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Elevated oxidative stress in skin of B6C3F1 mice affects dermal exposure to metal working fluid

ANNA A. SHVEDOVA, ELENA KISIN, JULIA KISIN, VINCENT CASTRANOVA AND CHOUDARI KOMMINENI

Health Effects Laboratory Division, Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, West Virginia 26505

Metal working fluids (MWFs) are widely used in industry for metal cutting, drilling, shaping, lubricating, and milling. Potential for dermal exposure to MWFs exists for a large number of men and women *via* aerosols and splashing during the machining operations. It has been reported earlier that occupational exposure to MWFs causes allergic and irritant contact dermatitis. Previously, we showed that dermal exposure of female and male B6C3F1 mice to 5% MWFs for 3 months resulted in accumulation of mast cells and elevation of histamine in the skin. Topical exposure to MWF also resulted in elevated oxidative stress in the liver of both sexes and the testes in males. The goal of this study was to evaluate the interaction between oxidative stress in the skin and topical application of MWF. Oxidative stress in skin of B6C3F1 mice of both sexes was generated by intradermal injection of the hydrogen peroxide (H_2O_2)-producing enzyme, glucose oxidase with polyethylene glycol (GOD+PEG). In mice given GOD+PEG, topical treatment with MWF (200 μl, 30%, for 1, 3, or 7 days) resulted in a mixed inflammatory cell response, accumulation of peroxidative products, and reduction of GSH content in the skin. Such changes were not observed with MWF treatment alone. These data indicate that oxidative stress can enhance dermal inflammation caused by occupational exposure to MWF. *Toxicology and Industrial Health* (2000) **16,** 267–276.

Keywords: *glucose oxidase, GSH, inflammation, metal working fluid, oxidative stress, skin*.

Introduction

Skin diseases were reported as the leading occupational illnesses in the United States during 1973–1987 (DOL, 1988, 1989). Industries with the highest incidence rates for skin diseases or disorders include the fabrication of screw-machine products (33.3 per 10,000 workers) and general industrial machinery (22.0 per 10,000 workers) (DOL, 1993; NIOSH, 1998). Metal working fluids (MWFs) are widely used in machining operations to remove heat and swarf and to provide lubrication (Sprince et al., 1994, 1996). There are three major types of MWFs: (1) straight oils, (2) water-based oil emulsions, soluble oil or semi-synthetic, and (3) synthetic MWF. Many skin ailments have been associated with the use of MWFs, including irritant contact dermatitis, allergic contact dermatitis, folliculitis, oil acne, oil keratosis, squamous cell carcinoma, pigmentary changes (melanoderma and leukoderma), oil granuloma, and mechanical injuries from metal shavings (Alomar, 1994). Irritant and contact dermatitis of the hands and forearms in workers exposed to different types of MWFs, e.g., soluble oil, semisynthetic or synthetic, are of major concern (De Boer et al., 1988, 1989a,b). The most important factor in the development of dermatitis is direct contact of the skin with MWFs (Rycroft, 1990; Sprince et al., 1996). It has been reported that up to 80% of workers exposed to MWFs have irritant contact dermatitis (Alomar, 1994; Alomar et al., 1985). The alkaline emulsifiers and solvents contained in insoluble and semisynthetic oils are directly irritating to the skin. They may change the structure and function of the skin by denaturing keratin, defatting and dehydrating the skin, or causing dryness, fissures and eczema (Zugerman, 1986). These and other components of MWFs, including metal contaminants such as chromium, cobalt or nickel, and additives such as mercaptobenzothiazole (MBT), triazines (Grotan BK), *N*-methylolchloracetamide (Parmetol K50), Fordice 78, *p*-chloro-*m*-xylenol (PCMX), *o*-phenyl phenol (Dowicide 1), alkanolamine borate, 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (Kathon), paraphenylenediamine, benzisothiazolone (Proxel), polymethacrylate, ethylenediamine, triethylamine, colophony, dipentene, Bioban P CS-1246, P-1487, glycidyl ester of hexahydrophthalic acid and fragrances, may cause allergic and contact dermatitis (Dahlquist, 1984; De Boer et al., 1989b; Damstra et al., 1992; Camarasa et al., 1993; Alomar, 1994; Alomar et al., 1985).

Oxidative stress is thought to play an important role in the etiology of numerous pathological skin conditions, including

1. Address all correspondence to: Dr. Anna A. Shvedova, Health Effects Laboratory Division, Pathology and Physiology Research Branch, NIOSH m/s 2015, 1095 Willowdale Road, Morgantown, WV 26505. Tel.: +1-304-285-6177. Fax: +1-304-285-5938. E-mail: ats1@cdc.gov

contact/irritant dermatitis, burns, psoriasis, carcinogenesis, and others (Picardo et al., 1992; Applegate et al., 1994; Nachbar and Korting, 1995; Boh, 1996; Rezazadeh and Athar, 1997; Freitas et al., 1998; Fuchs, 1998; Passi et al., 1998; Cetinkale et al., 1999). Skin is potentially the target of significant oxidative damage due to exposure to oxidizable industrial chemicals. Neutrophils and macrophages in the inflamed area may produce high local concentrations of a variety of reactive oxygen species (ROS) (Trenam et al., 1991, 1992). In this study, we describe the effect of preexisting oxidant damage caused by intradermal glucose oxidase (GOD) on the dermal response to subsequent topical application of unused MWF at the same site.

