Effects of 4-Tertiary Butyl Catechol on Glutathione-Metabolizing Enzymes In Vivo and In Vitro

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4-Tertiary butyl catechol (TBC) causes depigmentation in humans and animals and stimulates formation of pheomelanosomes. In this study, we investigated the effects of noncytotoxic doses of TBC on glutathione S-transferase (GST) activity in the skin of Uscd strain mice and B16 murine melanoma cells in culture, in relation to changes in activities of glutathione reductase (GR) and γ-glutamyl transpeptidase (GGT) reported to be involved in pheomelanogenesis. Occurrence of pheomelanosomes in skin melanocytes was demonstrated by electron microscopy and reduction (25%) of eumelanin content in melanoma cells was shown by spectrophotometry. Topical application of 1 M TBC-DMSO-acetone solution on the ear skin elevated GST activity about 27%, and activities of GGT and GR to 35% and 19%, respectively, within 1 week. Melanoma cells cultured in 10^{-4} M TBC-containing medium for 2 h showed no changes in GST and GGT activities, but 12% increase of GR activity during the first 12 h. Activities of all 3 enzymes was elevated (11–17%) 24 h later. The elevation detected by 48 h was 25% for GST, 26% for GGT, and 14% for GR. The findings were interpreted to show that depigmentation produced by the antioxidant results from stimulated pheomelanogenesis through activation of glutathione-metabolizing enzymes and suppressed oxidation of eumelanin intermediates.

4-Tertiary butyl catechol (TBC) is an antioxidant that causes depigmentation in humans [1,2]. Electron microscopic studies have shown that pheomelanosomes appear in melanocytes of pigmented mouse and guinea pig ear skin after TBC treatment, indicating that TBC depigmentation may result from stimulation of pheomelanogenesis and inhibition of eumelanogenesis [3,4]. Cysteinyldopa is a key intermediate of pheomelanin [5]. Although cysteine and dopa or dopaquinone combine nonenzymatically to form cysteinyldopa [6,7], enzymatic conversion of glutathionedopa to cysteinyldopa by γ-glutamyltranspeptidase (GGT) has been reported [8]. Hu and Buxman histochemically demonstrated the presence of GGT in normal and transformed melanocytes engaged in pheomelanogenesis [9,10]. Prota and his coinvestigators proposed that glutathione reductase (GR) and the reduced form of glutathione (GSH), may

also play a regulatory role in pheomelanogenesis [6,11]. Confirming this view, Benedetto et al detected higher levels of both GSH and GR activity in skin with pheomelanogenesis than in skin with eumelanogenesis [12]. Yonemoto et al found that TBC-induced pheomelanogenesis also was associated with an elevation of GR activity in the skin and both GR and GGT activities in tissue-cultured human melanoma cells [13,14].

In order to further elucidate enzymatic mechanisms involved in chemically stimulated pheomelanogenesis, we investigated effects of TBC on glutathione S-transferase (GST), another glutathione-metabolizing enzyme. Antioxidants are known to activate GST which conjugates GSH with a wide variety of intracellular electrophilic agents [15,16]. Both pigmented mouse ear skin and melanoma cells were treated with TBC, and GST activity was measured simultaneously with GR and GGT activities. The enzymatic changes were correlated with pheomelanosome formation in melanocytes of the ear skin and reduction of eumelanin content in the tissue-cultured cells.

MATERIALS AND METHODS

Materials

Male, 2-month-old Uscd strain mice were raised and maintained at the University of California, San Francisco. Murine B16 (HFH18) melanoma cells were kindly provided by Dr. F. Hu, University of Oregon. TBC, purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, was dissolved in a mixture of dimethyl sulfoxide (DMSO) obtained from Sigma Chemical Co., St. Louis, Missouri, and acetone (3v:7v) at the concentration of 1 M for in vivo study and in DMSO at 10^{-1} M for in vitro study. Eagle's minimum essential medium with Hanks' salts (MEM), basal medium Eagle (BME), fetal bovine serum, penicillin, and streptomycin were obtained from Grand Island Biological Co., Grand Island, New York. Isotonic diluent was purchased from Curtain Matheson Co., Brisbane, California, and Soluene-100, from Packard Instrument Co., Downers Grove, Illinois. All substrates and cofactors came from Sigma Chemical Co., except for 1-chloro-2,4-dinitrobenzene (CDNB) which was purchased from Aldrich Chemical Co.

