidase was incubated with H_2O_2 and GSH in the absence of phenol (**Fig 3*b***). Moreover, in myelogenous human

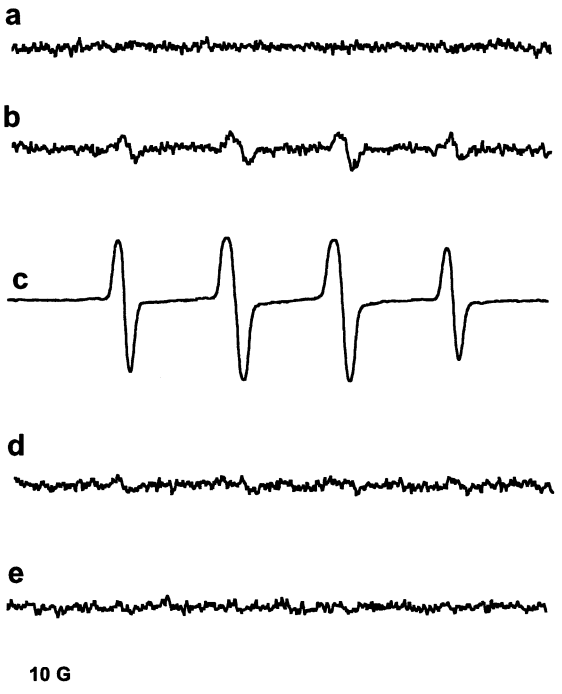

Figure 3. EPR signals of DMPO spin-adducts with GS· radicals (DMPO-GS·) generated by in a model system (containing myeloperoxidase/H_2O_2 in the presence of GSH and phenol) as well as in HL60 cells and normal human epidermal keratinocytes. Model system (*spectra a–c*). The reaction mixture contained 0.1 M phosphate buffer pH 7.4, 100 mM DMPO, GSH (4 mM) and: a myeloperoxidase (2 U per ml) + H_2O_2 (200 μM) + phenol (400 μM); b myeloperoxidase (2 U per ml) + H_2O_2 (200 μM) + GSH (4.0 mM); c myeloperoxidase (2 U per ml) + H_2O_2 (200 μM) + phenol (400 μM) + GSH (4.0 mM). HL60 cells (*spectrum d*). Cells (10^7 per ml) were incubated in 0.1 M phosphate buffer pH 7.4, containing 100 mM DMPO, phenol (400 μM), and H_2O_2 (200 μM). DMPO was added 2 min before addition of phenol. Normal human epidermal keratinocytes (*spectrum e*). Cells (10^7 per ml) were incubated in 0.1 M phosphate buffer pH 7.4, containing 100 mM DMPO, and phenol (400 μM). DMPO was added 2 min before addition of phenol. Spectra of GS-DMPO adduct were recorded at 3352G, center field; 50 G sweep width; 0.79 G, field modulation; 10 mW, microwave power; 0.1 s, time constant; 2 min time scan.

leukemia HL60 cells, known to contain relatively high concentrations of myeloperoxidase (Traweek *et al*, 1995), a very weak signal of DMPO-GS· adducts was discernible in the EPR spectrum (**Fig 3*d***).

It is likely that EPR detection of DMPO-GS· as a marker for phenol-induced formation of thiyl radicals was not sensitive enough for our purposes. Therefore, we decided to use a previously developed HPLC procedure for detection of the EPR-silent GS-DMPO adduct (GS-DMPO nitrone). This HPLC assay has sensitivity superior to that of EPR detection of DMPO-GS· adducts (Stoyanovsky *et al*, 1996). Typical HPLC tracings of the standard adduct as well as the adduct exogenously added to normal human epidermal keratinocytes incubated in the presence of 500 μM phenol are shown in **Fig 4**. No adduct formation, however, was detectable when DMPO-loaded normal human epidermal keratinocytes were incubated in the presence of either 50 μM or 500 μM phenol. Given that phenol/H_2O_2-induced accumulation of DMPO-GS· adducts were readily observed in myeloperoxidase-rich HL-60 cells (**Fig 4*c***), our results indicate that the formation of GS· radicals most likely did not occur in normal human epidermal keratinocytes exposed to phenol. Decrease of GSH content in normal human epidermal keratinocytes exposed to phenol may be due to either direct oxidation by phenoxyl radicals or result from enzymatically driven consumption of GSH (e.g., in GSH peroxidase-catalyzed reactions). In the latter case, no intermediate formation of free GS·-radicals would be expected to occur (Stoyanovsky *et al*, 1996). Our failure to detect GS·-DMPO adducts indicates that decreased GSH concentration in normal human epidermal keratinocytes was likely not due to its direct one-electron oxidation by phenoxyl radicals but rather to its enzymatic consumption. Alternatively, phenol-driven oxidation was not accompanied by the formation of free phenoxyl radicals. To determine whether phenoxyl radicals were produced in normal human epidermal keratinocytes in the presence of phenol, we performed experiments using ascorbate that is known to reduce phenoxyl radicals very effectively in a one-electron reaction to form EPR-detectable ascorbate radicals (Kagan *et al*, 1994).

