Carcinogenesis vol.28 no.7 pp.1582–1588, 2007
doi:10.1093/carcin/bgm001
Advance Access publication January 18, 2007

Pro/antioxidant status and AP-1 transcription factor in murine skin following topical exposure to cumene hydroperoxide

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Organic peroxides, widely used in the chemical and pharmaceutical industries, can act as skin tumor promoters and cause epidermal hyperplasia. They are also known to trigger free radical generation. The present study evaluated the effect of cumene hydroperoxide (Cum-OOH) on the induction of activator protein-1 (AP-1), which is linked to the expression of genes regulating cell proliferation, growth and transformation. Previously, we reported that topical exposure to Cum-OOH caused formation of free radicals and oxidative stress in the skin of vitamin E-deficient mice. The present study used JB6 P+ mouse epidermal cells and AP-1-luciferase reporter transgenic mice to identify whether exposure to Cum-OOH caused activation of AP-1, oxidative stress, depletion of antioxidants and tumor formation during two-stage carcinogenesis. *In vitro* studies found that exposure to Cum-OOH reduced the level of glutathione (GSH) in mouse epidermal cells (JB6 P+) and caused the induction of AP-1. Mice primed with dimethyl-benz[*a*]anthracene (DMBA) were topically exposed to Cum-OOH (82.6 μmol) or the positive control, 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 17 nmol), twice weekly for 29 weeks. Activation of AP-1 in skin was detected as early as 2 weeks following Cum-OOH or TPA exposure. No AP-1 expression was found 19 weeks after initiation. Papilloma formation was observed in both the DMBA–TPA- and DMBA–Cum-OOH-exposed animals, whereas skin carcinomas were found only in the DMBA–Cum-OOH-treated mice. A greater accumulation of peroxidative products (thiobarbituric acid-reactive substances), inflammation and decreased levels of GSH and total antioxidant reserves were also observed in the skin of DMBA–Cum-OOH-exposed mice. These results suggest that Cum-OOH-induced carcinogenesis is accompanied by increased AP-1 activation and changes in antioxidant status.

Introduction

Peroxide compounds yield free radicals and are widely used in industry as initiators for polymerization. Organic peroxides (OPs) have become the subject of occupational safety research interest due to possible genotoxic and carcinogenic risks in the workplace. Cumene hydroperoxide (Cum-OOH) is a bulk material for production of acetone and phenol. Cum-OOH is also used as a catalyst for rapid polymerization and production of styrene and acrylic monomers, curing agent for unsaturated polyester resins, and as an intermediate for cross-linking agents (1). Humans are exposed to Cum-OOH during manufacturing as well as in polluted urban air resulting from the photochemical reaction of nitrogen oxide with unsaturated hydrocarbons. Peroxides are also present in the exhaust fumes of gasoline, diesel and aviation fuels. Dermal exposure to Cum-OOH causes a number of toxic outcomes in the skin such as allergic and irritant dermatitis, rash, defattening of dermis and hair loss, burns and epidermal hyperplasia (2). Free radical production, which has been observed *in vivo* following dermal exposure to Cum-OOH (3), is considered to be the key factor contributing to skin tumor promotion by OPs.

Alteration of Ras proteins and/or elevated levels of their expression have drastic consequences for growth control and cause the development of tumors in humans (4). Recently, the role of the transcription factor activator protein-1 (AP-1) has been highlighted as a mediator of growth factors, oncogenes and the activity of the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). AP-1 converts extracellular signals into changes in the expression of specific target genes that harbor AP-1-binding sites in their promoter/enhancer regions. AP-1 consists of a family of Jun/Fos dimers including different Jun/Fos proteins (5–7). AP-1 and its regulated gene expression are involved in the pre-neoplastic to neoplastic progression in different cellular models (8–11).

Reactive oxygen species can regulate AP-1 binding to the DNA. The application of tumor promoters to the skin causes a significant reduction in antioxidant defense (12–16). In the present study, we attempted to evaluate whether topical exposure to Cum-OOH caused tumor promotion, induction of oxidative stress and activation of AP-1 protein in mouse skin. JB6 P+ mouse epidermal cells and AP-1-luciferase reporter transgenic mice were used to observe whether exposure to Cum-OOH caused activation of AP-1, depletion of antioxidants and skin tumor formation during two-stage carcinogenesis. Using a promotion-sensitive (P+) mouse epidermal cell line transfected with an AP-1-luciferase reporter (JB6 P+) which carries a stably transfected single AP-1-luciferase site (17–19), we found that exposure to Cum-OOH reduced the level of glutathione (GSH) and caused the induction of AP-1. Mice were topically exposed to the tumor initiator dimethyl-benz[*a*]anthracene (DMBA). Two weeks following initiation, mice were exposed to Cum-OOH (82.6 μmol) or the positive control, TPA (17 nmol), twice weekly for 29 weeks, to test tumor promotion. As early as 2 weeks following the start of Cum-OOH or TPA exposure, AP-1 activation was detected. Throughout the course of the experiment, papilloma formation was observed in both the DMBA–TPA- and DMBA–Cum-OOH-exposed mice, whereas skin carcinoma formation only occurred in the latter. Increased peroxidative products [thiobarbituric acid-reactive substances (TBARSs)], inflammation and decreased levels of GSH and total antioxidant reserves were also observed in the skin of DMBA–Cum-OOH-exposed mice. The results of this study suggest that oxidative stress and AP-1 activation accompany Cum-OOH-induced carcinogenesis.

Abbreviations: AAPH, 2,2′-azobis(2-aminodinopropane)-dihydrochloride; AP-1, activator protein-1; Cum-OOH, cumene hydroperoxide; DMBA, dimethyl-benz[*a*]anthracene; FBS, fetal bovine serum; GSH, glutathione; MEM, minimum essential medium; MPO, myeloperoxidase; OP, organic peroxide; TBARS, thiobarbituric acid-reactive substance; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Materials and methods

Chemicals

Fatty acid-free human serum albumin, luminol, sodium dodecyl sulfate, Cum-OOH, TPA, DMBA, acetone, guaiacol, cetylmethylammonium bromide, 3-amino-1,2,4-triazole, 2-thiobarbituric acid, malondialdehyde and GSH were obtained from Sigma Chemical Co. (St Louis, MO). Fetal bovine serum (FBS) and L-glutamine were purchased from Life Technologies (Rockville, MD). Eagle's minimum essential medium (MEM) was obtained from BioWhittaker (Walkersville, MD). Luciferase assay kit was purchased from Promega (Madison, WI). ThioGlo™-1 was obtained from Covalent (Woburn, MA). 2,2′-Azobis(2-aminodinopropane)-dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA).