TBC Application to Ear Skin and Electron Microscopy

Mice were divided into 2 groups. The ears of each group were painted 3 times with about 0.1 ml of either 1 M TBC solution or DMSO-acetone (3v:7v) only, at 48-h intervals. The ears were removed 24 h after the last application. Skin slices taken from the ears of 3 mice in both groups were prepared for electron microscopy. Photos magnified 45,000× were used for detection of pheomelanosomes according to the methodology reported by Nishimura et al [4].

TBC Treatment of B16 Melanoma Cells and Determination of Eumelanin Content

Cells (about 3×10^6) were seeded into each 75-cm² flask and grown in MEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin under a humidified atmosphere of 5% CO_2:95% air. By the 6th day after seeding the tissue-cultured melanoma cells, the medium in a set of 15–20 flasks was changed to BME containing TBC at a final concentration ranging from 10^{-3} M to 10^{-7} M. For controls, melanoma cells in a comparable number of flasks were treated with BME containing only DMSO in the appropriate concentration. Two hours later the BME with TBC or with DMSO was decanted and the cells were allowed to grow in MEM with serum for additional time periods. Eumelanin content was estimated colorimetrically using the methods described by Oikawa and Nakayasu [17].

Melanoma cells were detached from the flasks with 2 mM EDTA in phosphate-buffered saline at 0, 24, and 48 h after treatment; aliquots of 2×10^6 cells were precipitated with 5% trichloroacetic acid and washed with 0.1 N NaOH. The alkali-insoluble fraction was dissolved in 2.0 ml of Soluene-100 and the absorbance at 400 nm read on a Perkin-Elmer spectrophotometer. Cell numbers were counted in isotonic diluent using an electro Model ZBI Coulter Counter (Coulter Electronics Inc., Hialeah, Florida).

Enzyme Assays

About 6 mm × 4 mm of skin containing total epidermis and superficial dermis was excised from both ears of 14 and 12 mice treated with TBC or DMSO-acetone, repectively. Ear tissues were also obtained from 6 mice that received no treatment, as an additional control. They were washed in cold 25 mM sucrose in 50 mM Tris-HCl buffer, pH 7.0. Each tissue slice was minced, homogenized in the same buffer at 4°C 20 times with a glass homogenizer, and further with a Polytron homogenizer at maximum intensity for 15 s, and repeated 3 times at 5-s intervals. Culture was terminated by scraping cells in 20 mM Tris-HCl buffer, pH 7.4, containing 0.14 M sucrose at 12, 24, and 48 h after treatment. The cells treated with TBC and those treated with DMSO only were separately suspended in 10.0 ml of the same buffer. They were homogenized and centrifuged by the same technique used for ear tissues. The supernatants were used for assays of GR and GST, while the total homogenate was used for GGT assay. The enzyme assay was done with the tissue sample prepared from each ear, but with tissue-cultured cells, GST was measured in cells from 1 flask, while GGT and GR were assayed in cells from 2 combined flasks.

GGT assay: The cell homogenate, 0.1 ml, was added to 0.9 ml of the transpeptidase substrate solution containing 2.5 mM L-γ-glutamyl-*p*-nitroanilide, 20 mM glycyl-glycine, 75 mM NaCl, and 50 mM Tris-HCl buffer, pH 8.0, and incubated for 1 h at 37°C with constant stirring. The reaction was terminated by the addition of 1.0 ml of 3 N acetic acid and the absorbance of *p*-nitroaniline released was measured spectrophotometrically at 410 nm [18]. The activity was expressed as nmoles of *p*-nitroaniline released/min/mg protein.

