Biochemical Pharmacology, Vol. 34, No. 19, pp. 3517–3521, 1985
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

STIMULATION OF PHEOMELANOGENESIS IN CULTURED B16 MELANOMA CELLS BY 4-*TERTIARY* BUTYLCATECHOL

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(*Received* 20 *November* 1984; *accepted* 18 *February* 1985)

Abstract—Intermediates of pheomelanin in tissue cultured B16 melanoma cells were analyzed by high performance liquid chromatography, and reduced glutathione (GSH), L-dopa, 2-[(L)-*S*-cysteinyl]-L-dopa (2-SCD) and 5-[(L)-*S*-cysteinyl]-L-dopa (5-SCD) were quantified. The effects of 4-*tertiary* butylcatechol (TBC), an antioxidant which causes skin depigmentation, on the levels of the intermediate were then examined. A concentration of 10^{-4} M TBC increased the intracellular levels of GSH, 2-SCD and 5-SCD, whereas the L-dopa level was unchanged. The time-course of the increased intermediates corresponded to the elevation of glutathione-metabolizing enzyme activities previously reported by Kawashima *et al.* [*J. invest. Derm.* **82**, 53 (1984)] in the same cell line exposed to 10^{-4} M TBC. The findings establish chemical evidence that TBC stimulates pheomelanogenesis in melanocytes.

Melanocytes in mammalian epidermis are capable of synthesizing two polymeric pigments, eumelanin and pheomelanin, which are derived from the catechol amino acid L-dopa and which differ, in part, by the incorporation of glutathionyl moieties into pheomelanin but not eumelanin. It has been reported that the type of melanin which is formed is under genetical control [1]. However, stimulation of the production of pheomelanin and its precursors like 5-[(L)-*S*-cysteinyl]-L-dopa (5-SCD) has been found to occur as a phenotypic change not only during malignant transformation [2–5] but also in eumelanin-producing melanocytes that have been exposed to ultraviolet radiation [6–8]. Recently, an increased number of pheomelanosomes was reported to be present in melanocytes isolated from guinea pigs [9] and mice [10] that had been treated with 4-*tertiary* butylcatechol (TBC), an antioxidant which causes loss of skin color [11, 12]. These results suggested that TBC also may chemically stimulate the production of pheomelanin.

Prota [13] has proposed that glutathione (GSH)-metabolizing enzymes are involved in pheomelanogenesis. Therefore, as part of our evaluation of the effects of TBC on pheomelanogenesis, we [14, 15] measured the activities of GSH-metabolizing enzymes in cultured melanoma cells treated with TBC. It was found that the activities of *gamma*-glutamyltranspeptidase (GGT), glutathione reductase (GR) and glutathione *S*-transferase (GST) are elevated in TBC-treated cells in which the eumelanin content is decreased. In the present study, we have extended our observations on the effect of TBC on the phenotypic expression of melanogenesis by estimating the levels of the pheomelanin precursors 5-SCD, 2-[(L)-*S*-cysteinyl]-L-dopa (2-SCD), GSH and L-dopa in tissue-cultured B16 melanoma cells by high performance liquid chromatography (HPLC) and electrochemical detection.

MATERIALS AND METHODS

Cell cultures, treatment and sample preparation. A relatively pigmented cell line was established from murine B16 melanoma cells (HFH-18) originally provided by Dr. F. Hu, University of Oregon, U.S.A. The cells were dendritic, and their doubling time was around 2.6 days. About 2×10^6 cells were seeded in a 75-ml flask and cultured in Eagle's minimum essential medium (25 ml) with Hanks' salts (MEM) supplemented with 10% fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin, under a humidified atmosphere of 5% CO_2 and 95% air at 37°. TBC (Aldrich Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO) at 10^{-1} M, and the resulting solution was diluted with Eagle's basal medium (BME) to make a final concentration of 10^{-4} M TBC. Previous studies [15] had shown this concentration to be optimal for increasing the levels of the GSH-metabolizing enzymes and reducing the eumelanin content of these cells. The media, serum and antibiotics for cell culture were purchased from Gibco. The medium was changed every 48 hr and at day 7 of culture, 24 hr after the last medium change, the MEM used for cell growth was decanted and replaced with 25 ml of the BME containing 10^{-4} M TBC and 0.1% DMSO alone (control). Following an additional incubation period of 2 hr, the TBC–DMSO or DMSO culture medium was replaced with 25 ml of the original MEM medium. Incubations were continued for various time periods (0, 6, 12, 24 and 48 hr) following which the cells were isolated and then suspended in 10 ml of cold 0.1 M phosphate-buffered saline (PBS, pH 7.2) containing 2 mM EDTA. An additional set of control cells that had not been treated with TBC or DMSO also was pre-