Cell culture

The JB6 family of mouse epidermal clonal genetic variants (P^+/P^-) is a widely used model for studying critical gene regulation events that occur during carcinogenesis (17–19). The JB6 P+ mouse epidermal cell line transfected with the AP-1-luciferase reporter plasmid (JB6 P+/AP-1) was a kind gift from the laboratory of Dr Nancy Colburn (National Institutes of Health, Frederick, MD). JB6 P+/AP-1 cells were cultured in Eagle's MEM containing 5% FBS and 2 mM L-glutamine. The cells were grown at 37°C in a 5% CO_2 atmosphere.

Animals

The AP-1 transgenic female mice used to study tumor promotion by Cum-OOH were provided by the laboratory of Dr Min Ding (National Institute for Occupational Safety and Health, Morgantown, WV). AP-1-luciferase reporter transgenic mice were originally established by Rincon *et al.* (17). A C57BL/6 male mouse carrying the 2× TPA response element-luciferase transgene was crossed with a DBA2 female (20). The offspring were screened for the presence of luciferase activity. Each mouse was housed in an individual ventilated cage under controlled environmental conditions in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Food and water were provided *ad libitum*. All animal procedures were performed in accordance with an approved Animal Care and Use Committee protocol.

Assay of AP-1 activity in vitro

The JB6 P+/AP-1 cells (5×10^4 cells/ml) were placed in 96-well plates in 200 µl per well of Eagle's MEM supplemented with 5% FBS and 2 mM L-glutamine. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. Twelve hours later, cells were cultured in Eagle's MEM supplemented with 0.5% FBS and 2 mM L-glutamine for 24 h to minimize basal AP-1 activity. The cells were then exposed to Cum-OOH (50, 100 or 200 µM) or TPA (40 nM) in the same medium for 24 or 48 h to monitor the effects on AP-1 induction. The cells were extracted with 200 µl of 1× lysis buffer provided in luciferase assay kit by the manufacturer, and the luciferase activity was measured using a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA). The results are expressed as relative AP-1 activity compared with respective controls.

Tumor promotion experiment

Eight-week-old AP-1-luciferase reporter-bearing female mice were randomly divided into six groups with each group consisting of six mice. The dorsal skin in the inter-scapular area was shaved with a surgical clipper 2 days before initiation, and animals showing no hair re-growth were used in the experiment. DMBA (51.2 µg dissolved in 100 µl of acetone for each mouse) was used as a tumor initiator and applied to the skin of mice in groups 4, 5 and 6 (Table I). In groups 1, 2 and 3, 100 µl of acetone vehicle was applied to the mouse skin. Fourteen days following initiation, the mice were dermally exposed to the promoters 17 nmol TPA (groups 2 and 5, positive control) or 82.6 µmol Cum-OOH (groups 3 and 6) dissolved in 100 µl of acetone twice weekly for the next 29 weeks. Negative control mice were treated with a comparable quantity of the vehicle, acetone (groups 1 and 4). The body weight of each animal and papillomas/carcinomas appearing on the shaved area of the skin were recorded at weekly intervals. A board certified veterinary pathologist confirmed the presence of papillomas and carcinomas and differentiated between papillomas and carcinomas via examination of histological sections at the completion of the experiment. Tumor promoting activity was evaluated by both the ratio of tumor-bearing mice and the number of tumors, >1 mm in diameter, per mouse. Animals were killed by inhalation of an excess of carbon dioxide after the termination of the treatments. Skin flaps from the inter-scapular area of the back of mice (1.5×2.0 cm^2) were excised and samples were saved for histopathology and biochemical analyses. Skin for histology was fixed in 10% neutral buffered formalin until processing. Skin for biochemical studies was immediately frozen at −80°C until homogenized. The skin homogenates were prepared from frozen tissues with ice-cold phosphate-buffered saline (pH 7.4) using a tissue tearer (model 985-370, Biospec Products, Racine, WI). Homogenates were stored at −80°C until further processed. The tumor promotion experiments were replicated two times.

Assay of AP-1 activity in vivo

AP-1-luciferase activity was measured in dorsal skin punch biopsy samples (1.5 mm, Acuderm, Ft Lauderdale, FL). Lysis buffer (Promega, 100 µl) was added to each skin biopsy, and the tissues were lysed overnight at 4°C. The luciferase activity of the tissue supernatant obtained after lysis was measured with a luminometer (Monolight 2010, Analytical Luminescence Laboratory). AP-1 activity was expressed relative to the level of luciferase activity in control groups. Samples for AP-1 activity assay were collected every 4 weeks.

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).

Determination of inflammatory biomarkers

The modifying effect on inflammation induced by Cum-OOH or TPA topical application was determined by two biomarkers, edema formation and myeloperoxidase (MPO) activity. To assess the extent of Cum-OOH- or TPA-induced edema in mice skin 24 h after last treatment, a dial caliper (The Dyer Company, Lancaster, PA) was used to measure the skin bi-fold thickness at three random locations per mouse. Edema formation was expressed as net increase in skin bi-fold thickness between experimental and control (acetone–acetone) groups. For the MPO activity determination, a spectrophotometric assay (Shimadzu UV 160U spectrometer, Kyoto, Japan) was used in which guaiacol oxidation was monitored by changes of absorbance at 470 nm ($\epsilon = 26.6$/mM/cm) (21). Skin homogenates were mixed with phosphate-buffered saline (100 mM, pH 7.4) containing cetylmethylammonium bromide (0.02%), guaiacol (13 mM) and 3-amino-1,2,4-triazole (3.75 mM). The reaction was started by the addition of H_2O_2 (0.6 mM). Activity of MPO was calculated and expressed in nanomoles of tetraguaiacol per minute per milligram of protein.

Determination of peroxidative products (TBARSs)

Peroxidative products were determined using the procedure described by Buege *et al.* (22). The formation of TBARSs was measured in skin homogenates. Tissue homogenates (0.5 mg of protein) were mixed with 1 ml 0.67% (w/v) thiobarbituric acid and 30% (w/v) trichloroacetic acid (1:1). The samples were heated for 20 min at 100°C and centrifuged for 15 min at 5000*g*. The absorbance of the supernatant was determined at 535 nm using Shimadzu UV 160U spectrophotometer. A standard curve was generated using malondialdehyde. A molar extinction coefficient of $\epsilon = 1.56 \times 10^5$/M/cm was used for calculations.