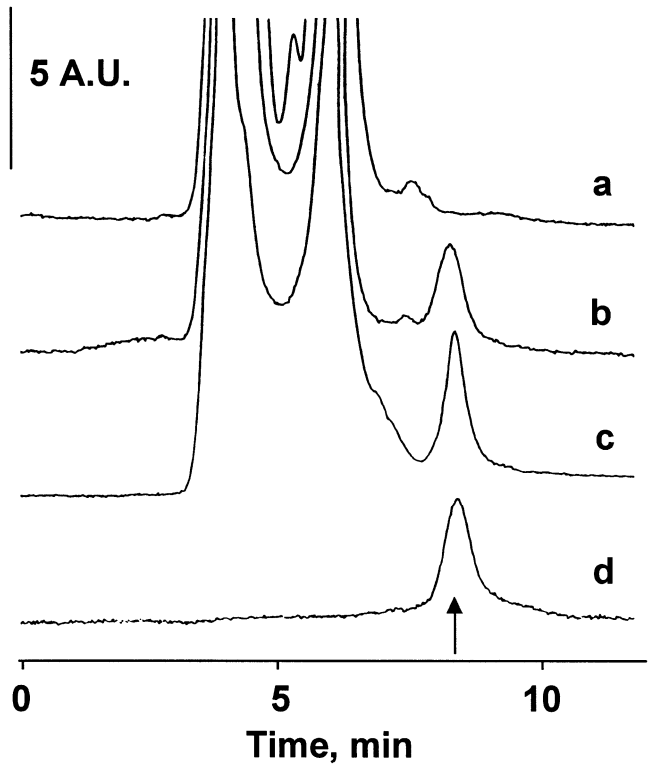

Figure 4. HPLC tracings of GS-DMPO nitrone. Normal human epidermal keratinocytes were treated with phenol (500 μM) at 37°C for 1 h. 100 mM DMPO was added 2 min before addition of phenol. After normal human epidermal keratinocytes were washed twice with phosphate-buffered saline and harvested by trypsinization, pellets of cells were resuspended in 100 mM Na-phosphate buffer, disrupted by sonication, and centrifugated at 10,000 × *g* for 5 min. Supernatant was analyzed by HPLC as described in the *Materials and Methods* section. HL60 cells grown in RPMI 1640 medium containing 10% fetal bovine serum were incubated in 100 mM phosphate buffer (pH 7.4 at 37°C) in the presence of phenol, DMPO and H_2O_2 (as indicated below). (*a*) Normal human epidermal keratinocytes treated with 500 μM phenol. (*b*) Normal human epidermal keratinocytes treated with 500 μM phenol + GS-DMPO nitrone standard (0.53 nmol). (*c*) HL-60 cells treated with 1 mM phenol in the presence of 400 μM of H_2O_2. (*d*) GS-DMPO nitrone standard (0.53 nmol).

Phenol-induced ascorbate radicals in ascorbate-preloaded normal human epidermal keratinocytes As phenoxyl radicals of phenol are too short-lived to be directly detectable by conventional EPR spectroscopy, we used ascorbate which readily reduces phenoxyl radicals to form ascorbate radicals that can be easily monitored by EPR. As normal human epidermal keratinocytes are usually grown in ascorbate-free medium, sodium ascorbate (120 nmol per 10^6 cells) was added to the cells before EPR measurements. Exposure of normal human epidermal keratinocytes to phenol in the presence of ascorbate produced EPR-detectable signal of ascorbate radicals (**Fig 5**). This EPR signal was hardly detectable in ascorbate-preloaded normal human epidermal keratinocytes in the absence of phenol. Predictably, no ascorbate radical signal was discernible in the EPR spectra of normal human epidermal keratinocytes nonsupplemented with ascorbate. These results suggest that redox-cycling of phenoxyl radicals by ascorbate occurs in normal human epidermal keratinocytes.

HPTLC of normal human epidermal keratinocyte phospholipids In our initial experiments aimed at the detection of oxidative stress in phospholipids, we attempted to determine whether changes in phospholipid composition occurred in normal human epidermal keratinocytes exposed to phenol. To