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pared for HPLC analysis. The experiments were repeated three times in duplicate. The resulting cell suspensions were centrifuged at 1000 rpm for 10 min, and the cell pellets (about 2×10^7 cells) in 1 ml of 0.4 N HCl containing 0.1 mM EDTA and 400 ng of *alpha*-methyldopa (Merck Sharp & Dohme) used as internal standard (IS) were sonicated with three 15-sec bursts of Fisher's sonic dismembrator at 40% maximal power. The homogenates were centrifuged at 37,000 rpm for 30 min, and the supernatant fractions were subjected to HPLC analysis. The extraction procedure described above was performed at 40°. Three additional incubations (each run in duplicate) were carried out using 10^{-4}, 10^{-5} or 10^{-6} M TBC–DMSO in order to study the dose-dependent effects of TBC. Only the 24-hr time point was obtained in these studies. At a concentration higher than 10^{-4} M, TBC was cytotoxic, and the post-treatment culture of treated cells was not possible. Finally, the amount of excreted 5-SCD during 24 hr post-treatment by cells exposed to 10^{-4} M TBC–DMSO and to DMSO alone was also measured. An aliquot of medium (4.5 ml) was mixed with 0.5 ml of 4 N HCl containing 400 ng *alpha*-methyldopa. After centrifugation of the solution at 3000 rpm for 15 min, the supernatant fraction was analyzed by HPLC.

HPLC system. The HPLC assay for the pheomelanin precursors was modified from the method described by Hansson *et al.* [16, 17]. A Beckman model 110A solvent pump and a Rheodyne model 7125 syringe loading sample injector equipped with a 200 μl loop were used. A precolumn packed with LiChrosorb RP-2 (30 μm, 50 × 4.6 mm, E. Merck) was placed in the line to protect the C18 LiChrosorb analytical column (10 μm, 250 × 4.6 mm, Alltech). Detection of the separated precursors was done by an LC-3A electrochemical detector (Bioanalytical System) operated at +750 mV vs a Ag/AgCl reference electrode and a glassy carbon as working electrode. A mixture of 62.5 mM methanesulfonic acid (Eastman Kodak Co.), 30.6 mM phosphoric acid (Fisher Scientific Co.) and 0.1 mM Na_2EDTA in Millipore-filtered water, adjusted to pH 2.9 with 5 N NaOH, served as the mobile phase. The chromatogram was run at ambient temperature with a flow rate of 1.5 ml/min.

The two isomeric cysteinyldopas, L-dopa and GSH in the samples were identified not only by the retention times of peaks in comparison with those of the reference compounds, but also by their affinity to phenyl boronate gel (PBA)-60 by the method described by Kagedahl and Pettersson [18]. Standard 5-SCD, 2-SCD, 6-[(L)-*S*-cysteinyl]-L-dopa (6-SCD) and 2,5-[(L,L)-*S*-*S*-dicysteinyl]-L-dopa (2,5-S,SCD) were synthesized in our laboratory following the methods described by Agrup *et al.* [19] and Ito and Prota [20]. A reference standard of 5-SCD, provided by Dr. H. Rorsman, University of Lund, Sweden, proved to be identical to our product. The references of GSH and L-dopa were purchased from the Sigma Chemical Co. The amounts of precursors in the samples were calculated by comparing their peak areas with that of *alpha*-methyldopa in each run. Mean values and standard deviations were calculated and statistically examined by Student's *t*-test. P values lower than 0.05 were considered as significant.

RESULTS

Identification of pheomelanin precursors in B16 melanoma cells and cultured medium. Typical chromatograms of controls and 10^{-4} M TBC–DMSO-treated cell extracts are shown in Fig. 1, panels A and B. Eleven peaks were observed. The retention times of peaks 2, 3, 5 and 7 corresponded with the reference compound 2-SCD, GSH, L-dopa and 5-SCD respectively. The peak corresponding to *alpha*-methyldopa appeared between peaks 7 and 8. Neither 6-SCD nor 2,5-S,SCD was detected in any samples under the conditions used. The identity of the compounds giving rise to the remaining peaks remains unknown. Chromatography of a cell extract (pH adjusted to 8.0 with 1 N NaOH) on a PBA-60 column followed by elution with 0.1 N HCl (6 ml) provided material which, upon chromatography on the C18 LiChrosorb column, gave the tracing shown in Fig. 1C. Recovery of peaks 2, 5 and 7 suggested that these peaks contain compounds with a *cis*-diol structure, supporting the chemical analysis of the catechol intermediates. As expected, peak 3 (GSH) was not present, presumably because it was not retained on the PBA-60 column. Of the unknown peaks, only No. 11 displayed chromatographic properties expected of catechol containing compounds, while peaks 1, 4, 6, 8, 9 and 10 did not bind to the PBA-60 column. Since all of the unknown compounds are electrochemically active, however, they may be related to GSH or catechol-type structures and hence are worthy of further investigation.