Table I. Biomarkers of inflammation and oxidative stress in skin of AP-1 transgenic mice after Cum-OOH-induced or TPA-induced tumor promotion (29 weeks)

	Acetone–acetone (group 1)	Acetone–TPA (group 2)	Acetone–Cum-OOH (group 3)	DMBA–acetone (group 4)	DMBA–TPA (group 5)	DMBA–Cum-OOH (group 6)
Net increase in skin bi-fold, thickness (mm)	0.00 ± 0.01	0.40 ± 0.03[a]	0.43 ± 0.04[a]	0.04 ± 0.01	0.94 ± 11[a,b,c]	1.39 ± 0.25[a,b,d]
MPO (nmol/min/mg protein)	ND	1.93 ± 0.28	2.05 ± 0.51[a]	ND	4.83 ± 0.2411[a,b,c]	12.67 ± 1.90[a,b,d]
TBARS (nmol/mg protein)	0.30 ± 0.01	0.32 ± 0.01	0.29 ± 0.01	0.33 ± 0.01	0.44 ± 0.0311[a,b,c]	0.54 ± 0.0325[a,b,d]
Peroxyl radicals scavenged by skin homogenates (nmol/mg)	173.6 ± 24.9	52.8 ± 3.1[a]	41.0 ± 6.5[a]	87.0 ± 8.7[a]	25.7 ± 3.811[a,b,c]	12.9 ± 3.0[a,b,d]
GHS (nmol/mg protein)	25.7 ± 0.2	18.7 ± 0.6[a]	18.0 ± 0.3[a]	16.8 ± 0.2[a]	14.8 ± 0.111[a,b,c]	10.9 ± 0.3[a,b,d]
Protein thiols (nmol/mg protein)	39.6 ± 2.2	38.6 ± 0.4	36.9 ± 0.9	36.9 ± 0.5	34.4 ± 0.911[a,b,c]	32.8 ± 0.7[a,b,d]

ND, not detectable.
[a]$P < 0.05$ versus acetone–acetone-treated mice.
[b]$P < 0.05$ versus DMBA–acetone-treated mice.
[c]$P < 0.05$ versus acetone–TPA-treated mice.
[d]$P < 0.05$ versus acetone–Cum-OOH-treated mice.

Chemiluminescence measurement of total antioxidant reserve

A water-soluble azo-initiator, AAPH, was used to produce peroxyl radicals (23). Oxidation of luminol by AAPH-derived peroxyl radicals was assayed by the chemiluminescence response. A delay in the chemiluminescence response caused by interaction of endogenous antioxidants with AAPH-derived peroxyl radicals was observed upon addition of homogenates. Based on 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 phosphate 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 luminescence analyzer 633 (Coral Biomedical, San Diego, CA) was employed for these determinations.

GSH and protein thiols assay in cells and tissue

Total protein sulfhydryl concentration in homogenates of skin or cells was determined using ThioGlo™-1, a maleimide reagent which produces a highly fluorescent product upon reaction with sulfhydryl groups (24). A standard curve was established by addition of GSH (0.02–1.0 μM) to 0.1 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 or cell homogenate. Total protein sulfhydryls were determined from the augmentation of the fluorescence response after addition of sodium dodecyl sulfate (4 mM) to the same homogenate. A spectrofluorophotometer (Shimadzu RF-5000 U, Kyoto, Japan) was employed in the assay (excitation 388 nm and emission 500 nm).

Protein assay

Measurement of protein in homogenates of tissue and cells was conducted using a Bio-Rad protein assay kit, cat. no. 500-0006 (Richmond, CA).

Statistics

Data were expressed as the mean ± SEM for each group. One-way analysis of variance with Tukey's test was employed to compare the responses between treatments. Statistical significance was set at $P < 0.05$.

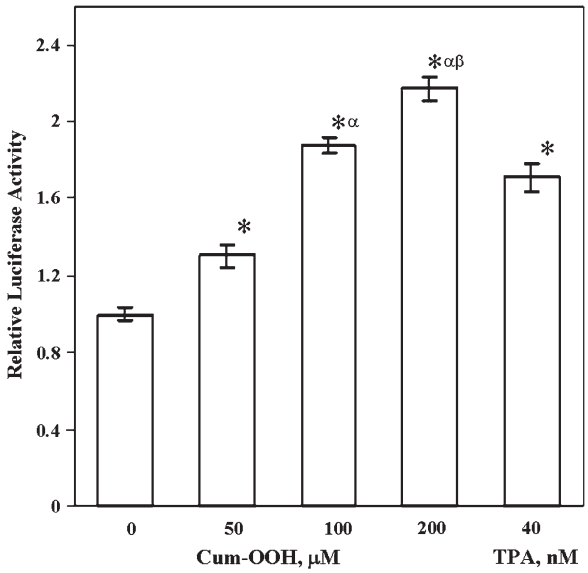

Fig. 1. Cum-OOH-induced AP-1 activation in JB6 P+ cells. JB6 P+ cells (5×10^4 cells/ml) were cultured in Eagle's MEM supplemented with 0.5% FBS and 2 mM L-glutamine for 24 h to minimize basal AP-1 activity, and then exposed to Cum-OOH (0, 50, 100 or 200 μM) or TPA (40 nM) in the same medium for 24 h to monitor the effects on AP-1 induction. The cells were extracted with 200 μl of 1× lysis buffer provided in luciferase assay kit by the manufacturer. Values are means ± SEMs of three experiments. $*P <$ 0.05 versus control cells; $^{\alpha}P < 0.05$ versus 50 μM Cum-OOH and $^{\beta}P < 0.05$ versus 100 μM Cum-OOH.

Results

AP-1 activity in vitro after Cum-OOH exposure

To determine whether Cum-OOH induced AP-1 activation, we incubated the JB6 P+ cells with Cum-OOH or TPA (positive control). Exposure of JB6 P+ cells to Cum-OOH (24 h) causes a dose-dependent activation of AP-1 (Figure 1). JB6 P+ cells incubated with 50, 100 and 200 μM of Cum-OOH for 24 h produced a significant 30, 80 and 120% increase in luciferase activity as compared with vehicle-treated control cells, respectively ($P < 0.05$). No further AP-1 activation was observed after incubation of JB6 P+ cells with Cum-OOH for 48 h (data not shown).

