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16. Abstract (Limit: 200 words)

The effects of noncytotoxic doses of 4-tertiary-butyl-catechol (98293) (TBC) on glutathione-S-transferase (GST) activity in the skin of male, 2 month old Uscd-mice and B16-murine-melanoma cells in culture were investigated. These effects were studied in relation to changes in the activities of glutathione-reductase (GR) and gamma-glutamyl-transpeptidase (GGT) which are involved in pheomelanogenesis. Electron microscopic findings in the in-vivo studies demonstrated an altered form of melanosomes, classified as pheomelanosomes, in the TBC treated animals. Ear tissues demonstrated increased activities for all enzymes tested following TBC application. These increases were 27, 19, and 35 percent for GR, GST, and GGT, respectively. In-vitro studies demonstrated a dose dependent decrease in the eumelanin content. GR activity in TBC treated cells demonstrated a 12 percent elevation already at 12 hours. This continued to be higher than controls throughout the study period. No significant difference in GGT was noted at 12 hours, but it began to elevate at 24 hours, reaching a 26 percent increase after 48 hours. GST also did not increase during the first 12 hours, but showed a 25 percent increase by 48 hours after exposure. The authors suggest that these findings may aid in the construction of an experimental model to investigate the molecular action of other more commonly used chemical depigmenting agents.

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EFFECTS OF 4-TERTIARY BUTYL CATECHOL ON GLUTATHIONE-METABOLIZING ENZYMES IN VIVO AND IN VITRO

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Running head: TBC and glutathione-metabolizing enzymes

ABBREVIATIONS

TBC - 4-tertiary butyl catechol

GST - glutathione S-transferase

GGT - γ-glutamyl transpeptidase

GR - glutathione reductase

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Abstract

4-tertiary butyl catechol (TBC) causes depigmentation in man and animals and stimulates formation of pheomelanosomes. In this study, we investigated the effects of non-cytotoxic 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 wk. Melanoma cells cultured in 10^{-4} M TBC containing medium for 2 hr showed no changes in GST and GGT activities, but 12% increase of GR activity during the first 12 hr. Activities of all 3 enzymes was elevated (11-17%) 24 hr later. The elevation detected by 48 hr 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.

Introduction

4-Tertiary butyl catechol (TBC) is an antioxidant which causes depigmentation in man (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 et al. 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 (HFH1B) melanoma cells were kindly provided by Dr. F. Hu, University of Oregon. TBC, purchased from Aldrich Chemical Co.,

Milwaukee, Wis., was dissolved in a mixture of dimethyl sulfoxide (DMSO) obtained from Sigma Chemical Co., St. Louis, Mo., 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 Hank's salts (MEM), Basal Medium Eagle (BME), fetal bovine serum, penicillin and streptomycin were obtained from Grand Island Biological Co., Grand Island, N.Y. Isotonic diluent was purchased from Curtain Matheson Co., Brisbane, Ca. and Soluene-100, from Packard Instrument Co., Downers Grove, Ill. 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 hr intervals. The ears were removed 24 hr after the last application. Skin slices taken from the ears of 3 mice in both groups were prepared for electron microscopy. Photos magnified at x 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 cm^2 flask and grown in MEM supplemented with 10% fetal bovine serum, 50 u/ml

penicillin and 50 ug/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 to 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 hr 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 (17). Melanoma cells were detached from the flasks with 2 mM EDTA in phosphate buffered saline at 0, 24 and 48 hr after treatment; aliquots of 2 x 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, Fla.).

Enzyme assays: About 6 mm x 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, and 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 sec and repeated 3 times at 5 sec 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 hr 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 one flask, while GGT and GR were assayed in cells from two combined flasks.

1) 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 hr 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.

2) 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.

3) 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 method of Lowry et al. (22) using bovine serum albumin as the standard.

Results

1) In vivo studies

a. 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 site (Fig. 1).

b. Enzyme activities: All enzyme activities were elevated in ear tissues after TBC application as compared with those in DMSO-acetone treated ear tissues (Table 1). 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 nmoles/min/mg protein and GR: 131±5 nmoles/min/mg protein).