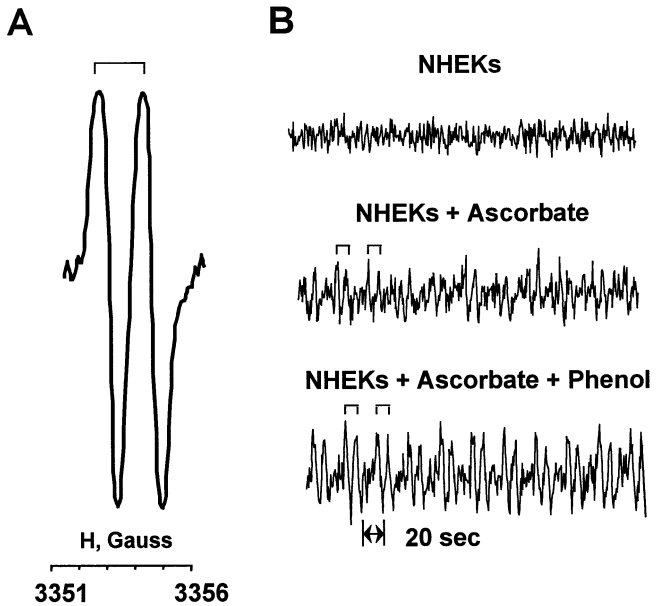

Figure 5. Typical EPR spectrum and time course of ascorbate radicals generated during incubation of normal human epidermal keratinocytes with ascorbate in the absence and in the presence of phenol. (*A*) EPR spectrum; (*B*) time course. EPR spectra were recorded under the following reaction conditions: normal human epidermal keratinocytes (10^6 cells) in 50 mM Na-phosphate buffer, pH 7.4, were incubated with ascorbate (120 nmol per 10^6 cells) in the absence or in the presence of phenol (500 nmol per 10^6 cells) at 37°C. EPR settings are given in *Materials and Methods*.

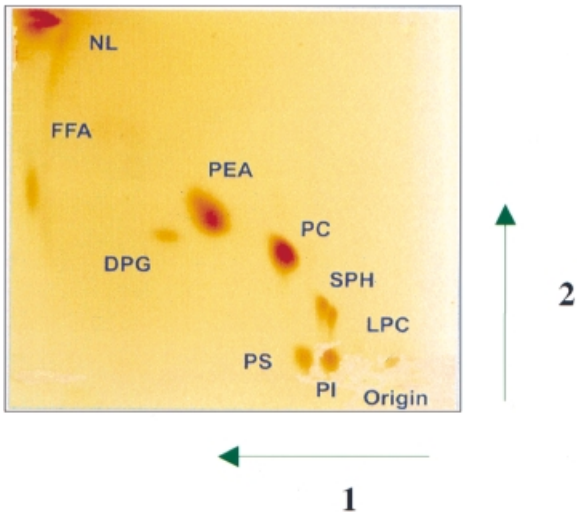

Figure 6. A typical HPTLC two-dimensional chromatogram of total lipid extract from normal human epidermal keratinocytes stained by exposure to iodine vapor. The identities of lipids are: NL, neutral lipids; FFA, free fatty acids; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

Table I. Phospholipid composition of normal human epidermal keratinocytes[a]

Phospholipids	Phospholipid content, % of total phospholipids		
	Control	Phenol	AMVN
Phosphaidylcholine	43.3 ± 1.1	42.3 ± 1.3	44.6 ± 1.5
Phosphatidylethanolamine	29.9 ± 1.0	29.6 ± 1.2	28.6 ± 1.1
Phosphatidylserine	6.6 ± 0.4	7.1 ± 0.6	6.1 ± 0.6
Sphingomyelin	10.0 ± 0.5	10.8 ± 0.5	9.8 ± 0.6
Phosphatidylinositol	8.1 ± 0.7	7.8 ± 0.6	8.2 ± 0.6
Diphosphatidylglycerol	1.5 ± 0.4	1.6 ± 0.5	1.7 ± 0.5
Lysophosphatidylcholine	0.5 ± 0.4	0.8 ± 0.2	1.0 ± 0.5

[a]Normal human epidermal keratinocytes were incubated in the absence and in the presence of phenol (50 μM) or AMVN (500 μM) for 2 h at 37°C in medium 154. After incubations, lipids were extracted and resolved by HPTLC as described in *Materials and Methods*. All values are means of percent total phospholipids values ± SEM (n = 4, control; n = 3, phenol and AMVN treatment).

this end, we used a sensitive HPTLC assay for different classes of membrane phospholipids. Normal human epidermal keratinocytes were incubated for 2 h at 37°C in the presence of 50 μM phenol or a lipophilic azo-initiator of radicals, AMVN (500 μM). **Figure 6** shows a typical chromatogram of phospholipids from control normal human epidermal keratinocytes. Seven different phospholipid spots were detected by HPTLC of lipids extracted from either control or phenol-exposed normal human epidermal keratinocytes (**Fig 6**). Phosphatidylcholine (PC) represented 43.4 ± 1.1% and 42.3 ± 1.3% of the total phospholipids in the control and exposed cells, respectively, with phosphatidylethanolamine (PEA) being the next most prominent phospholipid (29.9 ± 1.0% and 29.6 ± 1.2%, respectively). Additionally, the other phospholipids in the order of their abundance – sphingomyelin > phosphatidylinositol > phosphatidylserine (PS) >> diphosphatidylglycerol >> lysophosphatidylcholine – were detectable on the HPTLC plates. No significant difference in phospholipid distribution was detected in normal human epidermal keratinocytes following exposure to phenol and AMVN (**Table I**). The slight increase in content of lysophosphatidylcholine, a relatively minor phospholipid, in phenol-treated cells may be due