Levels of GSH and protein thiols in vitro after Cum-OOH exposure

Levels of GSH and protein thiols were dramatically reduced after 24 or 48 h incubation of JB6 P+ cells with Cum-OOH (Figure 2). Treatment of JB6 P+ cells with 50, 100 and 200 μM of Cum-OOH for 24 h resulted in a 47, 85 and 91% decrease, respectively, in the level of GSH when compared with cells treated with vehicle. Exposure of JB6 P+ cells to Cum-OOH (50, 100 and 200 μM) for 48 h resulted in a 59, 93 and 95% reduction, respectively, in GSH (Figure 2A) as compared with vehicle-treated controls. As shown in Figure 2B, incubation of JB6 P+ cells with 50, 100 and 200 μM of Cum-OOH for 24 h caused the oxidation of protein sulfhydryls as observed by a 45, 56 and 67% decrease in the level of protein thiols compared with controls, whereas exposure to Cum-OOH for 48 h resulted in a 50, 70 or 73%, respectively, as compared with vehicle-treated cells.

Effects of Cum-OOH or TPA on morphological alterations in the skin of AP-1-luciferase transgenic mice

To determine whether AP-1 activation is involved in Cum-OOH-induced cancer promotion, we used AP-1-luciferase reporter transgenic mice. Initially, the histopathological changes in the skin of AP-1-luciferase reporter transgenic mice were evaluated by a board certified veterinary pathologist 24 h after the last topical application following 29 weeks of Cum-OOH or TPA. We observed epidermal hyperplasia in DMBA–Cum-OOH (Figure 3C and E) and DMBA–TPA (Figure 3B) groups but not in control groups given DMBA–acetone (Figure 3A and D) or acetone–acetone (data not shown). In DMBA-initiated mice, topical treatment with Cum-OOH resulted in increased skin thickness as compared with DMBA–acetone-treated mice. Histological observation of mouse skin exposed to DMBA–Cum-OOH displayed a marked increase in the amount of melanin, a moderate increase in the number and size of sebaceous gland epithelial cells and the hair follicles show an activated epithelium (Figure 3C, E and F). Both DMBA–Cum-OOH and DMBA–TPA applications resulted in an increase in the number of blood vessels and inflammatory infiltrates as compared with control (DMBA–acetone) as determined histopathologically by a board certified veterinary pathologist. However, accumulation of inflammatory cells in the skin of mice given DMBA–Cum-OOH (Figure 3C, E and F) was greater compared with that seen in the skin of mice treated with DMBA–acetone (Figure 3A and D).

Effect of Cum-OOH or TPA treatment on tumor promotion

We found the appearance of tumors on the skin of AP-1 transgenic mice beginning at 7 weeks of treatment (Figure 4A). At the termination of the experiment (29 weeks), DMBA–Cum-OOH treatment resulted in the formation of skin carcinomas (75%) as well as skin papillomas (20%; Figure 4A) in AP-1 transgenic mice. No carcinomas were observed on the skin of AP-1 transgenic mice treated with DMBA–TPA; however, 80% of the mice exhibited papillomas in this group (Figure 4A). DMBA–Cum-OOH treatment caused the formation of 2.5 ± 0.2 (mean ± SEM, $n = 6$) carcinomas per mouse and 2.0 ± 0.2 papillomas per mouse (Figure 4B). After DMBA–TPA treatment, 7.0 ± 1.5 (mean ± SEM, $n = 6$ mice) papillomas per mouse were observed (Figure 4B, inset).

Effect of Cum-OOH or TPA on AP-1 activation in the skin of AP-1-luciferase reporter transgenic mice

Skin samples of transgenic mice were punch biopsied (1.5 mm) at the beginning of the experiment to determine the basal luciferase activity

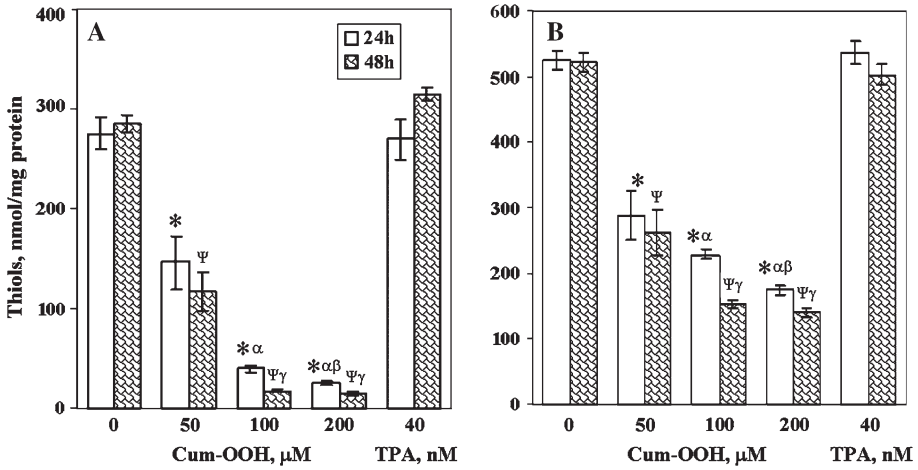

Fig. 2. Effect of Cum-OOH on the levels of GSH (**A**) and protein thiols (**B**) in JB6 P+ cells. JB6 P+ cells (5×10^4 cells/ml) were cultured in Eagle's MEM supplemented with 0.5% FBS and 2 mM L-glutamine for 24 h to minimize basal AP-1 activity, and then exposed to Cum-OOH (0, 50, 100 or 200 μM) or TPA (40 nM) in the same medium for 24 or 48 h. Following exposure, cells were washed with phosphate-buffered saline and frozen at −80°C. ThioGlo™-1 was used to measure changes in GSH and protein thiol levels as a result of exposure. White bars: 24 h after exposure and pattern bars: 48 h after exposure. Values are means ± SEMs of three experiments. $*P < 0.05$ versus 24 h control cells; $^{\Psi}P < 0.05$ versus 48 h control cells; $^{\alpha}P < 0.05$ versus 24 h 50 μM Cum-OOH; $^{\gamma}P < 0.05$ versus 48 h 50 μM Cum-OOH and $^{\beta}P < 0.05$ versus 24 h 100 μM Cum-OOH.

