Phenol-Induced *in Vivo* Oxidative Stress in Skin: Evidence for Enhanced Free Radical Generation, Thiol Oxidation, and Antioxidant Depletion

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A variety of phenolic compounds are utilized in industry (e.g., for the production of phenol (PhOH)-formaldehyde resins, paints and lacquers, cosmetics, and pharmaceuticals). They can be toxic to skin, causing rash, dermal inflammation, contact dermatitis, depigmentation, and cancer promotion. The biochemical mechanisms for the dermal toxicity of phenolic compounds are not well understood. We hypothesized that topical PhOH exposure results in the generation of radicals, possibly via redox-cycling of phenoxyl radicals, which may be an important contributor to dermal toxicity via the stimulation of the induction and release of inflammatory mediators. To test this hypothesis, we (1) monitored *in vivo* the formation of PBN-spin-trapped radical adducts by ESR spectroscopy, (2) measured GSH, protein thiols, vitamin E, and total antioxidant reserves in the skin of B6C3F1 mice topically treated with PhOH, and (3) compared the responses with those produced by PhOH in mice with diminished levels of GSH. We found that dermal exposure to PhOH (3.5 mmol/kg, 100 μL on the shaved back, for 30 min) caused oxidation of GSH and protein thiols and decreased vitamin E and total antioxidant reserves in skin. The magnitude of the PhOH-induced generation of PBN-spin-trapped radical adducts in the skin of mice with diminished levels of GSH (pretreated with BCNU, an inhibitor of glutathione reductase, or BSO, an inhibitor of γ-glutamylcysteine synthetase) was markedly higher compared to radical generation in mice treated with PhOH alone. Topical exposure to PhOH resulted in skin inflammation. Remarkably, this inflammatory response was accelerated in mice with a reduced level of GSH. Epidermal mouse cells exposed to phenolic compounds showed the induction of early inflammatory response mediators, such as prostaglandin E_2 and IL-1β. Since dermal exposure to PhOH produced ESR-detectable PBN spin-trapped signals of lipid-derived radicals, we conclude that this PhOH-induced radical formation is involved in oxidative stress and dermal toxicity *in vivo*.

Introduction

Occupational exposure to PhOH[1] and phenolic compounds is estimated to affect over a half a million people in the United States annually (*1*). The major route of exposure is through skin contact (*2*) during the production of phenol-formaldehyde resins, paints and lacquers, cosmetics, and pharmaceuticals. This exposure to phenolic compounds is known to cause skin rashes, burns, ulceration, dermal inflammation, necrosis, irritant and allergic contact dermatitis, eczematous black-spot dermatitis, and leukoderma, and is believed to play a role in cancer promotion. The mechanism underlying the toxic effects of PhOH in the skin is unknown.

The cytotoxic and genotoxic effects that result from occupational exposure to PhOH may be due to the one-electron oxidation of phenolic compounds to free radical intermediates known as phenoxyl radicals (*3–10*). Free radical generation may be an important contributor to the dermal toxicity of phenolic compounds. Enzymes expressed in the skin, such as cyclooxygenase (COX-2), prostaglandin synthase, lipoxygenase, and tyrosinase (*11–16*), may provide a favorable redox environment for the oxidation of phenolic compounds. We hypothesize that topical PhOH exposure induces free radical formation via the enzymatic metabolism of phenolic compounds, which is capable of inducing oxidative stress and causing an inflammatory response in the skin.

ROS play an important role in the initiation and progression of many conditions where inflammatory mediators are implicated (*17, 18*). The presence of ROS causes an upregulation in the release and production of various pro-inflammatory mediators, such as IL-1, IL-6, IL-8, TNF-α, leukotrienes, and prostaglandins, which may be responsible for the inflammation seen following exposure to an irritant chemical such as PhOH (*19*). The release of such inflammatory mediators stimulates the production of other cytokines and further amplifies the inflammatory response (*20*).

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[1] Abbreviaitions: PhOH, phenol; PBN, α-phenyl-*N*-*tert*-butylnitrone; GSH, glutathione; BCNU, 1,3-bis(2chloroethyl)-1-nitrosourea; BSO, l-buthione-[*S*,*R*]-sulfoximine; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; 2,2′-DP, 2,2′-dipyridyl; AAPH, 2,2′-azobis(2aminodinopropane)-dihydrochloride; ELISA, enzyme linked immunosorbent assay.

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GSH, a sulfur-containing antioxidant, is involved in maintaining intracellular redox status by regulating and controlling oxidative stress (*21–23*). GSH directly scavenges ROS, and the loss of GSH is associated with an augmented pro-inflammatory state (*24*). Depletion of GSH upregulates ROS and results in enhanced cytokine secretion in tissue (*25*). The reduction of intracellular low molecular weight thiols prevents cytokine production (*26–29*).

Exposure of normal human epidermal keratinocytes (NHEK) to a variety of phenolic compounds causes oxidative stress and cytotoxicity (*30*). The observed cytotoxicity of phenols correlated with decreases in GSH (*30*). To study the role of GSH in the phenol-induced radical formation and inflammation in skin *in vivo*, we used BCNU (an inhibitor of glutathione reductase) and BSO (an inhibitor of γ-glutamylcysteine synthetase) to decrease basal GSH levels. In particular, we determined: (1) whether pre-exposure to BCNU or BSO induced accelerated oxidative stress and augmented the depletion of antioxidants in the skin of B6C3F1 mice after topical exposure with PhOH, (2) whether topical exposure of B6C3F1 mice to PhOH induced the formation of free radical adducts assessed *ex vivo* by ESR spectroscopy in the skin of animals with normal and reduced levels of GSH achieved by pretreatment with BCNU or BSO, and (3) whether cyclooxygenase (COX-2) activation is involved in the generation of the inflammatory mediators, IL-1β and prostaglandin E_2, following PhOH or BSO/PhOH exposure in murine epidermal JB6 cells.

Materials and Methods

Chemicals. Fatty acid-free fetal bovine serum, luminol, SDS, 2,2′-DP, PBN, PhOH, GSH, BCNU, and BSO were purchased from Sigma Chemicals Co. (St. Louis, MO). Methanol, ethanol, chloroform, hexane, and water (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Thio-Glo-1 was obtained from Covalent Inc. (Wobum, MA). AAPH was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Prostaglandin E_2 ELISA Kits were obtained from Cayman Chemical Co. (Ann Arbor, MI). IL-1β ELISA Kits were obtained from BioSource International (Camarillo, CA).