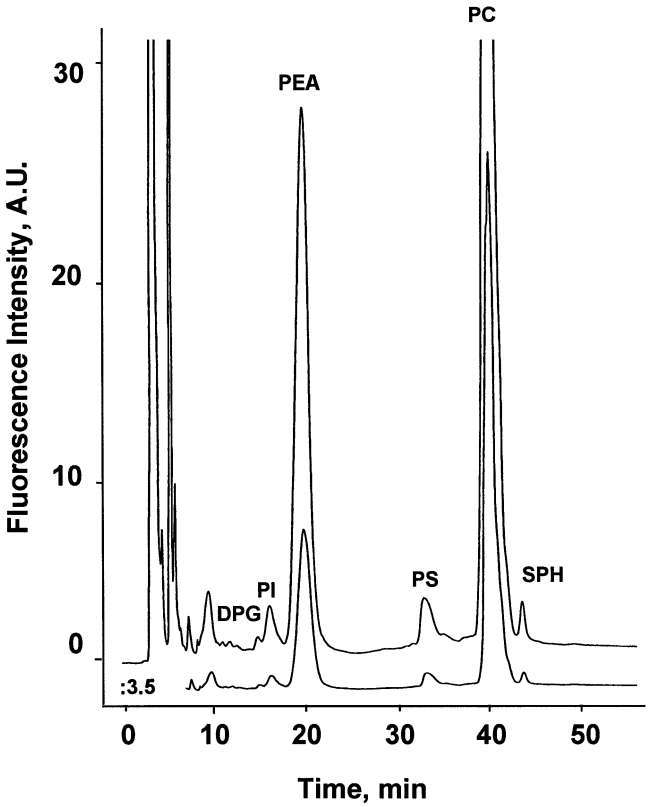

Figure 7. Normal phase HPLC chromatograms of total lipids extracted from normal human epidermal keratinocytes. Fluorescence emission intensity, excitation at 324 nm, emission at 420 nm. Normal human epidermal keratinocytes were incubated with hSA-Pna complex (5 μg of PnA/0.5 mg hSA/10^6 cells) in medium 154 for 2 h at 37°C then washed with medium 154 with and without hSA (0.5 mg per ml). After incubation, cells were scraped and lipids were extracted and resolved by HPLC as described in *Materials and Methods*. DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SPH, sphingomyelin.

to PC hydrolysis that is known to be exacerbated by oxidative stress (van Kuijk *et al*, 1985; Pacifici *et al*, 1994). As with phenol, incubation with AMVN did not produce any significant changes of phospholipid composition (Tyurina *et al*, 1997), with the exception of a slight increase in the content of lysophosphatidylcholine.

PnA-based assay of oxidative stress in different classes of normal human epidermal keratinocyte phospholipids Oxidatively modified phospholipids are known to undergo rapid and effective remodeling that involves phospholipase A_2-catalyzed hydrolysis with subsequent acyltransferase-catalyzed reacylation of peroxidized phospholipids (Kagan *et al*, 1978; Rashba-Step *et al*, 1997). Hence, the lack of HPTLC-detectable changes in the phospholipid composition of normal human epidermal keratinocytes exposed to either phenol or AMVN might be due to effective repair of phospholipids via deacylation/reacylation pathways (Van der Vliet and Bast, 1992; McLean *et al*, 1993). Therefore, we applied PnA-based assay that permits sensitive detection of specific oxidative stress (oxidative "hits") in selected membrane phospholipids of cells without interference from repair mechanisms. During last 2 y, the method has been extensively used for the assessment of oxidative stress in viable cells by our laboratory as well as by others (McGuire *et al*, 1997; Fabisiak *et al*, 1998; Kagan *et al*, 1998; Drummen *et al*, 1999; Dubey *et al*, 1999).

Typical fluorescence emission profile of the column eluate of the constituent phospholipids is shown in **Fig 7**. Values for specific incorporation of PnA into membrane phospholipids of normal human epidermal keratinocytes suggest that incorporation of PnA

in the various phospholipids is different, and the amount of PnA incorporated can be ranked in the following order: PC > PEA > PS > PI (**Table II**). We found that phenol (50 μM) caused substantial oxidation of PnA-labeled phospholipids. Two major classes of phospholipids in normal human epidermal keratinocytes, namely PEA and PC, were primary targets for phenol-induced peroxidation with relatively minor contribution by the acid phospholipid, phosphatidylinositol. Phenol produced essentially no oxidation in PS despite the fact that this phospholipid is oxidized by AMVN. The phenol induced peroxidation of the major phospholipids was significant (**Table III**). It should be noted that based on the known rate constant for AMVN decomposition at 37°C (1.36×10^{-6} [AMVN] $\times M^{-1} s^{-1}$) (Niki, 1990), 2 h incubation of 500 μM AMVN yields about 6.4 μM radicals. In comparison with peroxidation induced by this well known oxidant, phenol (50 μM) was somewhat less effective than AMVN (500 μM).