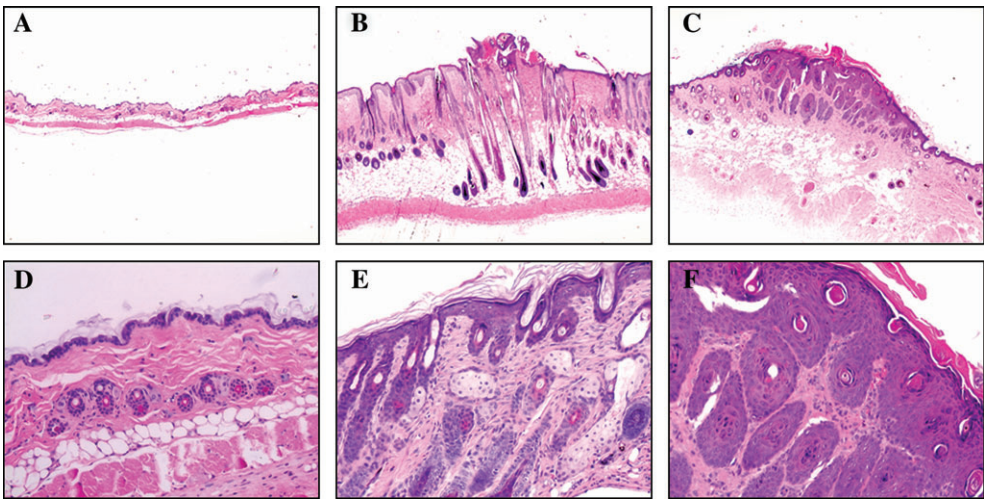

Fig. 3. Morphological alterations and tumor promotion in skin of AP-1 transgenic mice treated with DMBA–Cum-OOH or DMBA–TPA (29 weeks). Mice were exposed to DMBA (51.2 μg dissolved in 100 μl of acetone for each mouse), which was used as a tumor initiator. Fourteen days following initiation, the mice were promoted twice a week with acetone, 17 nmol TPA or 82.6 μmol Cum-OOH dissolved in 100 μl of acetone for the next 29 weeks. Skin sections were collected in formalin for histological evaluation at the completion of the experiment. (**A**) DMBA–acetone, (**B**) DMBA–TPA and (**C**) DMBA–Cum-OOH; magnification ×40. (**D**) DMBA–acetone, (**E**) DMBA–Cum-OOH and (**F**) DMBA–Cum-OOH; magnification ×200.

and then every 4 weeks throughout the experiment. As shown in Figure 5, AP-1 activity peaked in mouse skin exposed to DMBA–Cum-OOH or DMBA–TPA after 4 weeks of promotion. Maximal AP-1 activation was increased 22.4-fold (DMBA–Cum-OOH) or 45.2-fold (DMBA–TPA) as compared with the DMBA–acetone-treated group. AP-1 activation gradually declined after 8 weeks of DMBA–Cum-OOH or DMBA–TPA exposure. Interestingly, although AP-1 activity and papilloma formation were markedly higher in mice treated with DMBA–TPA (4–16 weeks of exposure), we did not observe carcinoma formation in this group (Figure 5). After 29 weeks of topical application with DMBA–Cum-OOH or DMBA–TPA, no differences in AP-1 activation were detected from control (Figure 5).

Inflammatory response in the skin of mice exposed to Cum-OOH or TPA

We found that exposure to DMBA–Cum-OOH or DMBA–TPA caused skin inflammation as determined by skin thickness and measurement of MPO activity in mouse skin. Topical application of DMBA–Cum-OOH or DMBA–TPA to mouse skin caused a 34.8-fold or a 23.5-fold net increase, respectively, in the skin bi-fold thickness at the termination of the experiment (29 weeks) as compared with the DMBA–acetone group (Table I). Topical exposure to DMBA–Cum-OOH or DMBA–TPA resulted in a significant elevation in MPO activity (12.7 and 4.83 nmol/min/mg protein, respectively) in mouse skin as compared with the levels seen in the DMBA–acetone group (Table I).

Biomarkers of oxidative stress in the skin of mice exposed to Cum-OOH or TPA

Topical application of DMBA–Cum-OOH- or DMBA–TPA-induced oxidative stress in mouse skin, as indicated by the accumulation of peroxidative products (TBARSs), depletion of total antioxidant reserve, a decrease in the levels of GSH and oxidation of protein sulfhydryls (Table I). After 29 weeks of treatment with DMBA–Cum-OOH or DMBA–TPA, the level of lipid peroxidative products in the

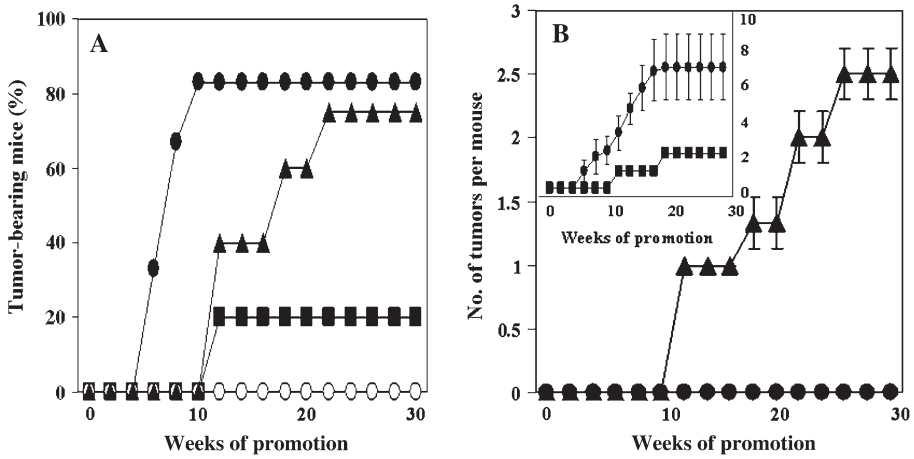

Fig. 4. **A**) Incidence of tumors in AP-1 transgenic mice treated with DMBA–Cum-OOH or DMBA–TPA. Mice were exposed to DMBA (51.2 μg dissolved in 100 μl of acetone for each mouse), which was used as a tumor initiator. Fourteen days following initiation, the mice were promoted twice a week with acetone, 17 nmol TPA or 82.6 μmol Cum-OOH dissolved in 100 μl of acetone for the next 29 weeks. The papillomas/carcinomas appearing on the shaved area of the skin were recorded at weekly intervals. Filled circles: percentage of animals having one or more TPA-induced papillomas; filled squares: percentage of animals having one or more Cum-OOH-induced papillomas; filled triangles: percentage of animals having one or more Cum-OOH-induced carcinomas and open circles: percentage of animals having acetone-induced papillomas/carcinomas. Values are means of six mice per group per experiment for two experiments. (**B**) Number of tumors in AP-1 transgenic mice treated with DMBA–Cum-OOH and DMBA–TPA; inset: number of papillomas per mouse. Filled circles: number of TPA-induced papillomas/carcinomas per mouse; filled squares: number of Cum-OOH-induced papillomas per mouse and filled triangles: number of Cum-OOH-induced carcinomas per mouse. Values are means of six mice per group per experiment for two experiments.