Animals. B6C3F1 mice (3–4 weeks; 16–18 g body weight) were obtained from Taconic (Germantown, NY). Each mouse was housed in an individual ventilated cage with Alpha-Dri cellulose chips and hardwood Beta-chips for bedding and provided HEPA-filtered air under controlled environmental conditions in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited, specific pathogen-free facility. Food and water were provided ad libitum. All animal procedures were performed in accordance with an approved Animal Care and Use Committee (ACUC) protocol.

Animal Exposures. B6C3F1 female mice ($n = 6$ per group for 3 experiments) were injected with BCNU or BSO to achieve a 30% decrease in the level of glutathione in the skin. BCNU (40 mg/kg, 100 μL) was injected intraperitoneally (i/p) 3 h prior to phenol exposure. BSO (2 mmol/kg, 100 μL) was injected i/p 2 times within 21 h. The dorsal area (1.5 × 2.0 cm^2) of the mouse skin was shaved 24 h prior to topical exposure of PhOH (3.5 mmol/kg, 100 μL). Thirty minutes following PhOH exposure, mice were sacrificed by inhalation of excess carbon dioxide. Six mice per group were used for three replicate experiments.

Skin Collections and Preparation of Homogenates. Skin flaps from the intrascapular area of the back of mouse were excised and samples taken for ESR studies, histopathology, and biochemical analysis. Skin for biochemical analysis was immediately frozen at −80 °C until further processed. The skin homogenates were prepared from frozen tissues with ice-cold phosphate-buffered saline (PBS, 7.4) using a tissue tearer (model 985–370, Biospec Products, Inc., Racine, WI).

Skin Histopathology and Examination. The skin was processed after fixation in 10% neutral buffered formalin. Hematoxylin and eosin stained histology slides were prepared for light microscopic examination. Photomicrographs were prepared using an Olympus 300 double-headed microscope (Tokyo, Japan).

Fluorescence Assay of Glutathione (GSH) and Protein Sulfhydryls. Total thiol concentration in homogenates of skin was determined using ThioGlo-1, a maleimide reagent that produces a highly fluorescent product upon its reaction with sulfhydryl groups (*30*). A standard curve was established by addition of GSH (0.04–2.0 μM) to 0.1 M phosphate buffer (pH 7.4) containing 10 μM ThioGlo-1. GSH content was estimated by an immediate fluorescence response registered upon addition of ThioGlo-1 to tissue homogenates. Total protein sulfhydryls were determined from the additional fluorescence response after the addition of SDS (4 mM) to the same homogenate. A Shimadzu spectrofluorometer RF-5000 U (Shimadzu, Japan) was employed in the assay; excitation 388 nm and emission 500 nm. The data were acquired using an excitation slit of 1.5 nm and an emission slit of 5 nm. The fluorescence signals were exported from the spectrofluorometer using RF-5000 U PC Personal Fluorescence software (Shimadzu, Japan).

Determination of Skin Bi-fold Thickness as an Inflammatory Biomarker. To assess the extent of PhOH-induced edema in mouse skin following treatment, a dial caliper (The Dyer Company, Lancaster, PA) was used to measure the skin bi-fold thickness. Changes in skin bi-fold thickness were determined by the measurements of three random locations within the area of exposure per mouse. Edema formation was expressed as net increase in skin bi-fold thickness between experimental (PhOH) and control groups.

ESR Study of Generation of Free Radicals in Skin. The skin used to assess the free radical generation was obtained from the intrascapular region of the mouse back. Equal amounts of the skin (1.5 × 2.0 cm) were obtained from the control and treated animals. Following BCNU or BSO exposure, the mice were injected subcutaneously in the dorsal lumbar area with the spin-trap PBN (1 mmol/kg, 100 μL) dissolved in saline. Ten minutes following the injection with PBN, the intrascapular area of the back was painted with saline (100 μL) or PhOH (3.5 mmol/kg, 100 μL). Thirty minutes following phenol or saline exposure, the animals were sacrificed, and skin flaps were collected. Samples were minced and homogenized with 15 mL of chloroform/methanol (2:1 mixture) and 1.0 mL of 2,2′-DP (30 mM) to prevent oxidation during lipid extraction (*31*). The skin homogenates were then centrifuged (2000 rpm, 10 min, 4 °C), and the chloroform layer was collected. The lipid extracts from the skin were dried under nitrogen. One milliliter of lipid extracts from the mouse skin was used to detect spin-trapped free radicals. ESR spectra were recorded immediately at room temperature using a quartz flat cell under static conditions using a Bruker EMX with a Super High Q cavity. Instrumental settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; conversion time, 0.6 s; time constant, 1.3 s. Spectra were recorded on an IBM-compatible computer interfaced to the spectrometer. The determination of the coupling constants was done in chloroform/methanol. Hyperfine coupling constants were determined using the Win-Sim program of the NIEHS public EPR software tools package, which is available on the Internet (http://EPR.niehs.nih.gov/). The program was allowed to systematically vary the hydrogen and nitrogen hyperfine coupling constants and the relative concentrations of each species to achieve the best fit to the experimental spectra.

HPLC Assay of α-Tocopherol. Extracts of α-tocopherol from skin homogenates were prepared using a procedure described by Lang et al. (*32*). A Waters HPLC system with a 717 auto sampler, a Hewlett Packard ODS Hypersil column (5 mm; 200 × 4.6 mm), a Waters 600 Controller pump, and a 474 fluorescence detector was used to measure α-tocopherol in the samples. The wavelengths employed in the assay were 292 nm (excitation) and 324 nm (emission). Both the excitation and emission slits were 5 nm. Eluent was methanol with a flow rate of 1 mL/min. Under these conditions,

Table 1. Antioxidant Depletion in Skin B6C3F1 Mice after Topical Treatment with Phenol[a]

antioxidant	control	phenol	decrease
vitamin E (pmol/mg)	286.3 ± 58.9	143.1 ± 20.9*	50.0%
glutathione (nmol/mg)	12.6 ± 1.0	5.0 ± 0.4*	60.5%
protein thiols (nmol/mg)	36.2 ± 2.2	6.5 ± 0.9*	82.0%
total antioxidant reserve, (nmol/mg)	694.9 ± 84.4	328.5 ± 12.7*	52.7%

[a] Mean ± SE of three experiments. * $p < 0.05$, vs control.

the retention time for α-tocopherol was 8.2 min. The minimum detection level for α-tocopherol in the samples was 0.1 pmol/mg of protein. The data acquired were exported from the Waters 474 detector using Millennium 2000 software (Waters Associates, Milford, MA).