In a separate series of experiments, we tested whether incubation of liposomes prepared from PnA-labeled phospholipids (extracted from normal human epidermal keratinocytes) with phenol would yield any oxidation of phospholipids. When phospholipids, equivalent to that in 2.5×10^5 cells, were incubated for 2 h with phenol (50 μM) no significant oxidation of any phospholipid class was observed (**Table IV**). In contrast, AMVN produced a significant oxidation in all phospholipid classes when liposomes prepared from PnA-labeled normal human epidermal keratinocyte phospholipids were incubated with the azo-initiator (500 μM, 2 h) (**Table IV**). Together these results suggest that enzymatic redox cycling of phenol in normal human epidermal keratinocytes was responsible for the development of oxidative stress in membrane phospholipids.

Table II. Specific incorporation of PnA into membrane phospholipids of normal human epidermal keratinocytes

Phospholipids	Specific incorporation (mol PnA/mol phospholipid)
Phosphatidylcholine	1:57
Phosphatidylethanolamine	1:78
Phosphatidylserine	1:152
Phosphatidylinositol	1:246

Effects of phenol and AMVN on cell viability normal human epidermal keratinocytes As shown in **Table V**, viability of normal human epidermal keratinocytes decreased by over 50% after incubation with AMVN. In contrast, exposure to either 50 μM or 500 μM phenol for 2 h at 37°C did not produce any measurable changes in viability of normal human epidermal keratinocytes. Additionally, 2 h exposure of normal human epidermal keratinocytes to phenol (500 μM) did not cause any apoptosis as measured by increased number of hypodiploid cells analyzed by flow cytometry (**Fig 8**). Flow cytometry analyzes of cells incubated for 2 h in the presence or absence of phenol always showed < 1% apoptotic cells.

Morphology Transmission electron microscopic examination of normal human epidermal keratinocytes incubated with 50 μM (400 nmol per 10^6 cells) phenol for 2 h at 37°C did not reveal any ultrastructural changes in cell morphology (**Fig 9**). The nucleus and cytoplasmic organelles of treated normal human epidermal keratinocytes did not show any changes compared with the controls. Mitochondria, tonofilaments, and other cytoplasmic organelles are present. We have consistently observed vacuolated cytoplasm in untreated (control) normal human epidermal keratinocyte cultures.

DISCUSSION

In this study, we used phenol as a prototype of phenolic compounds that are known to cause a variety of cytotoxic and genotoxic effects in skin (Adams, 1980; Kensler *et al*, 1995;

Table III. Effect of phenol and AMVN on oxidation of PnA-labeled phospholipids in normal human epidermal keratinocytes[a]

Phospholipids	PnA-labeled phospholipids (ng PnA/μg total lipid phosphorus)		
	Control	Phenol	AMVN
Phosphatidylcholine	66.64 ± 2.70	33.78 ± 5.10*	6.70 ± 0.13**
Phosphatidylethanolamine	34.00 ± 1.00	14.81 ± 2.50*	1.96 ± 0.10**
Phosphatidylserine	3.75 ± 0.20	4.02 ± 0.30	1.23 ± 0.48**
Phiosphatidylinositol	2.97 ± 0.20	2.25 ± 0.12***	0.28 ± 0.05**

[a]Normal human epidermal keratinocytes were incubated in medium 154 in the presence of PnA for 2 h at 37°C, then washed with this medium with and without hSA. Then PnA-labeled normal human epidermal keratinocytes were incubated in medium 154 in the absence and in the presence of phenol (50 μM) or AMVN (500 μM) for 2 h at 37°C. After incubation, lipids were extracted and resolved by HPLC as described in *Materials and Methods*. All values are mean ± SEM, n = 3; *p < 0.02, **p < 0.001 and ***p < 0.05 *versus* control (untreated cells).

Table IV. Effect of phenol on oxidation of PnA–labeled phospholipids in liposomes prepared from phospolipids of normal human epidermal keratinocytes[a]

Phospholipids	PnA-labeled phospholipids (ng PnA/μg total lipid phosphorus)		
	Control	Phenol	AMVN
Phosphatidylcholine	66.23 ± 5.70	64.66 ± 6.20	7.28 ± 0.38*
Phosphatidylethanolamine	32.58 ± 3.88	34.87 ± 3.50	2.60 ± 0.20
Phosphatidylserine	3.62 ± 0.63	3.77 ± 0.92	1.01 ± 0.10
Phiosphatidylinositol	2.94 ± 0.79	2.51 ± 0.28	0.29 ± 0.05

[a]Normal human epidermal keratinocytes were incubated in medium 154 in the presence of PnA for 2 h at 37°C, then washed with this medium with and without hSA. Then lipids were extracted and liposomes were prepared by sonication. Liposomes were incubated in HEPES buffer, pH 7.4 in the absence and in the presence of phenol (50 μM) or AMVN (500 μM) for 2 h at 37°C. After incubation, lipids were extracted and resolved by HPLC as described in *Materials and Methods*. All values are mean ± SEM, n = 3. *p < 0.001 *versus* control, untreated liposomes.