Materials and methods

The hydrogen peroxide (H_2O_2)-producing enzyme [GOD (195,000 U/g)] and polyethylene glycol [PEG (E200)], and 2-thiobarbituric acid were purchased from Sigma Chemical (St. Louis, MO). MWF was obtained from Milacron (Cincinnati, OH); ThioGlo-1 from Covalent Assoc. (Woburn, MA); and sodium dodecyl sulfate (SDS) from Bio-Rad Laboratories (Hercules, CA). All solutions were prepared in sterile isotonic saline.

Experimental design

Animals

B6C3F1 mice (n=240) of both sexes were obtained from Harlan Laboratories (Scottsdale, PA). Each mouse was housed in an individual cage in an AAALAC-accredited facility maintained at 70°F and about 60% humidity. The mice were given a 6% NIH diet and municipal water *ad libitum*. The 10- to 12-week-old mice were randomly grouped after 2 weeks of acclimatization as detailed below. The backs of mice were shaved (area 1.5×2.0 cm^2) with a hair clipper before treatments (Oster Professional Products, McMinnville, TN). H_2O_2 was generated by intradermal injection of GOD+PEG to induce oxidative stress in the skin of mice. GOD+PEG was prepared by addition of 12.3 mg of GOD to 100 μl of saline. Sterile PEG (10 ml) was added to the GOD solution and the mixture was stirred for 1 h. Mice from Group 1 were injected, intradermally, with 20 μl of saline (once) and were sacrificed 1, 3, and 7 days after the treatment. Mice from Group 2 were injected intradermally (once) with 20 μl of GOD+PEG (8 U/kg) and were sacrificed 24 h after the treatment. Mice from Group 3, were treated (once) with 20 μl of GOD+PEG (16 U/kg) and were sacrificed 24 h after the treatment. Mice from Group 4 were injected once with 20 μl of GOD+PEG (24 U/kg) and were sacrificed 24 h after the treatment. Mice from Group 5 were injected once with 20 μl of PEG (200E) and were sacrificed 1, 3, or 7 days after the treatment. Mice from Group 6 were painted with saline once a day for 1, 3, or 7 days, and were sacrificed 24 h after the last saline application. Mice from Group 7 were painted with 200 μl of 30% MWF (v/v in water, pH 7.6) once a day for 1, 3, or 7 days, and were sacrificed 24 h after the last MWF application. Mice from Group 8 were injected with 20 μl of GOD+PEG (24 U/kg) and 24 h after mice were painted once a day with 200 μl of saline for 1, 3, or 7 days and were sacrificed 24 h after the last saline application. Mice from Group 9 were given 20 μl of

Table 1. Experimental design.[a]

Treatment	Gender		Sacrifice day[b]						
	M	F	0	2	3	4	5	8	9
	Animals per group[a]								
1. Saline[c] given once on day 1	16	16	4/4	4/4	–	4/4	–	4/4	–
2. GOD+PEG[d] (8 U/kg) given once on day 1	8	8	4/4	4/4	–	–	–	–	–
3. GOD+PEG[d] (16 U/kg) given once on day 1	8	8	4/4	4/4	–	–	–	–	–
4. GOD+PEG[d] (24 U/kg) given once on day 1	8	8	4/4	4/4	–	–	–	–	–
5. PEG[c] given once on day 1	16	16	4/4	4/4	–	4/4	–	4/4	–
6. Saline[c] was applied once a day for 1, 3, or 7 days	16	16	4/4	4/4	–	4/4	–	4/4	–
7. MWF (30%)[f] was applied once a day for 1, 3, or 7 days	16	16	4/4	4/4	–	4/4	–	4/4	–
8. GOD+PEG[d] (24 U/kg) given once on day 1 +1 day later Saline[e] was applied once a day for 1, 3, or 7 days	16	16	4/4	–	4/4	–	4/4	–	4/4
9. GOD+PEG[d] (24 U/kg) given once on day 1 +1 day later MWF (30%)[f] was applied once a day for 1, 3, or 7 days	16	16	4/4	–	4/4	–	4/4	–	4/4

[a]B6C3F1 male and female mice.
[b]Mice were sacrificed 24 h post each application of saline or MWF.
[c]PEG (20 μl, 200E) or saline (20 ml) — given once, intradermally.
[d]GOD+PEG — given once, intradermally.
[e]Saline (200 μl) was applied once a day for 1, 3, or 7 days to the shaved back skin.
[f]MWF — 200 μl of 30% solution in water (v/v) at pH 7.6 was applied once a day for 1, 3, or 7 days to the shaved back skin.

GOD+PEG (24 U/kg) and 24 h after mice were painted once a day with 200 μl of 30% MWF (v/v in water, pH 7.6) for 1, 3, or 7 days and were sacrificed 24 h after the last MWF application. At least four males and four females were sacrificed at each time (Table 1).

Necropsy

Mice were euthanized using an excessive quantity of carbon dioxide. The back skin from the cervical to the sacral region (1.5×2.0 cm) was excised and equal pieces from the same area of mouse skin were collected for histology and biochemical assays.

Histopathology

The skin was processed after fixation in 10% neutral buffered formalin, following the standard operating procedures of our laboratory. Hematoxylin and eosin-stained histology slides were prepared for light microscopic examination. Photomicrographs were prepared using an Olympus 300 double-headed microscope (Tokyo, Japan).