Chemiluminescence Measurements of Total Antioxidant Reserve. A water-soluble azo-initiator, AAPH, was used to produce peroxyl radicals (*33*). Oxidation of luminol by AAPH-derived peroxyl radicals was assayed by the chemiluminescence response. A delay in the chemiluminescence response caused by the interaction of endogenous antioxidants with AAPH-derived peroxyl radicals was observed upon the addition of homogenates. On the basis of the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants was evaluated. The incubation medium contained 0.1 M phophate buffer (pH 7.4) at 37 °C, AAPH (50 mM), and luminol (0.4 mM). The reaction was started by the addition of AAPH. A luminescent analyzer 633 (Coral Biomedical, Inc., San Diego, CA) was employed for determination.

Cell Culture. The JB6 P^+ mouse epidermal cell line was a kind gift from the laboratory of Dr. Nancy Colburn. JB6/AP cells were cultured in Eagle's MEM containing 5% fetal bovine serum and 2 mM L-glutamine. The cells were plated at 37 °C in a 5% CO_2 atmosphere. Cells were grown in 96-well plates for 18 h prior to exposure to allow for adherence. Cells were exposed to BSO (10 μM) for 4 h. After 4 h, the media was changed, and the cells were exposed to PhOH (15 mM) and BSO for ½, 1, 2, or 18 h. At the completion of the experiment, cellular supernatant was stored for ELISA analysis, while cells were suspended in PBS. All samples were frozen at −80 °C until analyzed.

ELISA Measurements of Pro-inflammatory Mediators. ELISA was utilized to measure the pro-inflammatory mediators, interleukin-1β (IL-1β) and prostaglandin E_2, in supernatants from JB6 cells exposed to PhOH. The concentration of IL-1β in cultured supernatants was measured using a commercially available ELISA immunoassay kit (Biosource International, Camarillo, CA) with sensitivity for IL-1β concentrations ranging from 7.8 pg/mL to 1000 pg/mL. Concentration of prostaglandin E_2 in cultured supernatants was measured using prostaglandin E_2 EIA Kit-Monoclonal (Cayman Chemical, Ann Arbor, MI), which ranges in sensitivity from 7.8 pg/mL to 1000 pg/mL. Each supernatant was assayed at two dilutions, and each dilution was assayed in duplicate.

Protein Assay. Measurements of protein in homogenates from mouse skin were run using a Bio-Rad protein assay kit, catalog no. 500-0006 (Richmond, CA).

Statistics. Data were expressed as the mean ± SEM for each group. A one-way ANOVA test for multiple comparisons was employed. A Holm Sidak was used for pairwise comparisons as well as for comparisons versus a control group. Statistical significance was set at $p < 0.05$.

Results

PhOH-Induced Oxidative Stress. Dermal exposure to PhOH was found to cause a significant depletion in the overall antioxidant status of murine skin. A significant decrease in GSH (60.5%) in the skin of B6C3F1 mice was observed as early as 1 h post-exposure to PhOH. Protein thiol, vitamin E, and total antioxidant reserve levels were also significantly reduced by 82%, 50%, and 52.7%, respectively (Table 1).

Skin Inflammation of B6C3F1 Mice Following PhOH Exposure. Skin bi-fold thickness was assessed as a measure of skin inflammation following exposure to PhOH (1.75, 3.5, 7.0 mmol/kg; 0.5, 1, 2, 3, and 6 h). PhOH exposure resulted in significant dose-dependent increase in skin bi-fold thickness of B6C3F1 mice as compared to the control (Figure 1A). The observed increase in skin bi-fold thickness following exposure to PhOH (3.5 mmol/kg) was not time-dependent. A 90% increase in skin bi-fold thickness occurred as early as 0.5 h post-exposure and this increase did not change significantly through 6 h post-exposure (Figure 1B). No changes in skin bi-fold thickness were observed in vehicle (PBS)-exposed mice.

Depletion of GSH in the Skin of B6C3F1 Mice by Treatment with BSO and BCNU. We used BCNU, an agent that effectively inhibits glutathione reductase, to reduce glutathione levels in the skin of mice. The effects of BCNU were studied over a period of 18 h, and no significant differences in GSH were observed once a 30% decrease was achieved 3.5 h post-exposure (Figure 2A). BSO irreversibly inhibits the enzyme γ-glutamylcysteine synthetase, thereby decreasing GSH levels. Initially, GSH levels were reduced by 52.2% following 2 injections of BSO in a 21 h time period. BSO exposure longer than 21 h produced no further significant decreases (Figure 2B).

Formation of Lipid-Derived Radicals following PhOH Exposure. PhOH exposure resulted in the formation of lipid-

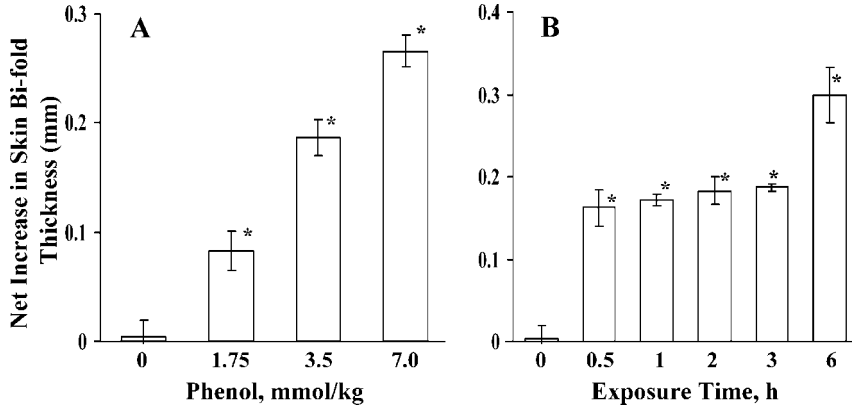

Figure 1. (A) Dose-dependent increase in skin bi-fold thickness as a result of dermal phenol exposure in B6C3F1 mice. Mice were painted on the dorsal area of the back with 1.75, 3.5, or 7.0 mmol/kg PhOH. Skin bi-fold thickness was measured 2 h following exposure. (B) Time-dependent increase in skin bi-fold thickness as a result of dermal phenol exposure in B6C3F1 mice. Mice ($n = 6$ per group) were painted on the dorsal area of the back with phenol (3.5 mmol/kg). Skin bi-fold thickness was measured 0.5, 1, 2, 3, or 6 h following phenol exposure. Values are the mean ± SE of three experiments. * $p < 0.05$ vs PBS treated control mice.