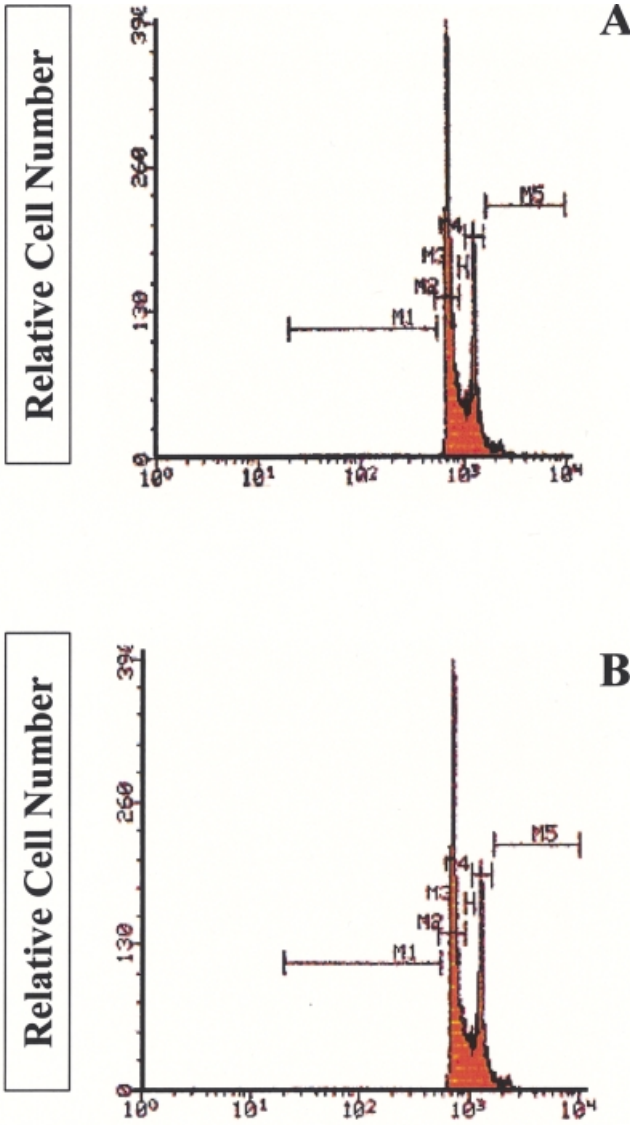

Figure 8. Flow cytometric profiles of DNA content in normal human epidermal keratinocytes following treatment with phenol. DNA content of normal human epidermal keratinocytes was determined by flow cytometry of propidium iodide-stained cells as described in *Materials and Methods*. Part (*A*) shows control untreated cells and part (*B*) depicts cells treated for 2 h with 500 μM phenol. The various cell populations defined by DNA content and cell cycle stage are given as M1 = hypodiploid (apoptosis), M2 = 1n DNA content (G_0, G_1), M3 = > n – < 2n DNA (S phase), M3 = 2n DNA (G_2/M). M5 contains events that are characterized by greater than 2n DNA content. Flow cytometric plots are shown on a log scale to amplify the region containing potentially apoptotic cells. Approximate percentage of cells in each cell cycle stage for control and phenol treatment, respectively, are G_0/G_1, 54.9% and 61%; S, 13.1% and 9.8%; G_2/M, 29% and 28.8%. Apoptotic cells were always less than 1% in any condition tested.