Sample preparation

The skin was excised promptly after the mice were sacrificed, and samples for biochemical analysis were frozen at −80°C until processed. The skin homogenates were prepared from frozen tissues with phosphate-buffered saline (pH 7.4) using a tissue tearer (model 985-370, Biospec Products, Racine, WI). Homogenates were stored at −80°C until processed further.

Fluorescence assay of GSH and protein sulfhydryls

Total protein sylfhydryl concentration in homogenates of skin was determined using ThioGlo-1, a maleimide reagent that produces a highly fluorescent product upon reaction with SH groups (Shvedova et al., 2000). A standard curve was established by addition of GSH (0.04–4.0 mM) to 0.1

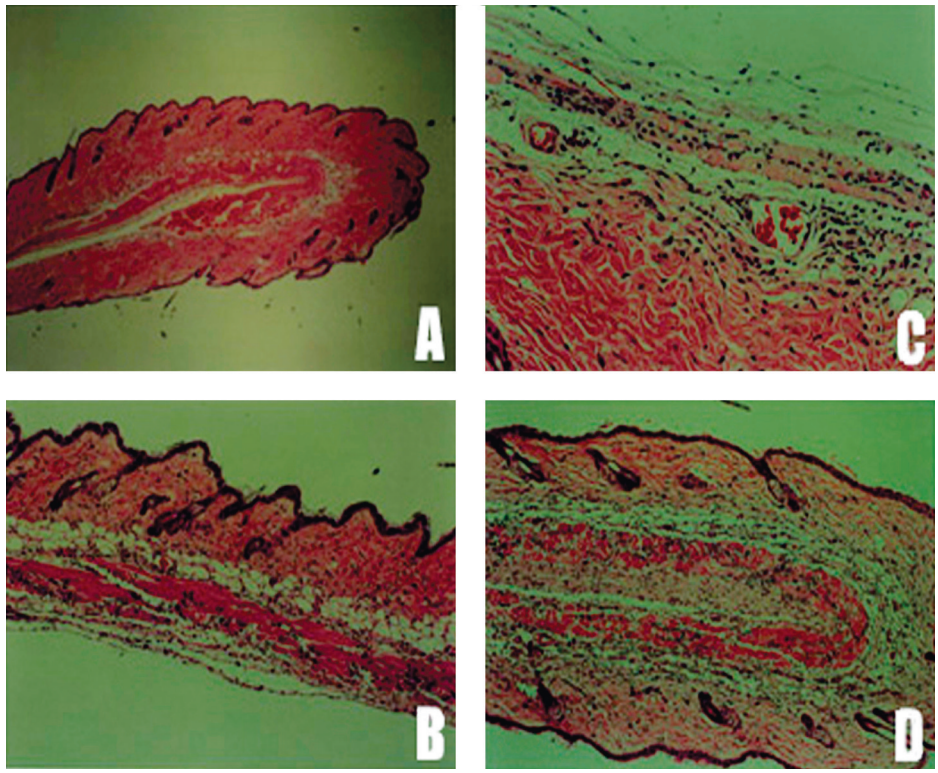

Figure 1. Skin histology of B6C3F1 female mice treated with GOD + PEG. (A) Twenty-four hours after intradermal injection of saline. Normal skin structure without inflammation. (B) Twenty-four hours after intradermal injection of 8 U/kg GOD + PEG. Inflammatory mixed cell infiltrates with neutrophils and mononuclear cells are present. (C) Twenty-four hours after intradermal injection of 16 U/kg GOD + PEG. Note mixed cell infiltration into deep layers. (D) Twenty-four hours after intradermal injection of 24 U/kg GOD + PEG. Intense mixed cell infiltration and myolysis. Edema in the adipose tissue is present. Magnification 10×. Conditions: Saline or GOD + PEG (8–24 U/kg) was injected intradermally on the shaved mouse back; total volume of saline or GOD + PEG was 20 μl. Skin samples were collected 24 h post exposure.