2) In vitro studies

a. Eumelanin content and cell counts: Table 2 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} M to 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 3. Treated cells contained less melanin than controls by both 24 and 48 hr. In some experiments we attempted to electron microscopically correlate 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.

b. Enzyme activities (Fig. 2): GR activity in TBC treated cells showed a 12±6% elevation already at 12 hr, and continued to be higher than DMSO-acetone treated cells throughout the experimental period (14±3% for 24 hr, and 14±6% for 48 hr). GGT showed no significant difference at 12 hr, but it began to elevate at 24 hr (11±6%) and reached a 26±14% increase after 48 hr. Similarly GST activity did not change during the first 12 hr, but it started to increase by 24 hr (17±11%) and showed a 25±9% elevation by 48 hr 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 wk. 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. support this possibility (4). They observed a statistically significant increase of non-melanized 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 lifespan and aging of experimental animals (24,25,26). 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. dopaquionone 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 cysteinyldopas. 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 Powelek 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.

REFERENCES

1. Gellin GA, Possick PA, Davis IH: Occupational depigmentation due to 4-tertiary butyl catechol (TBC). J Occup Med 12:386-389, 1970.

2. Horio T, Tanaka K, Komura J: Depigmentation due to para tertiary butyl catechol. Intl Arch Occup Environ Health 39:127-133, 1977.

3. Mansur J.D-S, Fukuyama K, Gellin GA, Epstein, WL: Effects of 4-tertiary butyl catechol on tissue cultured melanocytes. J Invest Dermatol 70:275-279, 1978.

4. Nishimura M, Gellin GA, Hoshino S, Epstein JH, Epstein WL, Fukuyama K: Ultrastructural demonstration of chemical modification of melanogenesis in hairless mouse skin. Anat Rec 202, 193-201, 1982.

5. Rorsman H, Agrup G, Falck B, Rosengren A-M, Rosengren E: Exposure to sunlight and urinary excretion of 5-S-cysteinyldopa. In, Pigment Cell, Vol. 2, Ed. by V Riley, S Karger, Basel, 1976, pp. 284-289.

6. Prota G: Recent advances in the chemistry of melanogenesis in mammals. J Invest Derm 75:122-127, 1980.

7. Mojamdar M, Ichihashi M, Mishima Y: Effect of DOPA-loading on glutathione-dependent 5-S-cysteinyldopa genesis in melanoma cells in vitro. J Invest Dermatol 78:224-226, 1982.

8. Agrup G, Falck B, Kennedy B-M, Rorsman H, Rosengren A-M, Rosengren E: Formation of cysteinyldopa from glutathionedopa in melanoma. Acta Dermatovener 55:1-3, 1975.

9. Hu F, Buxman, MM: Gamma-glutamyl transpeptidase in the pigment cells of rhesus eyes. J Invest Dermatol 76:371-375, 1981.

10. Hu F: Theophylline and melanocyte-stimulating hormone effects on gamma-glutamyl transpeptidase and DOPA reactions in cultured melanoma cells. J Invest Dermatol 79:57-61, 1982.

11. Benedetto J-P, Ortonne J-P, Voulot C, Khatchadourian C, Prota G, Thivolet J: Role of thiol compounds in mammalian melanin pigmentation: Part I. Reduced and oxidized glutathione. J Invest Derm 77:402-405, 1981.

12. Benedetto J-P, Ortonne J-P, Voulot C, Khatchadourian C, Prota G, Thivolet J: Role of thiol compounds in mammalian melanin pigmentation: Part II. Glutathione and related enzymatic activities. J Invest Dermatol 79:422-424, 1982.

13. Yonemoto K, Gellin GA, Epstein WL, Fukuyama K: Glutathione reductase activity in skin exposed to 4-tertiary butyl catechol. Intl Arch Occup Environ Health (in press).

14. Yonemoto K, Gellin GA, Epstein WL, Fukuyama K: Reduction in eumelanin by the activation of glutathione reductase and γ-glutamyl transpeptidase after exposure to a depigmenting chemical. Biochem Pharmacol (in press).