GR assay: The supernatant, 0.3 ml, was added to 1.7 ml of a reaction mixture consisting of 0.2 M Tris-HCl buffer, pH 7.02, 10 mM EDTA, 0.1 mM NADPH, 0.6 mM oxidized-form glutathione, and 0.5% bovine serum albumin. The oxidation rate of the cofactor NADPH was measured in a thermostatted cell compartment at 37°C by a decrease in absorption at 340 nm using the spectrophotometer connected to a Perkin-Elmer recorder (Model 56) [19,20]. The activity was expressed as nmoles of NADPH oxidized/min/mg protein.

GST assay: The reaction mixture contained a total volume of 2 ml consisting of 0.2 ml of the enzyme source, 0.1 M potassium phosphate buffer, pH 6.5, 1 mM CDNB, and 4 mM GSH. Since the substrate was poorly soluble in water, the stock solution was prepared as a 25 mM ethanol solution. It was placed in a thermostatted cell compartment at 25°C and changes in absorbance at 340 nm were monitored [21]. The rate of nonenzymatic conjugation was measured by the absorbance of the reaction mixture replacing the enzyme source with 0.1 M potassium phosphate buffer to be subtracted from the sample rate. The activity was expressed as nmoles of conjugated CDNB/min/mg protein.

Protein Concentration

Protein was measured by the method of Lowry et al [22] using bovine serum albumin as the standard.

RESULTS

In Vivo Studies

Electron microscopic findings: Different stages of eumelanosomes were observed in melanocytes of skin treated with DMSO-acetone alone, while an altered form of melanosomes, classified as pheomelanosomes by Nishimura et al [4], were seen in the TBC in DMSO-acetone-treated sites (Fig 1).

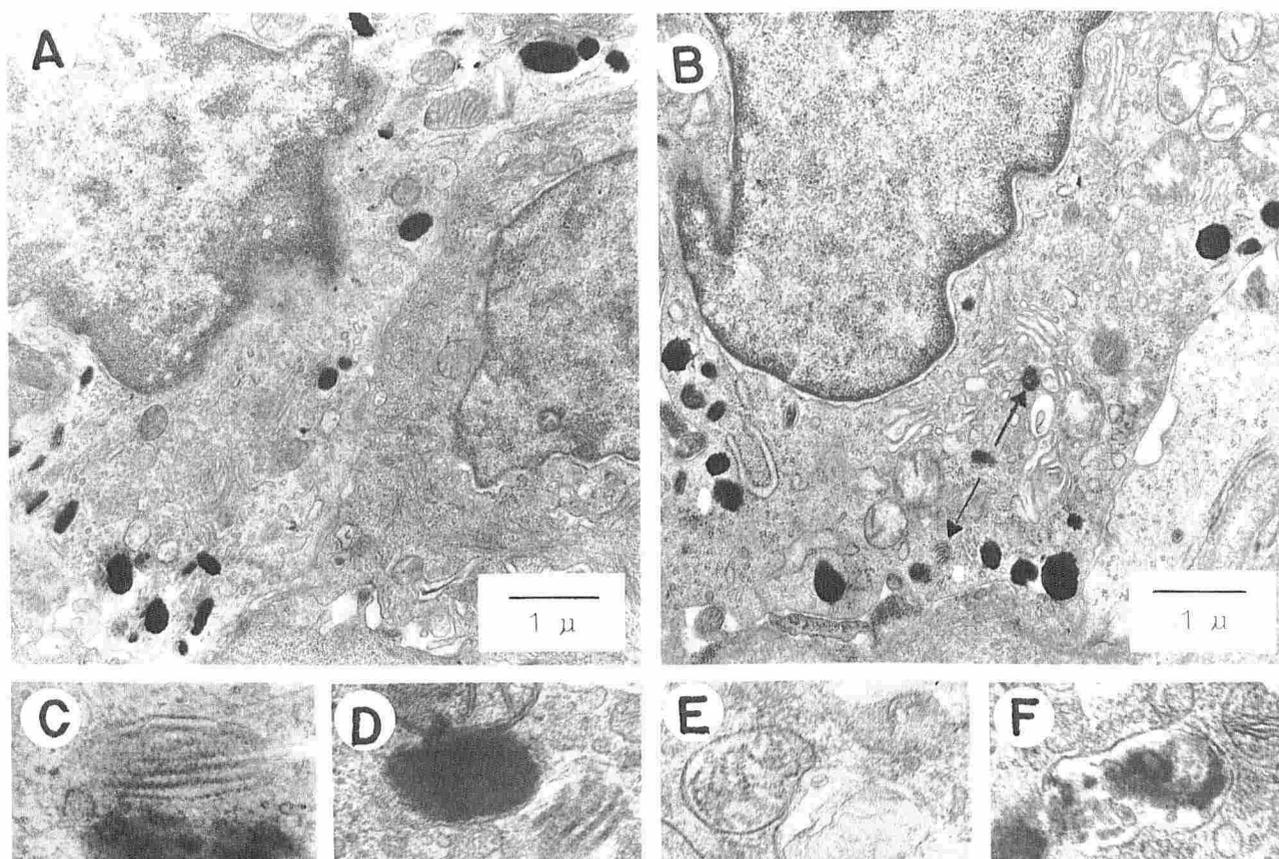*Enzyme activities*: All enzyme activities were elevated in ear