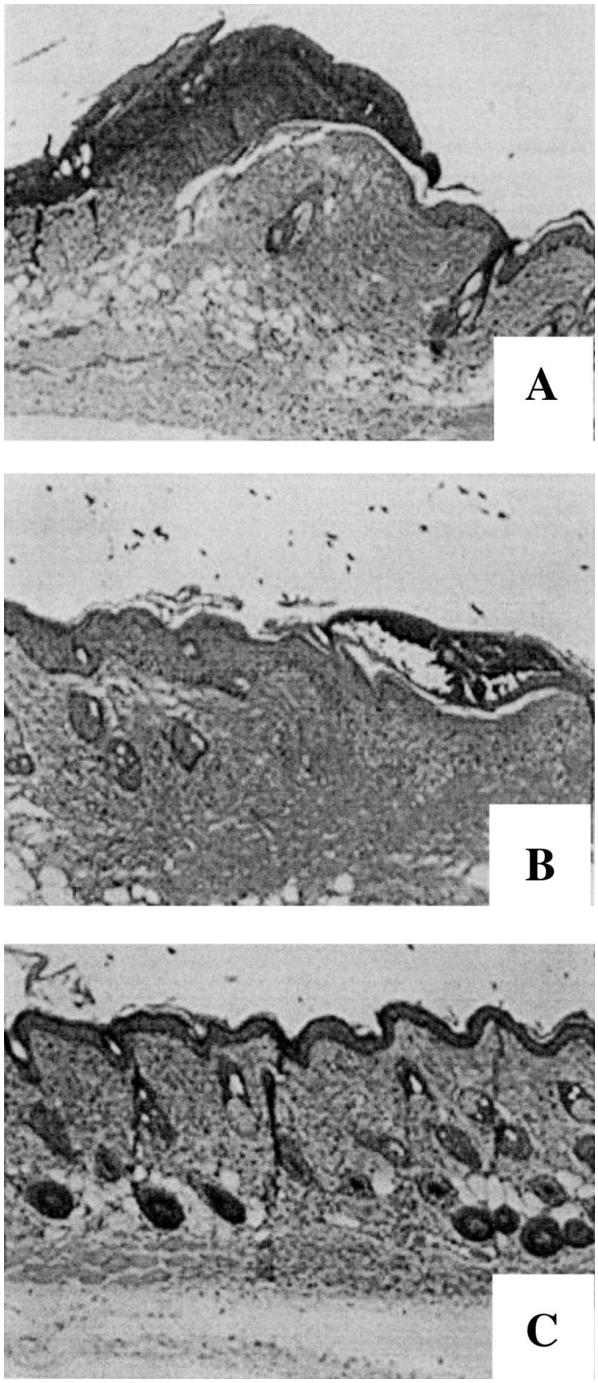

Figure 2. Skin histology of B6C3F1 female mice treated topically with MWF following intradermal administration of GOD + PEG. (A) GOD + PEG followed by 1 day of treatment with MWF: note ulcer with necrotic debris, mixed cell infiltration, myolosis and edema. (B) GOD + PEG followed by 3 days of treatment with MWF: note moderate thickening of the covering squamous epithelium with cellular debris. (C) GOD + PEG followed by 7 days of treatment with MWF: note very mild thickening of squamous epithelium with traces of hyperkeratosis and mild mixed cell infiltrates. Magnification 10×. Conditions: GOD + PEG (20 μl, 24 U/kg) was injected 24 h prior to topical application with unused MWF (200 μl, 30%, pH 7.6). Mice were painted with MWF once a day for 1, 3, or 7 consecutive days. Skin samples were collected 24 h post exposure.

Table 2. TBARS products (nmol/mg protein) in the skin of B6C3F1 mice treated with only PEG (20 μl, 200E).

Gender	Control	Days posttreatment	
		1	3
Female	0.18±0.01	0.17±0.02	0.22±0.02
Male	0.26±0.01	0.27±0.02	0.29±0.01

M phosphate buffer (pH 7.4) containing 10 μM ThioGlo-1. GSH content was estimated from the immediate fluorescence response registered upon addition of ThioGlo-1 to a tissue homogenate. Total protein sulfhydryls were determined from the augmentation of the fluorescence response after addition of SDS (4 mM) to the same homogenate. A spectrofluorophotometer (Shimadzu RF-5000 U, Kyoto, Japan) was employed in the assay (excitation λ=388 nm and emission λ=500 nm).

Measurements of peroxidative products

Peroxidative products were determined using the procedure described by Buege and Aust (1978). The formation of thiobarbituric acid-reactive substances (TBARS) was measured in skin homogenates. Tissue homogenates (0.5 mg of protein) were mixed (1:1) with 1 ml 0.67% thiobarbituric acid. The samples were heated for 20 min at 100°C and centrifuged for 15 min at 5000×g. The absorbency of the supernatant was determined at 535 nm using a UV 160 U Shimadzu spectrophotometer. A molar extinction coefficient of $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ was used for calculations.

Protein assay

Measurements of protein in homogenates of mouse skin were conducted using a Bio-Rad protein assay kit, cat. # 500-0006 (Richmond, CA).

Statistics

Data were expressed as the means±standard error of the mean for each group. One-way ANOVA was employed to

Table 3. TBARS products (nmol/mg protein) in the skin of B6C3F1 mice treated with only MWF (200 μl, 30%).

Gender	Control	Days posttreatment	
		1	3
Female	0.19±0.03	0.19±0.04	0.21±0.01
Male	0.30±0.05	0.29±0.04	0.28±0.02