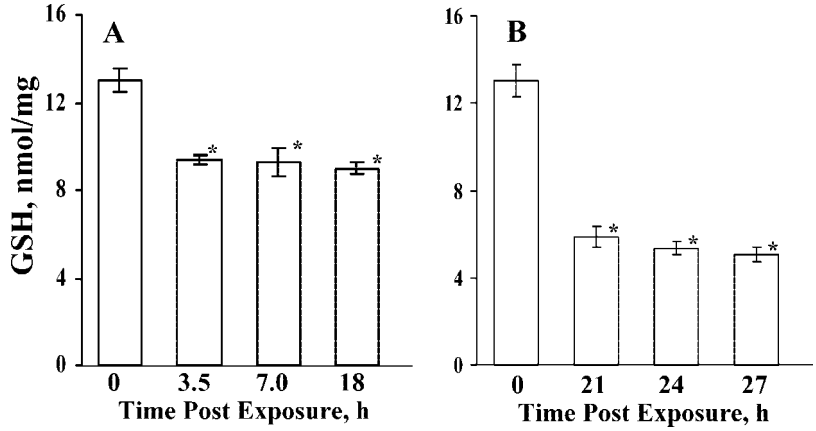

Figure 2. (A) Time-course depletion of glutathione by 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) in the skin of B6C3F1 mice. Mice were injected intraperitoneally with BCNU (40 mg/kg, 100 μL). Skin was collected for GSH analysis 3.5, 7.0, or 18 h post-exposure. (B) Time-course depletion of glutathione by L-buthione-[*S*,*R*]-sulfoximine (BSO) in the skin of B6C3F1 mice. Mice ($n = 6$ per group) were injected intraperitoneally 2 times with BSO (2 mmol/kg, 100 μL). Skin was collected for GSH analysis 21, 24, or 27 h post-exposure. Values are the mean ± SE of three experiments. * $p < 0.05$ vs PBS treated control mice.

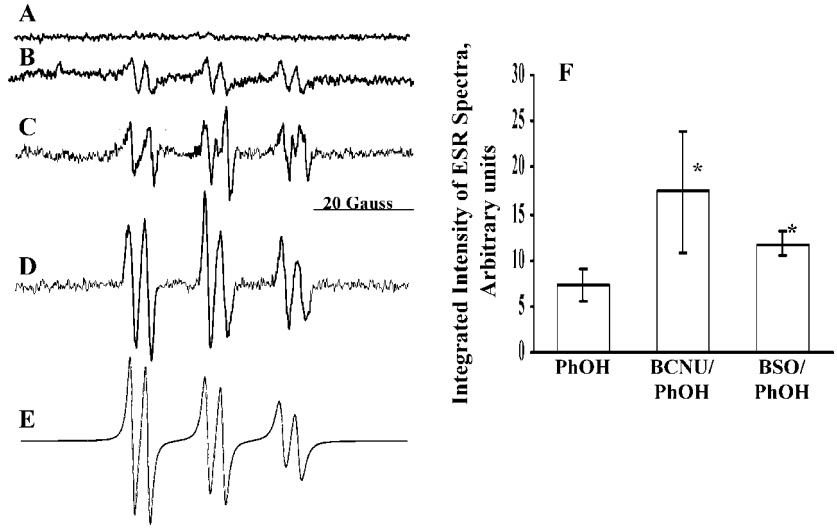

Figure 3. ESR detection of lipid-derived PBN spin-trapped free radicals formed *in vivo* in the skin of B6C3F1 mice topically treated with phenol. Mice ($n = 6$ per group) were intraperitoneally injected with vehicle (saline, 100 μL), BCNU (40 mg/kg, 100 μL), or BSO (2 mmol/kg, 100 μL), and then PBN was subcutaneously injected 10 min prior to phenol exposure. Thirty minutes following phenol exposure (3.5 mmol/kg), lipid extracts were isolated from the skin and used for ESR measurements. (A) PBN and saline exposed animals; (B) PBN and PhOH exposed animals; (C) BSO, PBN, and PhOH exposed animals; (D) BCNU, PBN, and PhOH exposed animals. (E) Complete computer simulation of the spectrum in (D) with hyperfine coupling constants: aN = 15.6 G and aH = 3.2 G. (F) Average integrated intensity of lipid-derived carbon-centered radicals obtained from B6C3F1 mice topically exposed to phenol as detected by ESR. Values are the mean ± SE of three experiments. * $p < 0.05$ vs phenol treated mice. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 1.3 s; conversion time, 0.6 s.

derived radicals in the skin of B6C3F1 mice. Using ESR spectroscopy, we found detectable signals 0.5 h following dermal exposure to PhOH (Figure 3A and B). In mice with a reduced level of GSH, topical exposure with PhOH resulted in a higher degree of free radical formation (Figure 3C and D).

Computer simulation of the detected radical adducts was consistent with the presence of two radical species in the skin of PhOH- and BCNU/ PhOH-exposed animals. The radical adducts had coupling constants of (*1*) $a^N = 15.6$ G; $a^H = 3.2$ G and (*2*) $a^N = 14.8$ G; $a^H = 2.7$ G. Radical 1 consisted of 60–75% of the obtained spectra and was identified as a carbon-centered lipid adduct, that is, a methyl radical (Figure 3E). Radical 2 was 25–40% of the obtained spectra and was determined to be an oxygen-centered lipid radical, that is, lipoxyl radical.

The spectra obtained from the BSO/PhOH-exposed animals were slightly different compared to those obtained from PhOH- and BCNU/PhOH-exposed animals. In particular, the ESR spectra from the BSO/PhOH-exposed animals consisted of two radical species. Radical 1 had coupling constants that corresponded to radical 1 from the PhOH- and BCNU/PhOH-exposed animals and was determined to be a carbon-centered lipid adduct ($a^N = 15.2$ G; $a^H = 3.35$ G). In contrast, radical 2 was an ascorbate radical ($a^H = 1.9$ G).

The major radical product detected was a carbon-centered radical adduct. Integration of the carbon-centered radical was done to determine the extent of the radical formation. PhOH exposure alone resulted in a carbon-centered radical with an integrated intensity of 7.3, while in BCNU/PhOH- or BSO/ PhOH-exposed animals, it resulted in significantly higher production of carbon-centered radicals with integrated intensities of 17.4 and 11.8 arbitrary units, respectively (Figure 3F).