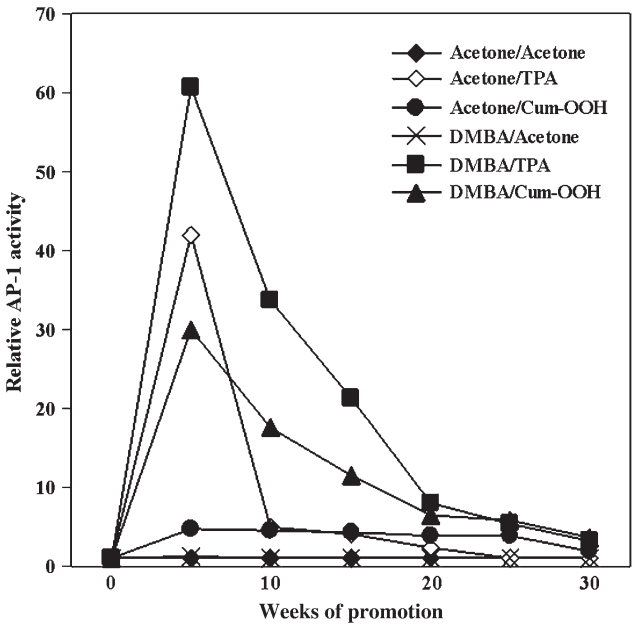

Fig. 5. AP-1 transactivation in the skin of AP-1-luciferase reporter transgenic mice treated with DMBA–Cum-OOH and DMBA–TPA. Mice were exposed to DMBA (51.2 μg dissolved in 100 μl of acetone for each mouse), which was used as a tumor initiator. Fourteen days following initiation, the mice were promoted twice a week with acetone, 17 nmol TPA or 82.6 μmol Cum-OOH dissolved in 100 μl of acetone for the next 29 weeks. AP-1-luciferase activity in the skin was measured by dorsal skin punch biopsy every 4 weeks. Filled diamonds: acetone–acetone-exposed mice; open diamonds: acetone–TPA-exposed mice; filled circles: acetone–Cum-OOH-exposed mice; cross symbols: DMBA–acetone-exposed mice; filled squares: DMBA–TPA-exposed mice and filled triangles: DMBA–Cum-OOH-exposed mice. Values are means of six mice per group per experiment for two experiments.

skin of AP-1-luciferase reporter transgenic mice were increased by 63.6 or 33.3%, respectively, above the levels seen in the control (DMBA–acetone) (Table I). Total antioxidant reserve was reduced remarkably by 85.2 or 70.5% in the skin of AP-1 transgenic mice treated with DMBA–Cum-OOH or DMBA–TPA, respectively, as compared with control groups exposed to DMBA–acetone (Table I).

DMBA–Cum-OOH or DMBA–TPA exposures also reduced the levels of GSH by 35.1 or 11.9% in the skin of AP-1 transgenic mice as compared with DMBA–acetone-treated controls. Along with GSH depletion, the levels of protein thiols were oxidized by 11.1 or 6.8% after DMBA–Cum-OOH or DMBA–TPA topical application as compared with controls treated with DMBA–acetone (Table I).

Discussion

Chemically induced skin cancer in mice has three chronological stages, initiation, promotion and progression (25,26). Tumor initiation is a rapid and irreversible process, whereas promotion is a long-term process that requires chronic exposure to a tumor promoter. A tumor promoter increases proliferation of initiated cells thereby accelerating neoplastic progression; however, the exact mechanism of promotion is more complicated (27). Conversion of benign papillomas to malignant carcinomas occurs spontaneously during the promotion stage of carcinogenesis; however, this conversion occurs at a very low rate in animals undergoing repetitive TPA exposures after DMBA initiation (28). The conversion frequency of a papilloma to a carcinoma can be increased by exposing papilloma-bearing animals to agents such as OPs or hydroperoxides (29) that are able to promote or enhance progression.

Free radical formation has been shown to play a role in both initiation and promotion of multi-stage carcinogenesis (30–33). The effectiveness of the OPs and hydroperoxides as promoters is related to the type of radicals produced (34). Previously, we have shown that exposure to Cum-OOH resulted in the production of two lipid-derived radical species that were detected as early as 30 min following topical exposure and determined to be methyl and methoxyl radicals derived from the metabolic oxidation of Cum-OOH (3). We speculate that these highly reactive radicals are able to interact with DNA and cause oxidative damage (35,36), which may contribute to increased malignant transformation (37). It has been shown that radical production could turn off redox-responsive genes such as JNK, Fos and CREB (38), triggering significant modifications in the mitogen-activated protein kinase/AP-1 pathways (19,39–41).

Changes in the level of AP-1 activity have been correlated with different stages of melanoma development and progression (42). Cum-OOH exposure has been shown to result in a concentration-dependent activation of cytosolic phospholipase A_2 in Her14 fibroblasts. Phospholipase A_2 has been implicated in cellular injury and hydrolyses of polyunsaturated arachidonic fatty acid (43). Enhanced conversion of arachidonic acid to prostaglandins via cyclooxygenase has been shown to be a potential contributor to the development of skin cancers (44). COX-2 over-expression has been linked to the pathophysiology of inflammation and cancer. COX-2 expression has been demonstrated to correlate with cell proliferation and tumor promotion (45). Benign papillomas have been shown to have COX-2 expression localized perinuclearly and cytoplasmically, whereas carcinomas have more intense and stronger COX-2 expression found within the tumor stroma and throughout the granular and spinous layers of tumors (44). We observed that Cum-OOH exposure of human keratinocyte cells (HaCat) induced the release of prostaglandin E_2. COX-2-specific inhibitors have been found to suppress the synthesis and release of prostaglandins induced by exposure to Cum-OOH (46). In this study, we found that animals exposed to DMBA–TPA had higher AP-1 activation along with increased papilloma formation compared with the animals treated with DMBA–Cum-OOH. Exposure to DMBA–Cum-OOH caused carcinoma formation not seen in mice treated with DMBA–TPA. The observed carcinomas in DMBA–Cum-OOH-treated mice appear to result from the malignant conversion of benign papillomas to carcinomas, which is possibly related to a COX-2-dependent pathway and was not entirely dependent upon AP-1 activation.