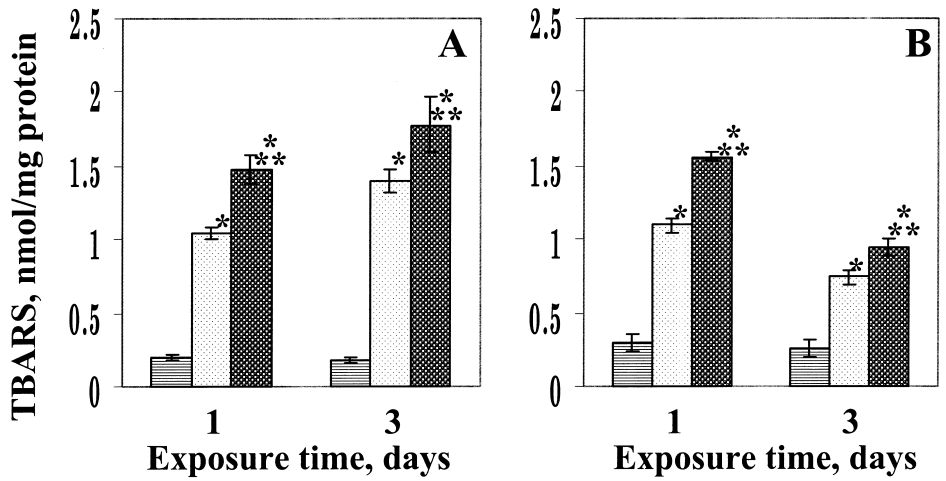

Figure 3. Accumulation of TBARS products in the skin of B6C3F1 mice treated topically with MWF or saline following intradermal administration of GOD + PEG. (A) Females. (B) Males. Striped bars: mice intradermally treated with saline (control). Light bars: mice given GOD + PEG intradermally then painted with saline. Dark bars: mice given GOD + PEG intradermally then painted with MWF. Conditions: GOD + PEG (24 U/kg, 20 μl, intradermally), prior to topical application of MWF or saline. Unused MWF (200 μl, 30%, pH 7.6) or saline (200 μl) were applied onto the shaved back 24 h later, once a day for 1 or 3 days. Skin samples were collected 24 h post exposure. Values are means ± SEM. *$p < 0.05$, vs. control mice; **$p < 0.05$, vs. mice treated with GOD + PEG and then saline.

compare the responses between treatments. Statistical significance was set at $p<0.05$.

Results

Subcutaneous injection of GOD+PEG in rats has been shown to produce skin inflammation and oxidant injury due to the generation of H_2O_2 by the enzyme, GOD (Trenam et al., 1991). Initially, we examined the histopatological changes in the skin of male and female B6C3F1 mice 24 h after intradermal injection of 8, 16, and 24 U/kg GOD+ PEG. Figure 1A is a photomicroscopy of skin from a B6C3F1 female mouse treated intradermally with saline, showing normal morphology. The skin from mice given 8 U/kg of GOD+PEG intradermally showed mild mixed cell infiltrate composed of neutrophils and mononuclear cells, extending from the epidermis to the subcutaneous skeletal

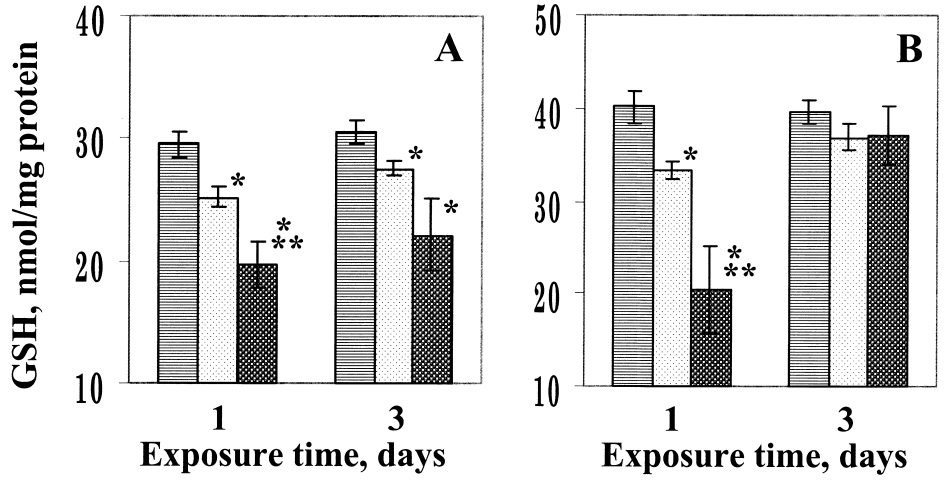

Figure 4. Level of GSH in the skin of B6C3F1 mice preinjected with GOD + PEG 24 h prior to topical exposure to MWF or saline. (A) Females. (B) Males. Striped bars: mice intradermally treated with saline (control). Light bars: mice given GOD + PEG intradermally then painted with saline. Dark bars: mice given GOD + PEG intradermally then painted with MWF. Conditions: GOD + PEG (24 U/kg, 20 μl, intradermally). After 24 h mice, were painted once a day for 1 or 3 days on the back with unused MWF (200 μl, 30%, pH 7.6) or saline (200 μl). Skin samples were collected 24 h post exposure. Values are means ± SEM. *$p < 0.05$, vs. control mice; **$p < 0.05$, vs. mice treated with GOD + PEG and then saline. GSH level in the skin of naive mice was not different from saline-treated controls (data not shown).