The amounts of GSH and L-dopa in the cells immediately after DMSO treatment varied somewhat from 10 to 60 $\mu g/2 \times 10^7$ cells and 766 to 3860 $ng/2 \times 10^7$ cells respectively. The corresponding range of values for 2-SCD and 5-SCD was narrower (60–90 ng 2-SCD and 350–530 $ng/2 \times 10^7$ cells). The 5-SCD level in the cells was always lower than that which appeared in the medium. The values of 5-SCD in a typical experiment in which we measured 5-SCD in both the cells and medium at 24 hr after DMSO or TBC–DMSO treatment were 470 ± 20 $ng/2 \times 10^7$ cells vs $72,400 \pm 2,300$ ng/25 ml medium (DMSO treatment) and 2180 ± 200 $ng/2 \times 10^7$ cells vs $211,000 \pm 12,000$ ng/25 ml medium (TBC–DMSO treatment).

Dose-dependent effects of TBC on GSH and 5-SCD in B16 melanoma cells. As summarized in Table 1, the amounts of both GSH and 5-SCD in 10^{-5} and 10^{-6} M TBC–DMSO-treated cells remained at the control levels after 24 hr. However, a 3-fold elevation of GSH and 5-SCD was observed in 10^{-4} M TBC–DMSO-treated cells.

Time-course studies on pheomelanin precursors in B16 melanoma cells (Fig. 2). The amount of GSH increased in the cells during a 2-hr exposure to DMSO and continued to rise after changing the medium to MEM for the first 8 hr but returned to normal levels thereafter. TBC–DMSO treatment caused a significant reduction of the GSH levels in the cells during the 2-hr exposure time. However, the cells recovered from the inhibition during the 48-hr post-treatment period when elevated levels of GSH were observed. L-Dopa levels showed a pattern similar to that observed with the GSH levels and

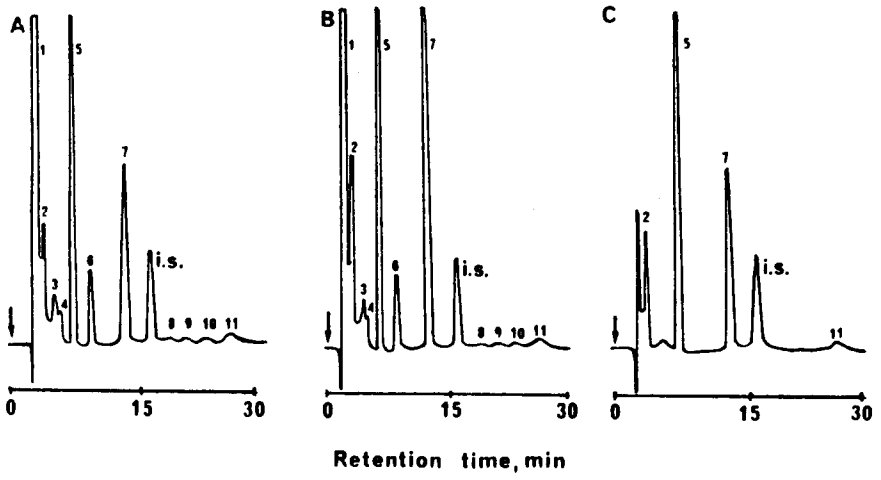

Fig. 1. HPLC separation of acid-soluble compounds extracted from cultured B16 melanoma cells at 14 hr after DMSO (A,C) or 10^{-4} M TBC–DMSO (B) treatment. The peaks seen from the DMSO-treated sample were compared before (A) and after (C) application to a PBA-60 column (2.5 × 1.0 cm). The affinity column was pre-equilibrated with 0.1 M phosphate buffer, pH 8.0. After thorough washing, catecholic amino acids were eluted with 0.1 M HCl containing 0.1 mM EDTA. I.S. is the peak of *alpha*-methyldopa used as an internal standard.