FIG 1. Melanocytes in the ear of Uscd strain mice treated with DMSO-acetone alone (*A*), and 1 M TBC in DMSO-acetone (*B*). All melanosomes in *A* are considered to be eumelanosomes, while many melanosomes in *B* are classified as pheomelanosomes; the *arrows* point out some examples. Insets are typical melanosomes of different stages observed in those melanocytes: melanized premelanosomes (*C*), mature eumelanosomes (*D*), premelanosomes which are round in shape, containing tubular structures (*E*), and altered melanosomes showing irregular deposition of electron-dense material (*F*).

tissues after TBC application as compared with those in DMSO-acetone-treated ear tissues (Table I). Increases were 27%, 19%, and 35% of the specific activities of GR, GST, and GGT, respectively. The differences were statistically significant as indicated by the p values. The enzyme levels detected in the DMSO-acetone-treated tissues did not differ from those detected in 6 nontreated mouse ears (GST: 211 ± 17 nmol/min/mg protein. GR: 131 ± 5 nmol/min/mg protein).

In Vitro Studies

Eumelanin content and cell counts: Table II summarizes the findings. With 10^{-7} M TBC, the eumelanin content of treated cells did not differ from that of control cells. As the concentration of TBC increased to the range of 10^{-5}–10^{-3} M, eumelanin content decreased. The maximum reduction was seen at the concentration of 10^{-3} M TBC and the eumelanin content became 60% of the control values. However this concentration was toxic to the cells and cell numbers were reduced to 65%. Therefore, for the enzyme assay studies, we treated the cells with 10^{-4} M TBC; this concentration causes a 25% depression in eumelanin content without detectable effects on cell number. Time course of decrease in eumelanin content in cells treated with 10^{-4} M TBC is shown in Table III. Treated cells contained less melanin than controls by both 24 and 48 h. In some experiments we attempted to electron microscopically correlate the appearance of pheomelanosomes. However ultrastructure of melanosomes in B16 melanoma cells was altered from melanocytes even without any chemical treatment. It was therefore concluded that data from a small number of cells are not reliable, and are not included in the present study.

Enzyme activities (Fig 2): GR activity in TBC-treated cells showed a $12 \pm 6\%$ elevation as early as 12 h, and continued to be higher than DMSO-acetone-treated cells throughout the experimental period ($14 \pm 3\%$ for 24 h, and $14 \pm 6\%$ for 48 h). GGT showed no significant difference at 12 h, but it began to elevate at 24 h ($11 \pm 6\%$) and reached a $26 \pm 14\%$ increase after 48 h. Similarly, GST activity did not change during the first 12 h, but it started to increase by 24 h ($17 \pm 11\%$) and showed a $25 \pm 9\%$ elevation by 48 h after treatment.