Glutathione and Protein Thiol Oxidation as a Result of PhOH Exposure. Topical exposure to PhOH (3.5mmol/kg; 30 min) reduced GSH levels in the skin of mice by 61%. Exposure to BCNU/PhOH resulted in a significantly lower level of GSH in the skin of mice (80% decrease vs control) compared to that in mice treated with PhOH alone (61% decrease vs control) (Figure 4). BSO/PhOH exposure also significantly decreased

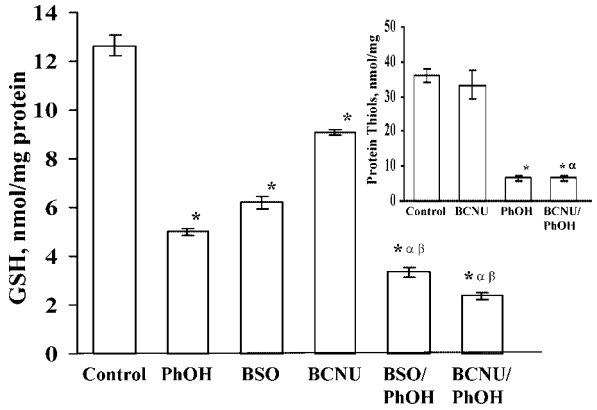

Figure 4. Effect of PhOH and/or BCNU or BSO exposure on the level of GSH. (Inset) Effect of phenol and/or BCNU on the level of protein thiols in the skin of B6C3F1 mice. Mice ($n = 6$) were intraperitoneally injected with vehicle (PBS, 100 μL), BCNU (40 mg/kg, 100 μL), or BSO (2 mmol/kg, 100 μL) prior to topical phenol exposure. Mice were then painted with phenol (3.5 mmol/kg) and sacrificed 0.5 h after exposure. Values are the mean ± SE of three experiments. *$p < 0.05$ vs PBS treated control mice; $^{\alpha}p < 0.05$ vs BCNU or BSO exposed mice; $^{\beta}p < 0.05$ vs PhOH exposed mice.

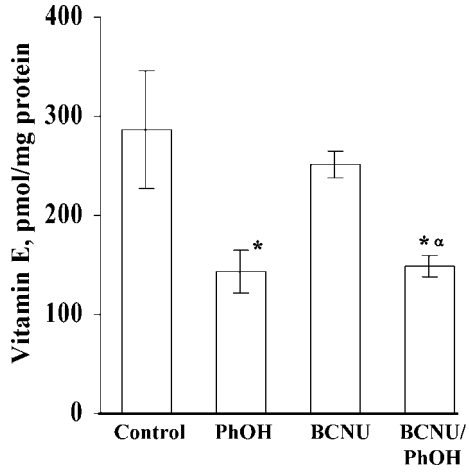

Figure 5. Effect of PhOH and/or BCNU exposure on the level of vitamin E in the skin of B6C3F1 mice. Mice ($n = 6$ per group) were intraperitoneally injected with vehicle (PBS, 100 μL) or BCNU (40 mg/kg, 100 μL) 3 h prior to topical exposure to PhOH. Mice were then painted with phenol (3.5 mmol/kg) and sacrificed 0.5 h after exposure. Values are the mean ± SE of three experiments. *$p < 0.05$ vs PBS treated control mice; $^{\alpha}p < 0.05$ vs BCNU exposed mice.

the level of GSH (75% decrease vs control) in the skin of mice as compared to those treated with PhOH alone.

Oxidation of protein thiols also occurred as a result of PhOH exposure. Topical exposure to PhOH resulted in a significant (82%) reduction in protein thiol levels. Exposure to BCNU/PhOH also resulted in a significant 82% reduction in protein thiol levels (Figure 4, inset).

Vitamin E Levels in Murine Skin following PhOH Exposure. Topical exposure to PhOH significantly decreased vitamin E levels by 45% in the skin of B6C3F1 mice exposed to PhOH (3.5 mmol/kg; 30 min). Glutathione depletion via BCNU administration also resulted in a significant decrease in vitamin E levels (45%) after PhOH as compared to that in control mice (Figure 5).

Total Antioxidant Reserve Status of Murine Skin following PhOH Exposure. Topical exposure to PhOH caused a significant 53% reduction in the total antioxidant status as compared to the skin of vehicle treated controls. In animals

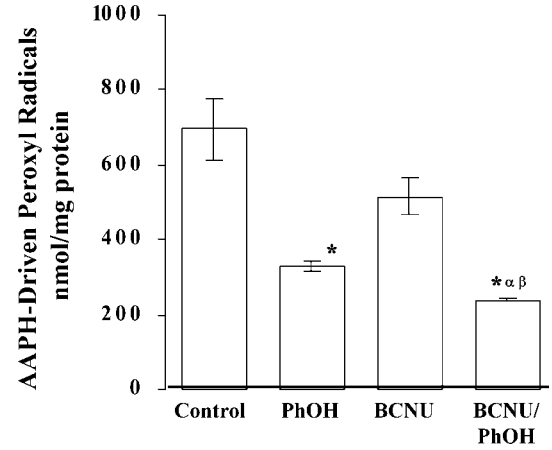

Figure 6. Effect of PhOH and/or BCNU on the level of total antioxidant reserve in the skin of B6C3F1 mice. Mice ($n = 6$ per group) were intraperitoneally injected with vehicle (PBS, 100 μL) or BCNU (40 mg/kg, 100 μL) 3 h prior to topical exposure to PhOH. Mice were then painted with phenol (3.5 mmol/kg) and sacrificed 0.5 h after exposure. Values are the mean ± SE of three experiments. *$p < 0.05$ vs PBS treated control mice; $^{\alpha}p < 0.05$ vs BCNU exposed mice; $^{\beta}p < 0.05$ vs PhOH exposed mice.

pretreated with BCNU, exposure to PhOH resulted in a 64% reduction in total antioxidant reserves as compared to the skin of vehicle-treated control mice (Figure 6).

Skin Histopathology following PhOH Exposure. Animals painted with vehicle (Figure 7A) as well as animals treated with BCNU (Figure 7E) or BSO (Figure 7C) had normal skin structure. Topical exposure to PhOH (1 h) resulted in the recruitment of inflammatory cells observed by the presence of polymorphonuclear leukocytes (PMNs) within the blood vessels of the skin (Figure 7B, inset). Exposure to BCNU/PhOH and BSO/PhOH caused more dispersed inflammation in the skin, that is, PMNs were present throughout the tissue (Figure 7D and F).