DMSO treatment and, unlike GSH, TBC–DMSO treatment did not affect the time–course. In DMSO-treated cells, the 5-SCD value remained almost constant while the 2-SCD level decreased gradually during the first 14 hr after changing the medium. In contrast, the levels of both 5-SCD and 2-SCD increased more than 2-fold in TBC–DMSO-treated cells. Additionally, the elevation of these precursors was retained in the cells throughout the experimental period.

DISCUSSION

Detection and quantitative estimation of pheomelanin precursors in tissue cultured B16 melanoma cells were possible using a reverse phase HPLC system employing an electrochemical detector. The assay method used was similar to that described by Hansson *et al.* [16, 17], except that the initial concentration of the catechols by chromatography on alumina [21] was omitted. This additional chromatographic step was found to be unnecessary for the detection of pheomelanin precursors. Furthermore, the recovery of 5-SCD from a sample treated with alumina was poor as was the experience of Morishima *et al.* [5] and GSH was totally lost after the purification process. On the other hand, the recovery of catechols, from the PBA-60 column described by Kagedahl and Pettersson [22] for purification of 5-SCD from urine, was essentially quantitative.

Table 1. Dose-dependent changes after 24 hr of GSH and 5-SCD in TBC–DMSO-treated B16 melanoma cells (per 2×10^7 cells)

TBC concn in BME (M)	GSH* (μg)	5-SCD* (ng)
0	28 ± 1	565 ± 15
10^{-6}	30 ± 12	583 ± 141
10^{-5}	29 ± 5	600 ± 12
10^{-4}	86 ± 4	1500 ± 9

* Mean ± S.D. was calculated from the data of three separate experiments.

A second analytical modification involved the use of *alpha*-methyldopa, instead of 5-[(D)-*S*-cysteinyl]-L-dopa (5-D-SCD) employed by Agrup *et al.* [19], as our internal standard. This was because the retention time of *alpha*-methyldopa did not correspond to any of the substances detected in the cell extracts whereas the retention time of 5-D-SCD coincided with that of unknown compound No. 6.

The range of concentrations of GSH and L-dopa in DMSO-treated cells was quite large among the different experiments as reported by Aubert *et al.* [23] for 5-SCD in cultured melanoma cells. On the other hand, 2-SCD and 5-SCD values in the DMSO-treated cells were rather constant in all experiments. At 24 hr after treatment with 10^{-4} M TBC, 5-SCD not only increased in the cells, but also increased in the medium most probably as a result of extracellular overflow of excess 5-SCD produced as suggested by Mojamdar *et al.* [24]. Since the increased rates of GSH and 5-SCD were observed with TBC at a concentration of 10^{-4} M but not with TBC at lower concentrations, the TBC effect was considered to correlate with other intracellular functional changes which occur with a similar concentration of TBC such as activation of glutathione-metabolizing enzymes [14, 15].

The time–course studies revealed that both GSH and L-dopa increased in DMSO-treated cells while TBC–DMSO significantly inhibited GSH, causing decreased levels during the 2-hr treatment. Depletion of GSH occurs in isolated hepatocytes treated with cytotoxic chemicals, i.e. *N*-acetyl-*p*-kenzoquinone amine [25] or 1-naphthol [26] and GSH most probably works as a scavenger for orthoquinones and free radicals in the cells [13]. TBC may be converted to a cytotoxic orthoquinone [27] and thus