DISCUSSION

GST activity increased in both ear skin and B16 melanoma cells treated with TBC. The elevation occurred after increase in GR activity, when GGT activity also increased. The treated cells in the skin revealed pheomelanosomes and those in vitro a decreased eumelanin content. Eumelanin content in the skin remained unchanged because TBC treatment was carried out for only 1 week. Similarly, clear identification of newly formed pheomelanosomes in B16 melanoma cells by electron microscopy was not possible and data are not included because the melanosomes of nontreated melanoma cells already showed much greater variation than normal melanocytes. Nevertheless we conclude that TBC causes two primary changes in cellular metabolism of pigmented cells: one is to reduce eumelanin content and the other is to increase glutathione-metabolizing enzyme activities and form pheomelanin.

Usami et al reported that TBC is not an inhibitor or a substrate for tyrosinase at the concentrations used [23]. The

reduction of eumelanin content therefore appears to result from an antioxidant effect of TBC on eumelanin precursors, although there has been no direct chemical study to demonstrate that TBC inhibits oxidation of eumelanin intermediates. However, quantitative electron microscopic analyses performed by Nishimura et al [4] support this possibility. They observed a statistically significant increase of nonmelanized pre(eu)melanosomes and a decrease of mature eumelanosomes in the TBC-treated skin.

The second metabolic change, elevation of glutathione-metabolizing enzymes detected in the present study, may also be a direct effect of TBC as an antioxidant. Benson et al reported induction of hepatic GST by feeding mice with 2(3)-tert-butyl-4-hydroxyanisole (BHA) or 1,2-dihydroxy-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquine) [15]. Sparnins and Wattenberg also showed that antioxidants including phenolic compounds increase GST activity and acid-soluble sulfhydryl levels in mouse forestomach [16]. More indirect evidences for antioxidant effects on glutathione-metabolizing enzymes have been reported by others investigating life span and aging of experimental animals [24–26]. We consider that TBC caused the antioxidant effects in pigmented cells and this nonspecific cell reaction led to the formation of pheomelanins due to the presence of unique cell metabolites, i.e., dopaquinone and other melanin intermediates. GSH generated by GR binds to the intermediates. Conjugation of GSH with dopaquinone to form glutathionedopas could occur without enzymatic intervention [27]. The presence of GST, however, enhances this binding because GST conjugates GSH with a wide variety of electrophilic substances including dopaquinone [28]. GGT, then, is involved in the conversion of glutathionedopas to cysteinyldo-

TABLE II. *Effects of different concentrations of 4-tertiary butyl catechol (TBC) on eumelanin content and cell counts of B16 melanoma cells, expressed as percent of the values obtained in B16 melanoma cells treated with DMSO alone*

Concentration of TBC	Eumelanin content (%)	Cell number (%)
10^{-7} M	106.1 ± 7.2^a	102.0 ± 4.1^a
10^{-5} M	89.8 ± 11.5	111.4 ± 14.2
10^{-4} M	74.3 ± 6.3	103.6 ± 9.1
10^{-3} M	59.7 ± 8.9	65.2 ± 12.4

^{a} Mean $\pm$ SE was calculated from data of 3 separate experiments.

TABLE III. *Eumelanin content in B16 melanoma cells at different times after 10^{-4} M 4-tertiary butyl catechol (TBC) treatment*

Treatment	Hours after treatment		
	0^a	24	48
DMSO	0.46 ± 0.03	0.44 ± 0.05	0.50 ± 0.04
TBC in DMSO	0.43 ± 0.05	0.33 ± 0.01	0.37 ± 0.02
p value	NS^b	<0.01	<0.05

Values represent absorbance at 400 nm detected in 2×10^6 cells. Mean $\pm$ SE calculated from data of 3 separate experiments.