Prostaglandin E_2 Levels in JB6 Mouse Epidermal Cells following PhOH Exposure. To reveal whether PhOH exposure induced the release of inflammatory mediators, JB6 mouse epidermal cells were exposed to PhOH (15 mM). Figure 8A reveals a time-dependent increase in the release of prostaglandin E_2 after PhOH exposure (15 mM; 1/2, 1, 2, and 18 h). In cells exposed to BSO/PhOH for 2 h, the amount of prostaglandin E_2 was 3.9-fold and 1.4-fold increased as compared to control and phenol-exposed cells, respectively. The observed changes in prostaglandin E_2 increased further following 18 h post-exposure (Figure 8B). To assess whether PhOH exposure to cells caused the activation of COX-2, we treated JB6 cells with aspirin, indomethacin, or NS-398 prior to PhOH addition. We found that the COX-2 inhibitor, aspirin, significantly reduced prostaglandin E_2 as assessed in the cell supernatant compared to the levels seen in absence of aspirin (Figure 9). Similar results were also observed using other COX inhibitors, for example, indomethacin and *N*-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398) (Figure 9, inset).

IL-1β release by JB6 Mouse Epidermal Cells following PhOH Exposure. Cellular exposure to PhOH for 2 h induced the release of IL-1β (2.3-fold vs control levels). A diminished level of GSH (BSO) prior to PhOH exposure further enhanced the release of IL-1β (3.3-fold; Figure 10A). These results were time-dependent with a further increase observed

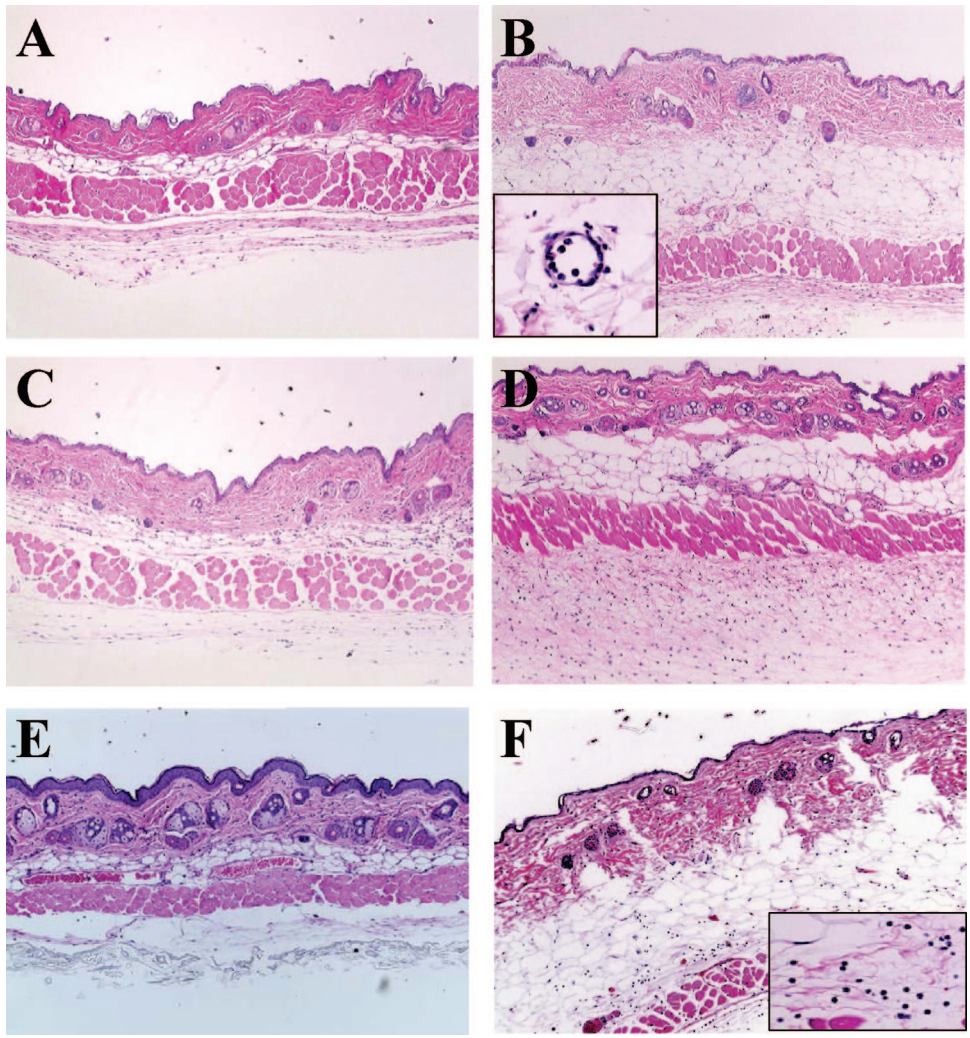

Figure 7. Skin histology of B6C3F1 female mice treated topically with phenol following the administration of BSO or BCNU. Mice ($n = 6$ per group) were intraperitoneally injected with vehicle (PBS, 100 μL), BCNU (40 mg/kg, 100 μL), or BSO (2 mmol/kg, 100 μL) prior to topical exposure to PhOH. Mice were then painted with phenol (3.5 mmol/kg) and sacrificed 1 h after exposure. (A) 1 h after painting with saline; (B) 1 h after painting with phenol; (C) 1 h after painting with saline following BSO administration; (D) 1 h after painting with phenol following the administration of BSO; (E) 1 h after painting with saline following the administration of BCNU; (F) 1 h after painting with phenol following the administration of BCNU. Magnification: 10×; inset magnification: 40×.