15. Benson AM, Batzinger RP, Ou SL, Bueding E, Cha YN, Talalay P: Elevation of hepatic glutathione S-transferase activities and protection against mutagenic metabolites of benzo(a)-pyrene by dietary antioxidants. Cancer Res 38:4486-4495, 1978.

16. Sparnins VL, Wattenberg LW: Enhancement of glutathione S-transferase activity of the mouse forestomach by inhibitors of benzo(a)pyrene-induced neoplasia of the forestomach. JNCI 66:769-771, 1981.

17. Oikawa A, Nakayasu M: Quantitative measurement of melanin as tyrosine equivalents and as weight of purified melanin. Yale J Biol Med 46:500-507, 1973.

18. Tate SS, Meister A: Interaction of γ-glutamyl transpeptidase with amino acids, dipeptides, and derivatives and analogs of glutathione. J Biol Chem 249:7593-7602, 1974.

19. Carlberg I, Mannervik B: Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 250:5475-5480, 1975.

20. Racker E: Glutathione reductase from baker's yeast and beef liver. J Biol Chem 217:855-865, 1955.

21. Habig WH, Pabst MJ, Jakoby WB: Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249: 7130-7139, 1974.

22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951.

23. Usami Y, Landau AB, Fukuyama K, Gellin GA: Inhibition of tyrosinase activity by 4-tertiary butyl catechol and other depigmenting agents. J Toxicol Environ Health 6:559-567, 1980.

24. Kohn RR: Effect of antioxidants on life-span of C57BL mice. J Gerontol 26:378-380, 1971.

25. Comfort A, Youhotsky-Gore I, Pathmanathan K: Effect of ethoxyquin on the longevity of C3H Mice. Nature 229:254-255, 1971.

26. Stohs SJ, Al-Turk WA, Angle C: Glutathione S-transferase and glutathione reductase activities in hepatic and extra-hepatic tissues of female mice as a function of age. Biochem Pharmacol 31: 2113-2116, 1981.

27. Rorsman H, Rosengren A-M, Rosengren E: Fluorometry of catechol thioethers. Acta Dermatovener (Stockholm) 52:353-356, 1972.

28. Jakoby WB, Ketley JN, Habig WH: 13. Rat glutathione S-transferases: binding and physical properties. In Glutathione: Metabolism and Function, ed. by IM Arias & WB Jakoby, Raven Press, New York, 1976.

29. Pawelek JM, Lerner AB: 5,6-Dihydroxyindole is a melanin precursor showing potent cytotoxicity. Nature 276:627-628, 1978.

30. Jimbow K, Obata H, Pathak M, Fitzpatrick T: Mechanism of depigmentation by hydroquinone. J Invest Dermatol 62:436-449, 1974.

31. Riley PA: Hydroxyanisole depigmentation: In-vitro studies. J Pathol 97:193-206, 1969.

Table 1. Effects of TBC on specific activities of glutathione S-transferase, glutathione reductase and γ-glutamyl transpeptidase in the ear skin of Uscd strain mice.

Enzymes	Specific activities (nmoles/min/mg protein)* DMSO-acetone	1 M TBC in DMSO-acetone	p value
glutathione S-transferase	218±46 (6)**	276±45 (10)	<0.05
glutathione reductase	134±7 (10)	159±8 (10)	<0.01
γ-glutamyl transpeptidase	35.0±4.0 (8)	47.4±10.2 (8)	<0.01

* Mean value ± standard error

( )** indicates number of ear tissues used for the enzyme assay.

Table 2. Effects of different concentrations of TBC on eumelanin content and cell counts of B16 melanoma cells. They are 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*	102.0±4.1*
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

* Mean ± standard error was calculated from data of 3 separate experiments.

Table 3. Eumelanin content in B16 melanoma cells at different times after 10^{-4} M TBC treatment.

Treatment	Hours after treatment		
	0*	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**	<0.01	<0.05

Values represent absorbance at 400 nm detected in 2 x 10^6 cells. Mean ± standard error was calculated from data of 3 separate experiments.