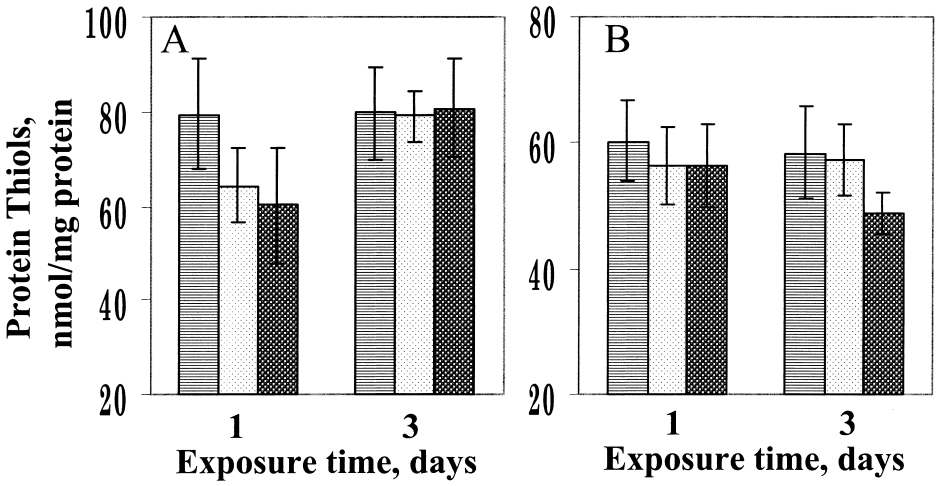

Figure 5. Level of protein thiols in the skin of B6C3F1 mice preinjected with GOD + PEG 24 h prior to topical exposure to MWF or saline. (A) Females. (B) Males. Striped bars: mice intradermally treated with saline (control). Light bars: mice given GOD + PEG intradermally then painted with saline. Dark bars: mice given GOD + PEG intradermally then painted with MWF. Conditions: GOD + PEG (24 U/kg, 20 μl, intradermally). After 24 h, mice were painted once a day for 1 or 3 days on the back with unused MWF (200 μl, 30%, pH 7.6) or saline (200 μl). Skin samples were collected 24 h post exposure. Protein thiols in the skin of naive mice were not different from saline-treated controls (data not shown).

muscle, which showed mild myolysis (Figure 1B). Skin from mice given 16 U/kg of GOD+PEG intradermally revealed moderate mixed cell infiltration with very mild edema (Figure 1C). The most severe inflammatory response was observed 24 h after administration of 24 U/kg GOD+PEG (Figure 1D). Marked mixed cell infiltration, myolysis, and mild edema in the adipose tissue was observed in the mice given 24 U/kg of GOD+PEG (Figure 1D).

The 24 U/kg treatment with GOD+PEG was chosen, due to severity of skin inflammation produced, to study the effect of preexisting inflammation on topically applied unused MWF (30%, 200 μl). No inflammation was observed in skin of mice painted alone with unused MWF (30%, 200 μl) for 1–7 days. Mice treated with GOD+PEG and unused MWF, sacrificed 24 h after a single MWF application (1 day), showed skin ulcers with attendant mixed cell infiltration that extended to the subcutaneous muscle with segmental myolysis (Figure 2A). Mice injected with GOD+PEG and then treated with MWF for 3 days (once a day) and sacrificed 24 h later, showed squamous cell bridging of the ulcer with thickened nucleated cells (acanthotic) of epidermis with a mild amount of nuclear debris on top and well-formed connective tissue with minimal mixed cell infiltrate (Figure 2B). Mice given GOD+PEG and then treated with MWF for 7 days (once a day) showed a two nucleated-cell-thick epidermis with very mild hyperkeratosis and full complement of adnexa in skin. The sebaceous glands appeared hypertrophic and the interfollicular distance seemed to be less than seen in the skin from mice sacrificed after a single topical MWF application (Figure 2C). The skin responses seen in mice similarly treated with GOD+PEG and then topical application with saline once a day for 1 or 3 days manifested exactly the same responses (not shown) as seen with GOD+PEG treatment alone (Figure 1D). Skin histology of mice treated with GOD+PEG and then topical application with saline for 1 day revealed mild myolysis, less mixed cell infiltration without necrotic debris, and ulcer compared to mice treated with GOD+PEG plus MWF (Figures 1D and 2A).

GOD+PEG has been used to induce oxidative stress in skin (Trenam et al., 1991). In the present study, we first evaluated whether MWF or PEG applied alone triggered oxidative stress in mouse skin. One group of mice was injected intradermally with PEG (20 μl, 200E). Accumulation of TBARS products was measured in skin homogenates of mice in 1 or 3 days after PEG application (Table 2). Another group of mice was painted once a day with MWF (200 μl, 30%) for 1 or 3 consecutive days (Table 3) and samples were collected 24 h post application for TBARS products. There was no differences in accumulation of TBARS products in skin of female and male treated with PEG or MWF alone (Tables 2 and 3). We observed accumulation of TBARS products in female and male B6C3F1 mice given GOD+PEG (20 μl, 24 U/kg, intradermal) and then painted with saline (200 μl) once a day for 1 or 3 consecutive days. Mice were sacrificed 24 h after treatment with saline. Changes in TBARS products in skin of female or male mice given GOD+PEG and then saline for 1 or 3 days are presented in Figure 3A and B, respectively (light and striped bars). In females intradermally exposed to GOD+PEG, levels of TBARS products increased 5.8- or 7.7-fold after 1 or 3 days of saline application compared to saline intradermally treated

controls, while in males treated similarly the level of TBARS products increased 4.1- and 2.7-fold compared to controls (Figure 3A and B, light bars). Application of MWF to the skin of mice intradermally treated with GOD+PEG (20 μl, 24 U/kg) resulted in increasing of oxidative stress in skin compared to the GOD+PEG then saline-treated group (Figure 3A and B, dark bars). In females and males given GOD+PEG and then treated topically with MWF for 1 or 3 days, accumulation of TBARS products in skin was 1.4- and 1.3-fold higher, respectively, compared with those treated with GOD+PEG and then painted with saline (Figure 3A and B, dark and light bars).