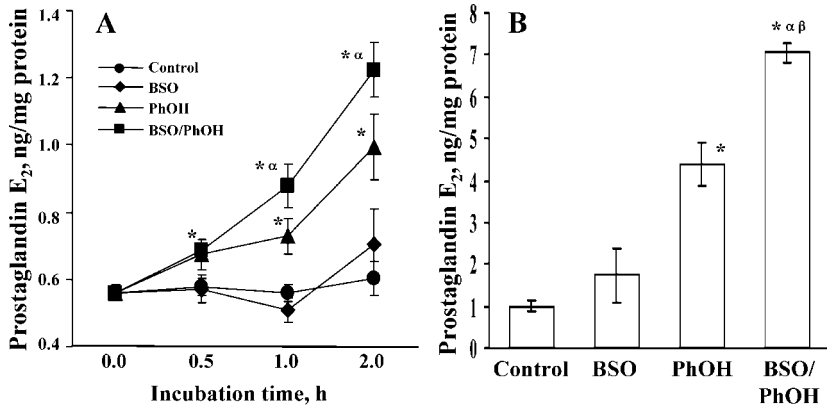

Figure 8. (A) Time course of prostaglandin E_2 release by JB6 cells following phenol/BSO exposure. (Circles) control cells; (diamonds) cells exposed to BSO (10 μM); (triangles) cells exposed to PhOH (15 mM); (squares) cells exposed to BSO/PhOH. (B) Level of prostaglandin E_2 in JB-6 cells following 18 h of PhOH/BSO exposure. Cells were exposed to BSO (10 μM). Three hours following BSO, cells were exposed to phenol (15 mM). 0.5, 1, 2, or 18 h following exposure, the cellular supernatant was collected and analyzed for prostaglandin E_2. Values are the mean ± SE of three experiments. $^*p < 0.05$ vs control cells; $^{\alpha}p < 0.05$ vs BSO exposed cells; $^{\beta}p < 0.05$ vs PhOH exposed cells.

18 h post-exposure (Figure 10B). At later time points (18 h), PhOH exposure led to a 4.5-fold increase in IL-1β released by the cells pretreated with BSO, while PhOH alone caused a 2.5-fold increase in IL-1β (Figure 10B).

Discussion

Dermal exposure to phenolic compounds are known to cause a number of inflammatory reactions in skin (*34, 35*). Topical

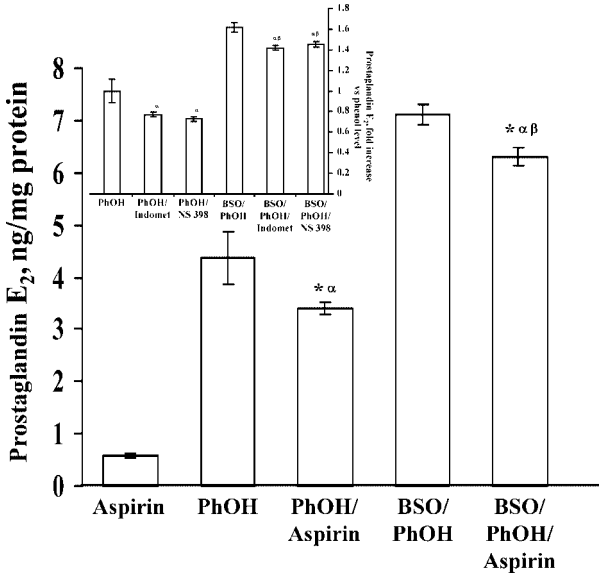

Figure 9. Effect of COX-2 inhibitor (aspirin) on the level of prostaglandin E_2 in JB6 cells following 18 h of phenol/BSO exposure. (Inset) Effect of indomethacin or NS-398 on the level of prostaglandin E_2 released from JB6 following 18 h of phenol/BSO exposure. JB6 cells were exposed to BSO (10 μM) and aspirin (50 μM), indomethacin (30 μM), or NS-398 (2 μM). Three hours following BSO, cells were exposed to phenol (15 mM). Eighteen hours following exposure, the cellular supernatant was collected and analyzed for prostaglandin E_2. Values are the mean ± SE of three experiments. $^{*}p < 0.05$ vs aspirin treated cells; $^{\alpha}p < 0.05$ vs phenol exposed cells; $^{\beta}p < 0.05$ vs BSO/PhOH exposed cells.

application of PhOH directly affects the skin by inducing inflammation and tissue damage (*36–38*). Severe edema, erythema, and necrosis occur as a result of the application of PhOH (*39, 40*).

A potential mechanism for the toxic effects of PhOH has been described as futile thiol pumping. The one-electron oxidation of PhOH and phenolic compounds by oxidative enzymes, such as peroxidases, prostaglandin synthetase, and tyrosinase, leads to the generation of phenoxyl radicals (*41*). Metabolism of PhOH has been shown to result in the formation of the phenoxyl radical (*42*). Myeloperoxidase-catalyzed formation of phenoxyl radicals has been shown previously to cause oxidative stress and modification of proteins, DNA, and lipids (*43*). These enzymatically formed phenoxyl radicals may then be reduced by thiols to regenerate the phenolic compound as a substrate for repeated enzyme-catalyzed one-electron oxidation. Oxidation of thiols generates thiyl radicals, which are subsequently able to interact with intracellular thiols and oxygen to initiate new oxidative cascades that generate new ROS, such as superoxide and hydroxyl radicals (*30*). If redox-cycling of PhOH was occurring, depletion of GSH prior to PhOH exposure would allow for an accumulation of free radicals during PhOH oxidation within the tissues. The results of this study show the increased formation of radicals in animals with depleted GSH prior to PhOH exposure. In addition, decreased GSH synthesis or the inhibition of GSH reductase prior to PhOH exposure also caused a significant depletion of the skin's antioxidants, that is, vitamin E and total antioxidant reserves (Figure 2), thereby making the animal's skin more susceptible to toxic agents and outcomes, such as increased inflammation and tissue damage due to PhOH exposure. Therefore, these results support the hypothesis that the redox cycling of free radicals formed in the skin reduces antioxidant capacity, thereby inducing significant oxidative stress. Insufficient antioxidant defense in the skin may lead to enhanced generation of ROS contributing to oxidative damage of protein, DNA, and lipids (*44*).

Thiols, such as GSH, and ascorbate have been shown to be major contributors to the antioxidant pool that participates in the reduction of phenoxyl radicals (*45*). Reduction of phenoxyl radicals by GSH and ascorbate results in the formation of thiyl and semidehydroascorbyl radicals (*30, 45*). We have shown that *in vitro* exposure of human keratinocytes to PhOH resulted in the formation of radicals most likely due to thiol oxidation (*30*). GSH plays a major role in monitoring the intracellular redox balance via regulation of the H_2O_2 content and lipid hydroperoxides via GSH peroxidase-catalyzed reactions (*46*). It is also important for the regulation of the oxidative stress signaling pathways (*21, 47, 48*). It has been shown that GSH is involved in maintaining intracellular protein integrity by the reduction of disulfide linkages, regulating their bio-synthesis, and regulating cellular sulfhydryl balance (*49*). Under physiological conditions, thiols are highly reductive. GSH acts as an antioxidant by detoxifing highly reactive peroxides (ROOH) via the conjugation of electophiles and metals (*50–53*) and results in the formation of a secondary glutathionyl radical (GS•; 46). GSH depletion enhances the formation of free radicals; therefore, GSH is a scavenger of free radicals preventing phenol-induced damage. The current studies found that PhOH exposure resulted in the production of carbon-centered lipid radical adducts.