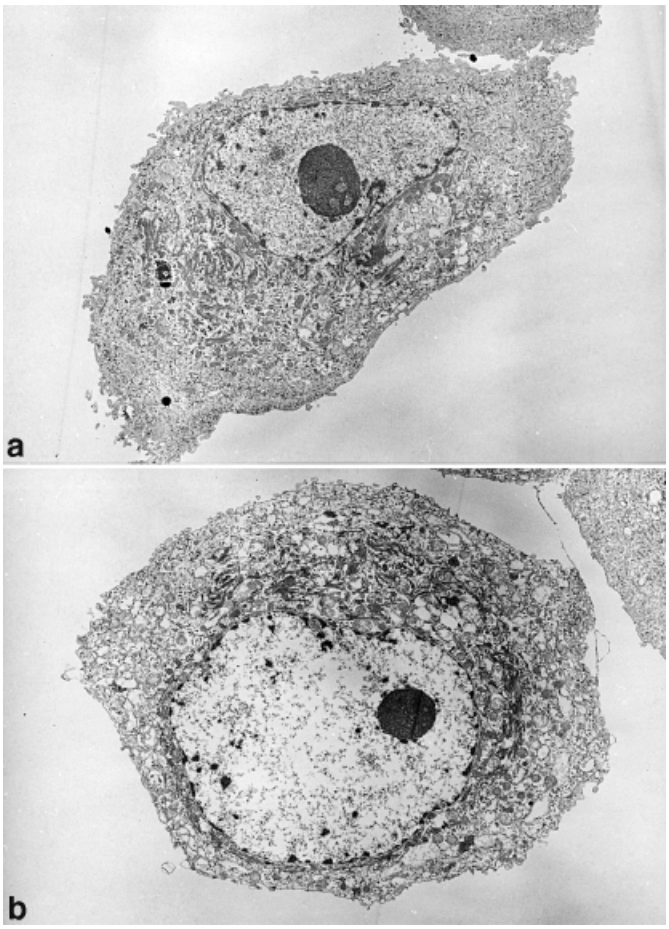

Figure 9. Electron microscopy of normal human epidermal keratinocytes. (*a*) Control normal human epidermal keratinocytes. (*b*) Normal human epidermal keratinocytes treated with 50 μM phenol. Distinctions between the phenol-treated and control keratinocytes are not evident either in the nucleolus or cytoplasm. Note normal ultrastructural appearance of nucleus and nucleolus. Cytoplasm along with other organelles contains tonofibrils. Magnification × 6750. Conditions: keratinocytes (5×10^5) were treated with 50 μM phenol at 37°C in CO_2 incubator in KGM-2 medium 1640 for 2 h. The cells were fixed immediately after treatment.

Table V. Effect of phenol and AMVN on viability of normal human epidermal keratinocytes[a]

Additions	Viability (%)
Control (no additions)	78.8 ± 4.3
Phenol, 50 μM	79.3 ± 5.5
Phenol, 500 μM	72.9 ± 15.4
AMVN, 500 μM	37.7 ± 2.4*

[a]Normal human epidermal keratinocytes were incubated in medium 154 in the absence and in the presence of phenol (50 μM) or AMVN (500 μM) for 1 h at 37°C. After incubation cells were scraped and viability was determined microscopically using a hemocytometer by Trypan Blue exclusion. All values are mean ± SEM, n = 5, *p < 0.001 *versus* control (untreated cells).

McCartney, 1996; Toro *et al*, 1996). We tested the hypothesis that redox cycling of one-electron oxidation intermediates of phenolic compounds, phenoxyl radicals, by intracellular reductants, such as GSH and protein sulfhydryls, triggers oxidative stress (see **Fig 10**). Our results demonstrate for the first time that phenol at subtoxic concentrations caused oxidative stress in normal human epidermal keratinocytes. Exposure of normal human epidermal keratinocytes to phenol decreased total antioxidant reserves, depleted GSH and protein SH groups and induced oxidation of membrane phospholipids. It is likely that these prooxidant effects were triggered by phenoxyl radicals enzymatically generated in normal human epidermal keratinocytes (**Fig 10**). In support of this, our EPR measurements revealed phenol-induced enhancement of ascorbate radical production in normal human epidermal keratinocytes preloaded with ascorbate. These data provide strong (although indirect) evidence for the phenoxyl radical generation in normal human epidermal keratinocytes as our previous work has

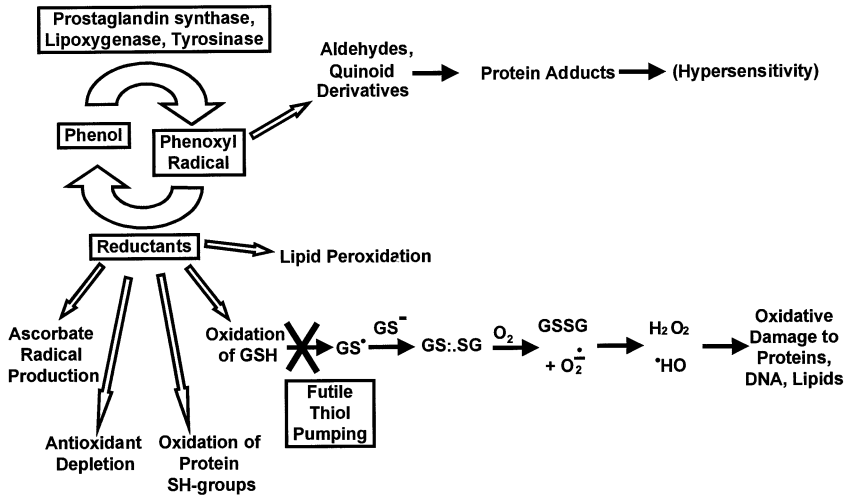

Figure 10. Enzymatic one-electron oxidation of phenol in normal human epidermal keratinocytes to phenoxyl radical and its potential interactions with major classes of intracellular biomolecules resulting in redox cycling, depletion of antioxidant reserves, ''futile thiol pumping'', accumulation of biomarkers of oxidative stress and oxidative damage.

established that ascorbate is the primary reductant of phenoxyl radicals in model systems and in cells (Kagan *et al*, 1994).