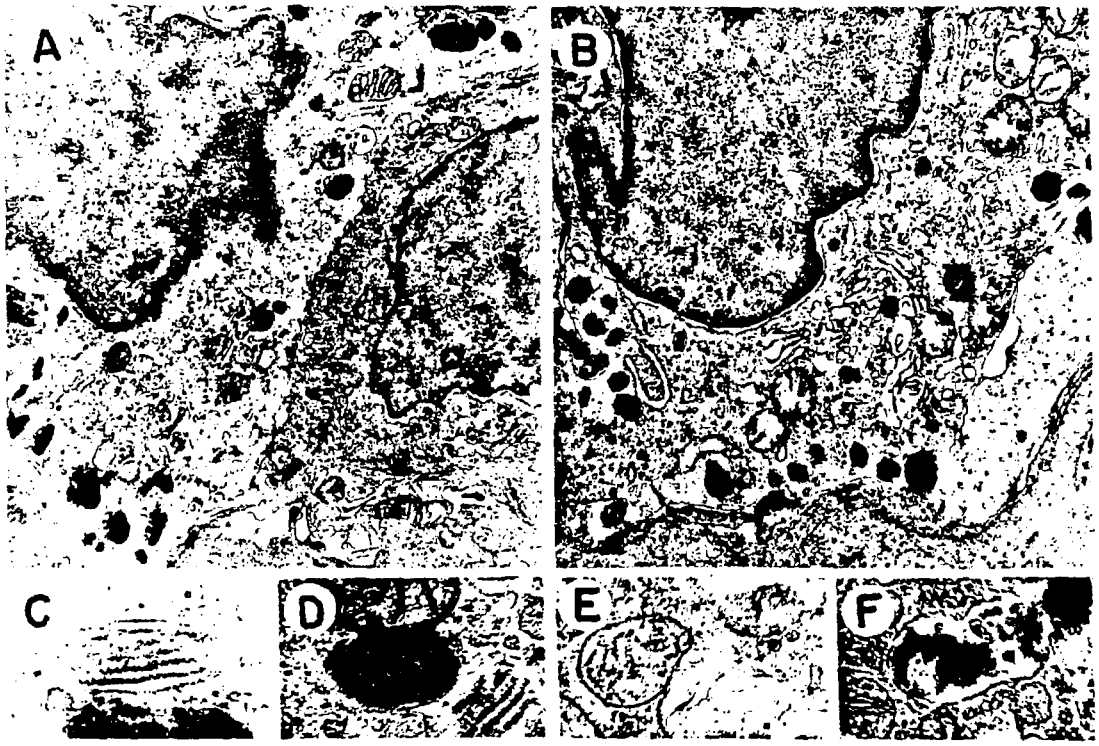
* Cells were collected immediately after 2 hr incubation in TBC containing medium.

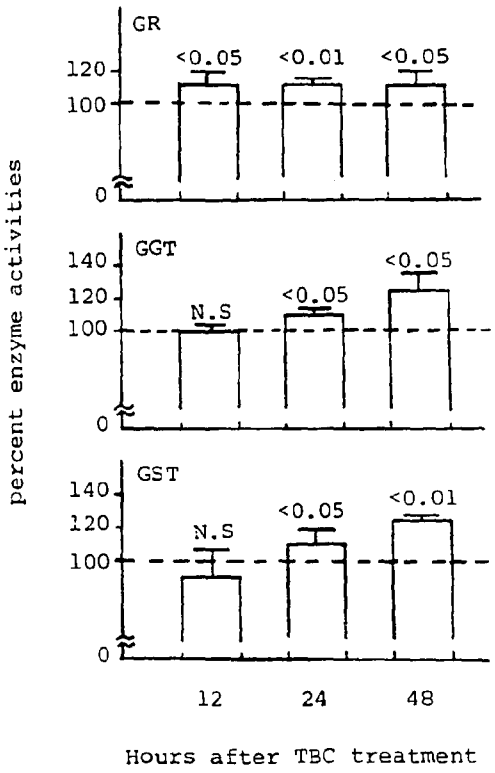
** not significant

LEGENDS

Figure 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. Magnification x 12,000. Inserts 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). Magnification x 50,000.

Figure 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 standard error calculated from 4 separate experiments. Statistical difference is indicated by p value on the top of each bar. N.S., not significant.

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percent enzyme activities
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