To reduce GSH levels in the skin, we used 2 agents, BCNU and BSO, which target the synthesis and recycling of GSSG to

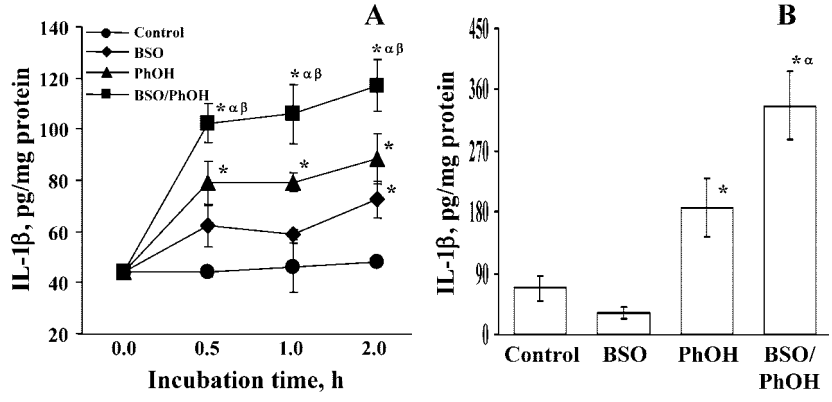

Figure 10. (A) Time course of IL-1β expression in JB6 cells following exposure to phenol. (Circles) control cells; (diamonds) cells exposed to BSO (10 μM); (triangles) cells exposed to PhOH (15 mM); (squares) cells exposed to BSO/PhOH. (B) IL-1β Expression by JB6 cells 18 h following phenol exposure. JB6 cells were exposed to BSO (10 μM) for 3 h. Following BSO exposure cells were exposed to phenol (15 mM) for 0.5, 1, 2, or 18 h. The cellular supernatant was collected and analyzed for IL-1β. Values are the mean ± SE of three experiments. $^{*}p < 0.05$ vs control cells; $^{\alpha}p < 0.05$ vs BSO treated cells; $^{\beta}p < 0.05$ vs PhOH treated cells.

GSH, respectively. It is interesting to point out that the nature of PhOH-induced free radicals as evaluated by ESR simulation showed the presence of differential radical species found in BCNU/PhOH- and BSO/PhOH-exposed animals. ESR from both BCNU/PhOH- and BSO/PhOH-exposed animals showed the presence of a carbon-centered lipid radicals. BCNU/PhOH ESR spectra also revealed the presence of oxygen-centered lipid-derived radicals. These observed differences are likely due to the transfer of the oxygen-centered radicals derived from the lipids of BCNU/PhOH-exposed mice to the aqueous phase. Although it is difficult to determine whether the trapped radicals are phenoxyl radicals, the presence of ascorbate radicals in BSO/PhOH-exposed animals provides indirect evidence for the phenol-induced formation of phenoxyl radicals. A study by Kagan et al. (*45*) provided evidence that ascorbate provides a line of defense against phenoxyl radical attack via its ability to reduce phenoxyl radicals, subsequently resulting in the formation of ascorbate radicals. It is also interesting to note that protein thiols serve as very good substrates for phenoxyl radical attack (*46*). It has also been shown that phenoxyl radicals have a relatively high oxidizing redox potential (+0.9 V), which results in the radical's ability to initiate lipid peroxidation (*46*). This study found the presence of lipid-derived free radical formation, which may be due to phenol-induced phenoxyl radicals initiating lipid oxidation. The animals with decreased levels of GSH have lipids that are more readily oxidized possibly by phenoxyl radicals, resulting in the greater presence of ESR lipid– radical adducts.

Oxidative conditions play a major role in modulating redox states by altering the dynamic equilibrium of GSH homeostasis (*54*). It has been shown that depletion of GSH can potentially enhance the inflammatory reaction via increased cytokine release. Cytokines, such as IL-1β and TNF-α, which are released by inflammatory cells, could activate oxidative stress (*55–57*), thereby affecting the GSH balance of cells and tissue and altering the redox equilibrium (*58*). GSH and GSH precursors are able to down-regulate cytokine synthesis and activation. Depletion of GSH has been shown to enhance cytokine secretion because of increased production of ROS (*25*).

The expression and activation of redox-sensitive/responsive transcription factors have been shown to be affected by GSH-redox status (*49*). An IκB-α/NF-κB-dependent pathway is responsible for mediating the redox-dependent regulation of inflammatory cytokines. Antioxidants, such as GSH, have been reported to inhibit cytokine production via an NF-κB dependent pathway (*59*). However, GSH depletion has been demonstrated to augment the oxidative stress-mediated inflammatory response via a mechanism that is not entirely NFκB dependent (*54*). GSH depletion results in an overabundance of ROS, which subsequently could lead to an increase in cytokine production, thus causing an acute inflammatory response in the skin. We found that topical exposure of B6C3F1 mice to phenol caused the development of oxidative stress and inflammation, which was accelerated in mice with a reduced level of GSH in the skin. Using murine epidermal cells, we observed that a reduced level of GSH caused increased production and release of inflammatory mediators, such as prostaglandin E_2 and IL-1β, as a result of PhOH exposure.

In conclusion, dermal exposure to PhOH caused the oxidation of GSH and protein thiols and decreased vitamin E and total antioxidant reserves in skin. The magnitude of the PhOH-induced generation of PBN-spin-trapped radical adducts in skin of mice with diminished levels of GSH (pretreated with BCNU, an inhibitor of glutathione reductase or BSO, an inhibitor of γ-glutamylcysteine synthetase) was markedly higher compared to radical generation in mice treated with PhOH alone. Topical exposure to PhOH resulted in skin inflammation. Remarkably, this inflammatory response was accelerated in mice with a reduced level of GSH. Epidermal mouse cells exposed to phenolic compounds showed the induction of early inflammatory response mediators, such as prostaglandin E_2 and IL-1β. Since dermal exposure to PhOH produced ESR-detectable PBN spin-trapped signals of lipid-derived radicals, we conclude that PhOH-induced free radical formation is involved in oxidative stress and dermal toxicity *in vivo.*

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Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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