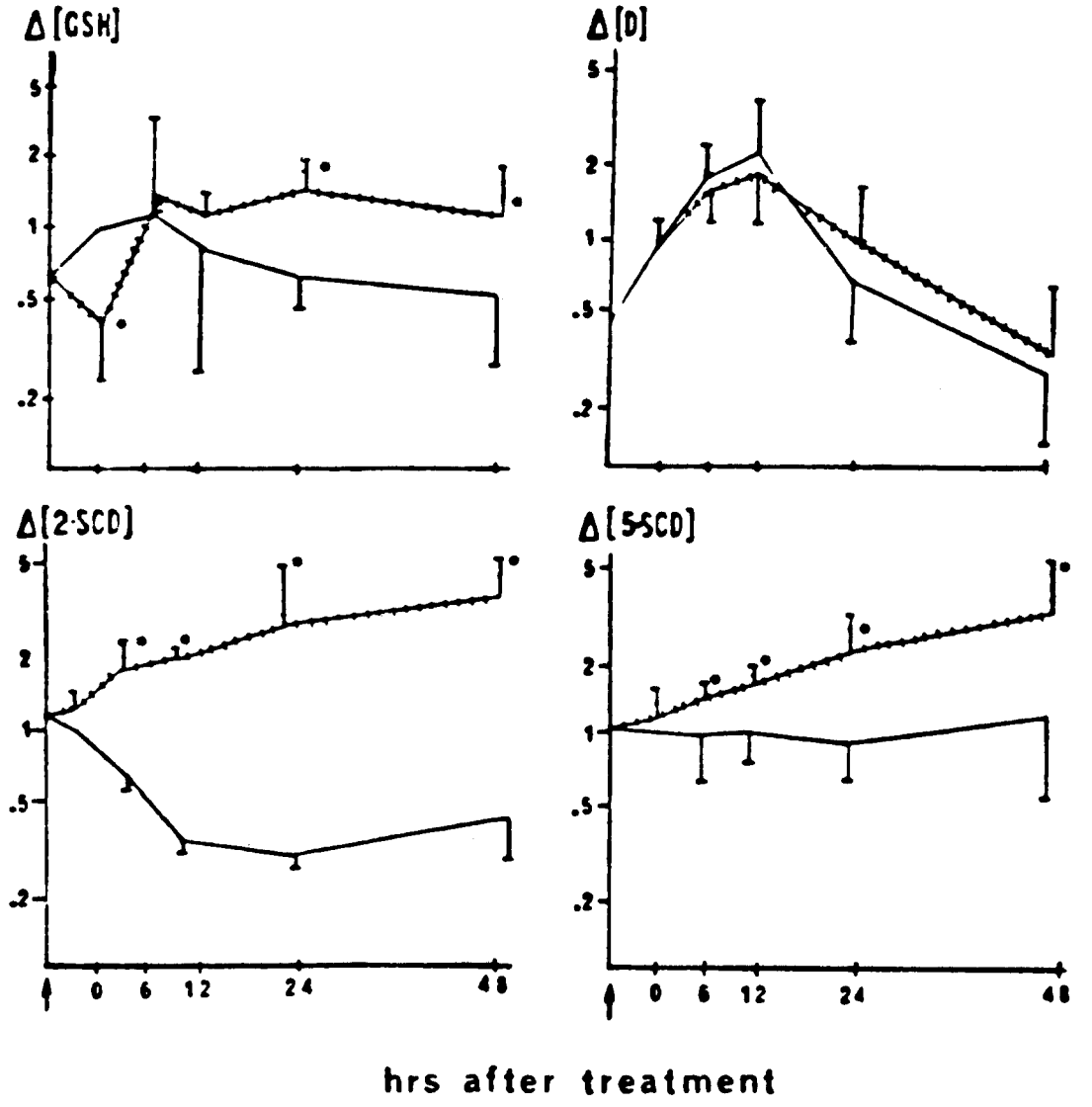

Fig. 2. Time-course of the levels of GSH, L-dopa (D), 2-SCD and 5-SCD in cultured B16 melanoma cells during and after treatment of DMSO (——) or 10^{-4} M TBC-DMSO (++++). The arrows indicate the beginning of the 2-hr treatment and 0 hr after treatment corresponds to the period when the treatment was ended and the culture medium changed. Values were calculated from the amount of each compound in DMSO-treated cells measured at 0 hr after treatment taken as 1. Bars are standard deviations of each mean value for DMSO-treated cells and 10^{-4} M TBC-DMSO-treated cells. An asterisk (*) indicates a P value of less than 0.05.

cause a reduction of GSH in melanoma cells. Recovery in the GSH level was noted by 6 hr after TBC–DMSO treatment. Since GR elevation was already detectable at a similar interval after treatment [15], we consider that GSH was supplied by enzyme activation. TBC did not show effects on L-dopa levels during the observation period, confirming the findings by Usami *et al.* [28] that 10^{-4} M TBC has no influence on tyrosinase activity. Increase in the 2-SCD and 5-SCD levels was gradual but continued for 48 hr and correlated with the changes of GGT and GST activities [14, 15]. The findings strongly support the proposal by Prota [13] that it is the biochemical pathway which is involved in pheomelanin formation, and our hypothesis that TBD is a chemical stimulator of pheomelanogenesis.

Acknowledgements—This study was supported by Grant 0H00714 from the National Institute for Occupational Safety and Health and Grant AM07175 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

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