Another possible mechanism by which DMBA–Cum-OOH papillomas may be transformed from benign to malignant carcinomas may be excessive production of radicals and subsequent continued DNA damage. This speculation has been drawn from a previous study showing that oxidative DNA damage occurred in the skin of older (32 weeks old) but not young mice (13 weeks) exposed to Cum-OOH (47). As the animals age throughout the duration of the experiment, increased oxidative DNA damage may be occurring thereby resulting in the increased malignant conversion of skin papillomas to carcinomas.

Inflammation and oxidative stress are probably associated with tumor development (48). Application of free radical-scavenging compounds, e.g. antioxidants, can affect the cancer promotion stage (31,32,49,50), indicating the role of free radicals in tumor promotion and development. *In vitro* exposure to Cum-OOH caused a dose-dependent reduction in GSH levels and a subsequent increase in AP-1 activity. This redox sensitive induction of AP-1 was prevented by the pre-treatment of cells with dehydroascorbic acid (30% inhibition when pre-incubated with 100 μM dehydroascorbic acid for 1 h), indicating the importance of the antioxidant network in AP-1 activation.

Cum-OOH has been shown to cause tumor promotion following topical exposure of murine skin (29,30,49). DMBA–Cum-OOH exposure reduced the antioxidative capacity in the skin of animals as indicated by decreases in GSH and total antioxidant reserves. Cum-OOH has been shown to be a substrate for GSH peroxidase-catalyzed reactions (51). Therefore, it is not surprising that Cum-OOH caused a more pronounced depletion of GSH and total antioxidant reserves than TPA. Interestingly, Cum-OOH induced AP-1 to a lesser extent as compared with TPA. Moreover, Cum-OOH induced carcinoma and papilloma formation whereas TPA induced higher yield of papillomas. These results suggest that additional factors along with oxidative stress were contributory to pro-carcinogenic activity of Cum-OOH. Increased inflammation, as revealed by neutrophil infiltration and elevated MPO activity in the skin, was also seen to a higher extent in DMBA–Cum-OOH-treated mice than in DMBA–TPA-treated animals.

It has been mentioned that papillomas appeared on the skin of AP-1 transgenic mice after 7 weeks of DMBA–TPA treatment, whereas in DMBA–Cum-OOH papillomas were found 11 weeks after exposure. The growth, external appearance and number of tumors were also different between the two groups (Figure 5). Distinctively, in the group of mice treated with DMBA–Cum-OOH, we found malignant carcinomas that were not detected in the DMBA–TPA-exposed animals. In conclusion, we found that activation of AP-1 in skin was elevated as early as 2 weeks following DMBA–Cum-OOH and DMBA–TPA exposures. Papilloma formation was observed in both the DMBA–TPA- and DMBA–Cum-OOH-exposed animals whereas skin carcinomas were found only in the DMBA–Cum-OOH-treated mice. Greater inflammation, increased accumulation of peroxidative products and reduced levels of GSH and total antioxidant reserves were observed in the skin of DMBA–Cum-OOH-exposed mice as compared with DMBA–TPA-exposed mice.

Acknowledgements

We thank Dr Min Ding for providing the AP-1 transgenic mice used in these experiments and Dr Nancy Colburn for providing the JB6 P+ cells used in these studies.

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.

Conflict of Interest Statement: None declared.

References

1. Lewis,R.J. (1993) *Hawley's Condensed Chemical Dictionary*, 12th ed. Van Nostrand Reinhold, New York, 12th ed133.
2. Adams,R.M. (1999) *Occupational Skin Disease*W.B. Saunders, Philadelphia, PA, 491–500.
3. Shvedova,A.A. *et al*. (2002) Antioxidant balance and free radical generation in vitamin E deficient mice after dermal exposure to cumene hydroperoxide. *Chem. Res. Toxicol.*, **15**, 1451–1459.
4. Barbasid,M. (1987) Ras genes. *Annu. Rev. Biochem.*, **56**, 779–827.
5. Angel,P. *et al*. (1987) Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell*, **49**, 729–739.
6. Angel,P. *et al*. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta*, **1072**, 129–157.
7. Hirai,S.I. *et al*. (1989) Characterization of junD: a new member of the jun proto-oncogene family. *EMBO J.*, **8**, 1433–1439.
8. Domann,F.E.Jr *et al*. (1994) Constitutive AP-1 DNA binding and transactivating ability of malignant but not benign mouse epidermal cells. *Mol. Carcinog.*, **9**, 61–66.
9. Dong,Z. *et al*. (1995) Transformation reversion induced in JB6 RT101 cells by AP-1 inhibitors. *Carcinogenesis*, **16**, 749–756.
10. Huang,C. *et al*. (1997) Inositol hexaphosphate inhibits cell transformation and activator protein-1 activation by targeting phosphatidylinositol-3′ kinase. *Cancer Res.*, **57**, 2873–2878.
11. Jochum,W. *et al*. (2001) AP-1 in mouse development and tumorigenesis. *Oncogene*, **20**, 2401–2412.
12. Perchellet,J.P. *et al*. (1987) Effects of combined treatments with selenium, glutathione, and vitamin E on glutathione peroxidase activity, ornithine decarboxylase induction, and complete and multistage carcinogenesis in mouse skin. *Cancer Res.*, **47**, 477–485.
13. Kisella,A.R. *et al*. (1983) Investigation of a possible role for superoxide anion production in tumour promotion. *Carcinogenesis*, **4**, 717–719.
14. Solanki,V. *et al*. (1981) Specific binding of phorbol ester tumor promoters to intact primary epidermal cells from Sencar mice. *Proc. Natl Acad. Sci. USA*, **78**, 2549–2553.
15. Amstad,P.A. *et al*. (1997) bcl-2 enhancement of malignant transformation in mouse epidermal JB6 cells. *Mol. Carcinog.*, **20**, 231–239.
16. Isbir,T. *et al*. (2000) The effects of *Brassica oleraceae var capitata* on epidermal glutathione and lipid peroxides in DMBA-initiated-TPA-promoted mice. *Anticancer Res.*, **20**, 219–224.
17. Rincon,M. *et al*. (1994) AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T-lymphocytes. *EMBO J.*, **13**, 4370–4381.
18. Huang,C. *et al*. (1996) Ultraviolet-B-induced activated AP-1 activation does not require epidermal growth factor receptor but is blocked by a dominant negative PKC. *J. Biol. Chem.*, **49**, 31262–31268.
19. Dhar,A. *et al*. (2002) The role of AP-1, NF-kB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. *Mol. Cell. Biochem.*, **234/235**, 185–193.