Depletion of antioxidants is associated with accelerated oxidative stress in mammalian tissues. We observed that topical exposure of B6C3F1 mice to 5% MWF for 13 weeks decreased levels of ascorbate and glutathione in the liver of both genders (Al-Humadi et al., 2000b). In the present work, we used GOD+PEG to induce oxidative stress in the skin before exposure to MWF. We observed that the level of GSH in the skin of B6C3F1 mice given GOD+PEG (20 μl, 24 U/kg, intradermal) and painted with saline (200 μl) once a day for 1 or 3 consecutive days was decreased, except in males after 3 days (Figure 4, light bars). There were no changes in GSH levels when mice were painted with MWF alone for 1 or 3 consecutive days (data not shown). Female and male mice treated topically with MWF for 1 day after GOD+PEG showed a significant decrease of GSH, 33% and 49%, respectively, compared with saline-treated controls ($p<0.05$). However, 3 days of MWF exposure of mice given GOD+PEG resulted in a significant decrease (27%) of GSH only in females, $p<0.05$ (Figure 4A). Furthermore, in mice given GOD+PEG and then painted with MWF, the level of GSH was decreased below that for the GOD+PEG/saline group, except in males after 3 days of MWF (Figure 4A and B, dark bars). There were no differences in oxidation of protein sulfhydryls at any point after intradermal treatment with GOD+PEG followed by topical exposure with saline or MWF in skin of mice (Figure 5A and B).

Discussion

Several cutaneous disorders, e.g., irritant and allergic contact dermatitis, folliculitis, oil granuloma, oil acne and keratosis, squamous cell carcinoma, leucoderma and melanoderma, have been associated with occupational exposures to MWFs (Alomar, 1994). Contact dermatitis of the hands and forearms of workers exposed to soluble, semisynthetic MWF is a widespread problem. Other factors contributing to irritant contact dermatitis among industrial workers include microtrauma to the skin by virtue of MWF contaminants, such as metal chips and shavings. Metal-induced abrasion of skin during manufacturing processes may result in bleeding and lysis of red blood cells with the release of heme into the surrounding tissues. This may contribute to the generation of ROS and skin inflammation (Mendelow et al., 1986; Shvedova et al., 1999). It is well known that ROS formed by leukocytes play an important role in continuous and excessive production of chemotactic factors, leading to skin inflammation (Nakamura et al., 1998). In our study, we employed a mouse model of skin inflammation induced by ROS generated by an H_2O_2-producing enzyme, GOD, to study the effects of MWF on skin. GOD is a flavoprotein containing two molecules of flavin adenine dinucleotide that oxidize glucose to glucose-lactone producing H_2O_2 (Figure 6, scheme). This GOD system provides a relatively low but continuous release of H_2O_2 within the dermis. High levels of glucose and oxygen (substrates for GOD) are present in all tissues and plasma (Muzykantov et al., 1988; Gow et al., 1999). H_2O_2, a common intermediate in oxidative metabolic processes, has been shown to play a role in the production of oxidative lesions (Salazar and Houten, 1997; Schallreuter et al., 1999). H_2O_2 reacting with transition metals, e.g., iron or copper, is able to trigger a cascade of free radicals leading to oxidative modification of cellular targets, e.g., lipids, DNA and proteins (Wardman and Candeias, 1996; Panayiotidis et al., 1999). Increased oxidative stress in skin after intradermal injection of an H_2O_2-producing enzyme may trigger release of transition metals from wounded and inflamed tissues causing of formation of ROS. Additional factors possibly contributing to accelerated oxidative stress in skin of mice given GOD+PEG and MWF are paraphenylenediamine and *o*-phenyl phenol, i.e., two reductants that are components of MWF capable of enhancing transition metal-catalyzed redox cycling (Figure 6).

We confirmed that intradermal injection of GOD+PEG to B6C3F1 mice of both sexes produced edema similar to that found in rats (Trenam et al., 1991). Injection of GOD+PEG also resulted in accumulation of inflammatory cells consisting of monocytes and neutrophils along with elevated oxidative stress in mouse skin. ROS, in particular superoxide, have been shown to play a major role in neutrophil-mediated inflammation and recruitment of neutrophils to the site of immune complex deposition in the dermal microvasculature (McCord et al., 1980; Petrone et al., 1980; McCord, 1993). It has been speculated that H_2O_2-mediated complement activation and cytokine-driven superoxide generation by skin fibroblasts triggered release of complement-derived chemotactic agents, such as C5a or C3b, causing dermal inflammation (Petrone et al., 1980; Shingu et al., 1989). Application of a well-known inflammatory agent, phorbol ester, to the skin of mice also has been shown to induce excessive production of ROS, e.g., superoxide, in mouse skin resulting in the recruitment of neutrophils and formation of edema (Nakamura et al., 1998).