^{a} Cells were collected immediately after 2-h incubation in TBC-containing medium.

^{b} Not significant.

TABLE I. *Effects of 4-tertiary butyl catechol on specific activities of glutathione S-transferase, glutathione reductase, and γ-glutamyl transpeptidase in the ear skin of Uscd strain mice*

Enzymes	Specific activities (nmol/min/mg protein)^a		p value
	DMSO-acetone	1 M TBC in DMSO-acetone	
Glutathione S-transferase	$218 \pm 46 \ (6)^b$	$276 \pm 45 \ (10)$	<0.05
Glutathione reductase	$134 \pm 7 \ (10)$	$159 \pm 8 \ (10)$	<0.01
γ-Glutamyl transpeptidase	$35.0 \pm 4.0 \ (8)$	$47.4 \pm 10.2 \ (8)$	<0.01

^{a} Mean value $\pm$ SE.

^{b} Number of ear tissues used for the enzyme assay are shown in parentheses.
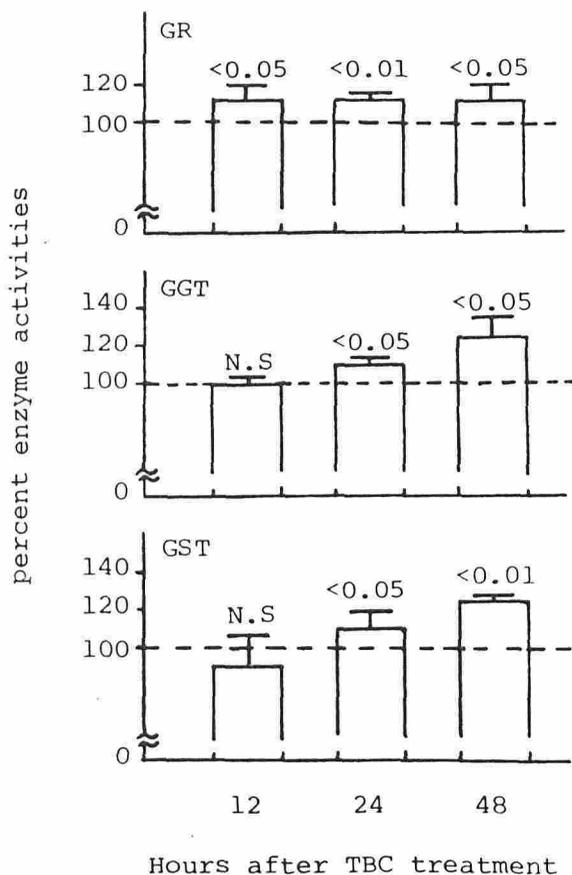
Hours after TBC treatment

FIG 2. Time course in changes of enzymatic activities—glutathione reductase (*GR*), γ-glutamyl transpeptidase (*GGT*), and glutathione S-transferase (*GST*)—in B16 melanoma cells after TBC treatment. The activities are expressed as percent activity of control cells treated with DMSO alone shown as *broken lines* at 100%. ▪ indicates mean and SE calculated from 4 separate experiments. Statistical difference is indicated by *p* value on the top of each bar. N.S. = not significant.

pas. As suggested by Prota, pheomelanogenesis may represent a scavenger mechanism [6]. It is even possible that pheomelanogenesis may be enhanced by the accumulation of eumelanin intermediates in cells by TBC. Cytotoxicity of eumelanin intermediates have been reported by Pawelek et al [29].

There are a number of chemical depigmenting agents, and some have been used as therapy for hyperpigmentation. The most representative chemical may be hydroquinone [30]. Despite their therapeutic efficacy, the role played by these chemicals remains unknown. Nevertheless, hydroquinone is also an antioxidant and not a tyrosinase inhibitor [31]. The present findings with TBC may provide an experimental model to investigate the molecular action of other more commonly used chemical depigmenting agents.

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