20. Huang,C. *et al*. (1996) Requirement for phosphatidylinositol 3-kinase in epidermal growth factor-induced AP-1 transactivation and transformation in JB6 P+ cells. *Mol. Cell. Biol.*, **16**, 6427–6435.
21. Nonaka,T. *et al*. (1992) Histamine-induced differentiation of HL-60 cells. The role of cAMP and protein kinase A. *Biochem. Pharmacol.*, **44**, 1115–1121.
22. Buege,J.A. *et al*. (1978) Microsomal lipid peroxidation. *Methods Enzymol.*, **52**, 302–310.
23. Niki,E. (1990) Free radical initiators as a source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol.*, **186**, 100–108.
24. Shvedova,A.A. *et al*. (2000) Redox cycling of phenol induces oxidative stress in human epidermal keratinocytes. *J. Invest. Dermatol.*, **114**, 354–364.
25. Bowden,G.T. *et al*. (1995) Molecular mechanisms involved in skin tumor initiation, promotion, and progression. In Mukhtar,H. (ed.) *Skin Cancer:-Mechanisms and Human Relevance*CRC Press, Inc., Boca Raton, FL, pp. 99–111.
26. Hennings,H. *et al*. (1993) FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. *Carcinogenesis*, **14**, 2353–2358.
27. Nguyen-Ba,G. *et al*. (1999) Epigenetic events during the process of cell transformation induced by carcinogens. *Oncol. Rep.*, **6**, 925–932.
28. Bhasin,G. *et al*. (2004) Free radical generating agents lead to the rapid progression of benign skin tumors to carcinoma in iron-overloaded mice. *Arch. Toxicol.*, **78**, 139–146.
29. Athar,M. *et al*. (1989) Malignant conversion of UV radiation and chemically-induced mouse skin benign tumors by free-radical-generating compounds. *Carcinogenesis*, **10**, 1841–1845.
30. Slaga,T.J. *et al*. (1981) Skin tumor promotion activity of benzoyl peroxide, a widely used free radical generating compound. *Science*, **213**, 1023–1025.
31. Taffee,B.G. *et al*. (1986) Generation of free radicals from organic hydroperoxide tumor promoter by mouse epidermial cells. *Pharmacologist*, **28**, 175.
32. Taffee,B.G. *et al*. (1986) Modification of cellular antioxidant defense mechanisms in mouse skin by multiple applications of TPA. *Proc. Am. Assoc. Cancer Res.*, **27**, 148.
33. Oberley,L.V.W. *et al*. (1986) Free radical, cancer, and aging. In Johnson,-J.E.Jr,Walford,R.,Harman,D. and Miquel,J. (eds.) *Free Radicals, Aging, and Degenerative Diseases*Alan R. Liss, Inc., New York, pp. 325–371.
34. Kensler,T. *et al*. (1995) Role of reactive intermediates in tumor promotion and progression. *Prog. Clin. Biol. Res.*, **391**, 103–116.
35. Floyd,R.A. (1982) *Free Radicals and Cancer*Marcel Dekker, Inc., New York.
36. Sultana,S. *et al*. (1995) Crude extracts of hepatoprotective plants *Solanum nigrum* and *Cichorium intybus* inhibit free radical-mediated DNA damage. *J. Ethnopharmacol.*, **45**, 189–192.
37. O'Connell,J.F. *et al*. (1986) Enhanced malignant progression of mouse skin tumors by free-radical generator benzoyl peroxide. *Cancer Res.*, **46**, 2863–2865.
38. Pugazhenthi,S. *et al*. (2006) Differential regulation of c-jun and CREB by acrolein and 4-hydroxynonenal. *Free Radic. Biol. Med.*, **40**, 21–34.
39. Remacle,J. *et al*. (1995) Low levels of reactive oxygen species as modulators of cell function. *Mutat. Res.*, **316**, 117–129.
40. Allen,R.G. *et al*. (2000) Oxidative stress and gene regulation. *Free Radic. Biol. Med.*, **28**, 463–499.
41. Cimino,F. *et al*. (1997) Gene regulation by reactive oxygen species. *Curr. Top. Cell. Regul.*, **35**, 123–148.
42. Angel,J.M. *et al*. (2001) Confirmation of the mapping of a 12-O-tetradecanoylphorbol-13-acetate promotion susceptibility locus, Psl1, to distal mouse chromosome 9. *Mol. Carcinog.*, **32**, 169–175.
43. van Rossum,G.S.A.T. *et al*. (2004) Activation of cytosolic phospholipase A2 in Her14 fibroblasts by hydrogen peroxide: a p42/44 MAPK-dependent and phosphorylation-independent mechanism. *Biochim. Biophys. Acta*, **1636**, 183–195.
44. An,K.P. *et al*. (2002) Cyclooxygenase-2 expression in murine and human nonmelanoma skin cancers: implications for therapeutic approaches. *Photochem. Photobiol.*, **76**, 73–80.
45. Chinery,R. *et al*. (1999) Prostaglandin J2 and 15-deoxy-delta12, 14-prostaglandin J2 induce proliferation and cyclooxygenase-depleted colorectal cancer cells. *Cancer Res.*, **59**, 2739–2746.
46. Shvedova,A.A. *et al*. (2004) Pro/antioxidant status in murine skin following topical exposure to cumene hydroperoxide throughout the ontogeny of skin cancer. *Biochemistry (Mosc.).*, **69**, 30–40.
47. Murray,A.R. *et al*. (2005) Role of vitamin E in the antioxidant defense system of skin in young and old mice exposed to cumene hydroperoxide. *Toxicologist*, **84**, 2188.
48. Nakamura,Y. *et al*. (2000) Search for naturally-occurring antioxidative chemopreventors on the basis of the involvement of leukocyte-derived reactive oxygen species in carcinogenesis. *Asian Pac. J. Cancer Prev.*, **1**, 115–120.
49. Timmins,G.S. *et al*. (1993) Free radical formation in isolated murine keratinocytes treated with organic peroxides and its modulation by antioxidants. *Carcinogenesis*, **14**, 1615–1620.
50. Perchellet,J. *et al*. (1992) Antioxidants and multistage carcinogenesis in mouse skin. *Free Radic. Biol. Med.*, **7**, 377–408.
51. Howard,S.A. *et al*. (1998) The relative effectiveness of human plasma glutathione peroxidase as a catalyst for the reduction of hydroperoxides by glutathione. *Biol. Trace Elem. Res.*, **61**, 127–136.

Received August 18, 2006; revised December 18, 2006; accepted January 4, 2007