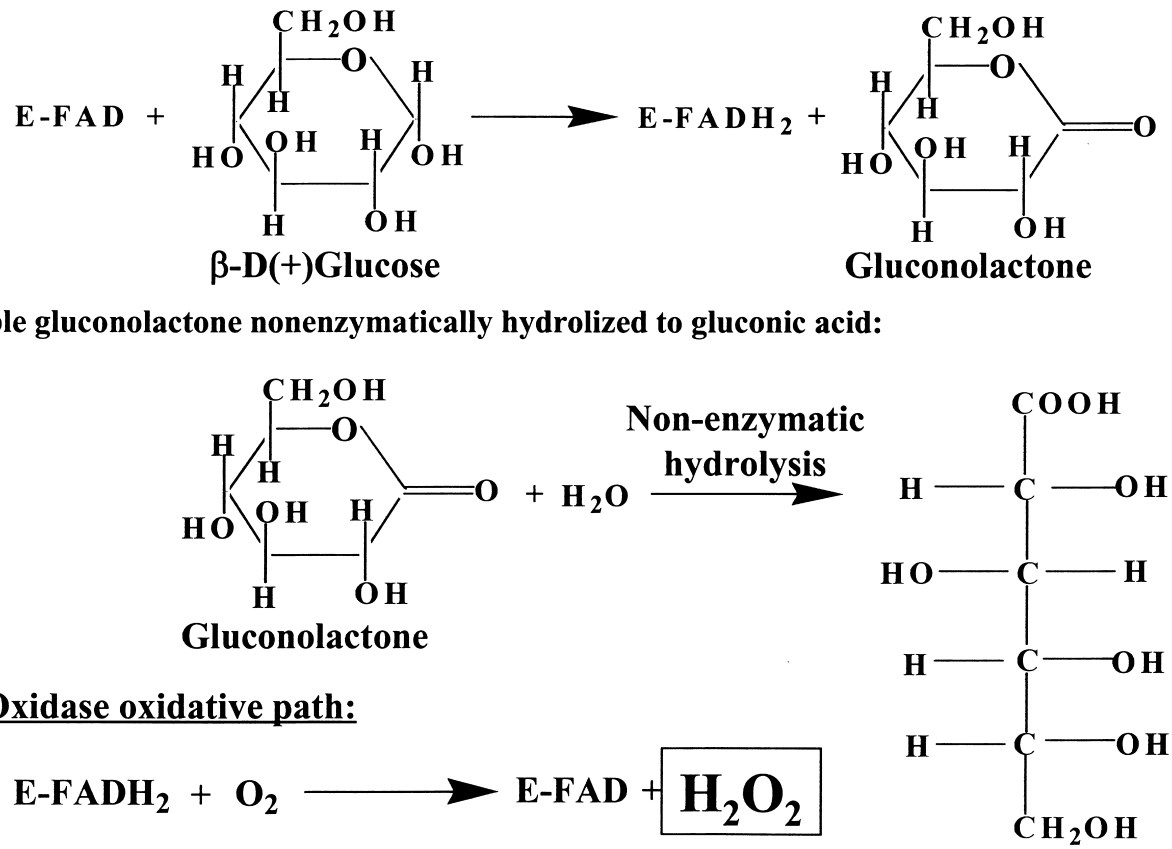

Figure 6. Scheme of formation of ROS in the presence of MWF *via* a GOD-catalyzed reaction. E-FAD, enzyme GOD containing two molecules of flavin adenine dinucleotide per molecules; E-$FADH_2$, enzyme GOD containing a reduced form of flavin adenine dinucleotide (dihydroflavin adenine dinucleotide); Me^{n+}, transition metals; OH^-, hydroxyl anion; $^{\cdot}OH$, hydroxyl radical.

The production of free radicals is essential to normal metabolism, but may become destructive if not controlled by endogenous defense mechanisms. When MWF was applied to inflamed skin, we observed an excessive accumulation of mononuclear and polymorphonuclear cells, which are sources of oxygen radicals. We observed accumulation of TBARS products after topical exposure to MWF in dermal tissue of mice treated with GOD+PEG. Elevated oxidative stress was accompanied by depletion of the sulfur-containing antioxidant, GSH, in skin. Possible involvement of GSH in inflammation was shown in the mice exposed to a chemical sensitizer, dinitrochlorobenzene (DNCB) (Hirai et al., 1997). When mice were topically challenged with DNCB, changes in GSH metabolism in skin and liver were evident. Histological examination of mice with an allergic response revealed that cutaneous inflammation was enhanced by buthionine sulfoximine (an inhibitor of GSH synthesis), suggesting that GSH may play an important role in the suppression of the immune inflammatory reactions in skin (Hirai et al., 1997). A preexisting compromised state of the mouse dermis, as in the case of GOD-injected skin, seems to make it more susceptible to ulceration. This compromised state does not seem to reduce the wound-healing properties of the skin, as seen in our mice at 3 days post MWF topical application following GOD+PEG treatment. This is not surprising as the wound healing does not solely depend on the water-soluble antioxidants, such as glutathione and vitamin C. In

fact, preexisting inflammation may have already induced the production of various cytokines (Moulin, 1995; Moore, 1999; Gallucci et al., 2000). The thickened squamous epithelium that bridged the two ends of the ulcer is also consistent with the role of various other factors (e.g., keratinocyte, fibroblast, platelet, vascular endothelial and transforming β1, β2, β3 growth factors, etc.) that play a pivotal role in skin wound healing (Benn et al., 1996; Singer and Clark, 1999). The enlarged sebaceous glands seen after 7 days of topical application of MWF probably represent the accumulation of MWF constituents in the regenerating sebaceous glands. We have previously described such hypertrophy of sebaceous gland epithelium in B6C3F1 mice of both sexes following application of as little as 5% unused MWF to intact skin (Al-Humadi et al., 2000a,b).

In conclusion, intradermal injection of GOD+PEG induced H_2O_2-generated ROS, and caused oxidative stress, accumulation of inflammatory cell infiltrates, and myofiber breakdown in skin of B6C3F1 mice. Unused MWF (30%, pH 7.6) applied topically by itself for 3 days did not induce oxidative stress or an inflammatory cell response in skin of B6C3F1 mice. Compromised skin, resulting from intradermal injection of GOD+PEG, was more susceptible to ulceration following the application of unused MWF. However, such preexisting compromise seems to help the skin wound to heal faster. These paradoxical events need to be reconciled. The skin of mice treated both with GOD+ PEG and the MWF showed elevated levels of TBARS and concomitant depletion of water-soluble antioxidant, GSH, that were not seen after MWF treatment alone. Therefore, these results indicate that oxidative stress can enhance dermal inflammation caused by topical exposure to MWF.

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