Loss of GSH indicates that GSH may play a specific part in phenol-induced oxidative stress in normal human epidermal keratinocytes. ''Futile thiol pumping'' has been described as a potential mechanism involved in toxic effects of phenolic compounds (Schreiber *et al*, 1989). During this process, enzymatically formed phenoxyl radicals may be reduced by thiols to regenerate the phenolic compound as a substrate for repeated enzyme-catalyzed one-electron oxidation (**Fig 10**). Oxidation of thiols (GSH) may generate thiyl radicals whose subsequent interactions with intracellular thiols and oxygen initiate new oxidative cascades and generate new reactive oxygen species – superoxide and (in the presence of transition metals) hydroxyl radicals. Thus, redox cycling of enzymatically formed phenoxyl radicals may induce severe oxidative stress, which ultimately leads to cytotoxicity and genotoxicity. In normal human epidermal keratinocytes, however, the thiyl radical-induced cascade is, most likely, not triggered by the phenoxyl radicals produced. This conclusion is based on the lack of both EPR-detectable DMPO-GS· adducts and HPLC-detectable GS-DMPO nitrone in the course of incubation of normal human epidermal keratinocytes with phenol despite the formation of EPR-detectable ascorbate radicals upon addition of ascorbate plus phenol to normal human epidermal keratinocytes.

Selective oxidation of two major membrane phospholipids, PC and PEA, occurred in normal human epidermal keratinocytes exposed to phenol. Interestingly, no phenol-induced peroxidation of PS was detectable in normal human epidermal keratinocytes. Our previous work has identified peroxidation of PS as an important lipid-signaling pathway in apoptosis (Fabisiak *et al*, 1997, 1998). The lack of PS oxidation suggests that phenol at subtoxic concentrations does not induce apoptosis. This indeed was confirmed by our flow cytometric assays of hypodiploid cells after exposure of normal human epidermal keratinocytes to phenol. Comparison of our results on phenol-induced peroxidation of membrane phospholipids with those on changes of phospholipid composition indicates that repair of oxidatively modified phospholipids was very effective in normal human epidermal keratinocytes as no significant changes of phospholipid composition were found.

Recently, a close link between generation of reactive oxygen species and changes in intracellular Ca^{2+} generated by growth factors and hormones has been demonstrated (Goldman *et al*, 1998) suggesting potential association of oxidative stress with proliferation, differentiation, and apoptosis. As endogenously formed nitric oxide may control peroxidation and oxidative stress (O'Donnell *et al*, 1999), Ca^{2+}-dependent regulation of nitric oxide production in keratinocytes (Heck *et al*, 1992; DeGeorge *et al*, 1997) may act as an important oxidative stress regulatory mechanism. One of the goals of our future studies will be to determine whether oxidative stress response to phenol is different in growing keratinocytes *versus* calcium-differentiated keratinocytes.

At the low concentrations of phenol used in this study, no significant cytotoxicity to normal human epidermal keratinocytes was observed. This is in contrast to myeloperoxidase-rich human myelogenous leukemia HL-60 cells, where phenol-induced severe oxidative stress accompanied by cytotoxicity (Goldman *et al*, 1999; Day *et al*, 1999). These results are consistent with the data in the literature that indicate that phenol concentrations in the range 50–500 μM are well below those that cause toxicity, necrosis, and skin discolorations (Pullin *et al*, 1978) or are used for ''phenol peeling'' on the pigmented areas of the skin (Bazex *et al*, 1995; Ryan and La Fourcade, 1995).

An important question is the identity of enzymatic pathways involved in one-electron oxidation of phenolic compounds. We found that a characteristic EPR signal of ascorbate radical was immediately detectable in ascorbate-preloaded normal human epidermal keratinocytes exposed to phenol. This effect of phenol was observed in the absence of exogenous peroxides – a necessary substrate for peroxidase-catalyzed reaction (Taffe *et al*, 1987) – suggesting that peroxidase(s) were not likely to be involved in the redox cycling of phenol in normal human epidermal keratinocytes. Earlier studies by Schreiber *et al* (1989) identified prostaglandin synthase as a potent source of phenol/arachidonic acid-induced thiyl radicals in murine keratinocytes (see **Fig 10**). Other potential candidates may include lipoxygenases and tyrosinases whose expression and activity may be regulated by differentiation as well as environmental and occupational factors (Janssen Timmen *et al*, 1995; Scholz *et al*, 1995; Jimenez and Garcia-Carmona, 1996; Leong *et al*, 1996; Datta *et al*, 1997). Experiments are now underway to characterize specific enzymatic pathways involved in bioactivation and redox cycling of phenolic compounds in normal human epidermal keratinocytes.

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