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Transcriptional activation of tyrosinase gene by human placental sphingolipid

Bidisha Saha · Suman Kumar Singh · Chinmoy Sarkar · Shampa Mallick · Rabindranath Bera · Ranjan Bhadra

Abstract The sphingolipids, a class of complex bioactive lipids, are involved in diverse cellular functions such as proliferation, differentiation, and apoptosis as well as growth inhibition. Recently sphingosylphosphorylcholine (SPC), sphingosine-1-phosphate (S1P), and C2-ceramide (C2-Cer), sphingolipid containing acetic acid are emerging as melanogenic regulators. A bioactive sphingolipid (PSL) was isolated from hydroalcoholic extract of fresh term human placenta and it induced melanogenesis in an *in vitro* culture of mouse melanoma B16F10 cells. Tyrosinase, the rate-limiting enzyme for melanogenesis, is required to be upregulated for the increased melanin production. The expression of tyrosinase, both at protein as well as mRNA level, was higher in the PSL treated B16F10 cells as evidenced by Western blot and RT-PCR analysis. Actinomycin D and cycloheximide, inhibitors of transcription and translation, respectively, inhibited PSL-induced tyrosinase activity and its protein expression showing decrease in melanogenesis, correspondingly. The activity of GFP coupled tyrosinase promoter was upregulated in transfected B16F10 cells after treating with PSL as determined by fluorescence microscopy, fluorometric analysis, and Western blot. These results, thus, suggested that PSL upregulated tyrosinase gene expression at transcription level through promoter activation to show increased melanogenesis. Therefore, PSL as an inducer of melanogenesis might account for the recovery of pigment in depigmentation disorder.

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Introduction

The sphingolipids are emerging as regulators of cellular functions ranging from proliferation, differentiation, and apoptosis to growth inhibition [1,2]. A recent review [3] illustrated such diverse functions of sphingolipids [4,5]. However, the role of sphingolipid on melanin synthesis has remained nearly unexplored. Melanin synthesis (melanogenesis) occurs through an enzyme cascade that converts tyrosine to melanin pigments, involving tyrosinase [6,7] and tyrosinase-related proteins TRP1 [8] and TRP2 [9]. Tyrosinase, the rate-limiting enzyme in the melanogenesis, catalyses hydroxylation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA) and oxidation of L-DOPA to dopaquinone [10]. Thus, the increase in melanin production demands up-regulation of tyrosinase [11], and its expression, being tissue-specific [12], is restricted to melanocytes. Tyrosinase promoter, with a highly conserved 10-bp motif (GTCATGTGCT) termed as M-box occurring in the mouse and human, plays a key role in the tissue-specific expression of tyrosinase [12,13].

We have reported that a prototype hydroalcoholic human placental extract [14–16] induced melanogenesis significantly in age-onset black mouse, having graying of hair and vehicle free extract in B16F10 mouse melanoma [17]. The chemical analysis of this extract showed the presence of small protein-peptides, nucleotides, carbohydrates, and a number of lipid constituents including cholesterol, triglycerides, lecithin, lysolecithin, phosphatidyl ethanolamine, phosphatidyl inositol, ganglioside (GD1b), β-carotene, lycopine, and at least four sphingolipids [18]. PTLF, the total lipids obtained from the vehicle-free extract, induced

melanogenesis in B16F10 [19] with corresponding upregulation of the key enzyme, tyrosinase [20,21].

In recent years, sphingosylphosphorylcholine (SPC), C2-ceramide (C2-Cer), and sphingosine-1-phosphate (S1P) have been reported as melanogenic regulators [22–24]. We have isolated and purified a placental sphingolipid (PSL) from placental total-lipid fraction and this study was undertaken to show that PSL induced melanogenesis by enhancing tyrosinase activity through higher gene expression at the promoter activity level in B16F10. SPC, C2-Cer, and S1P, the regulators of melanogenesis [22–24] and a well-established melanogenic hormone, α-melanocyte stimulating hormone (α-MSH) [25,26], were included in this study as controls.

Materials and methods

Cell culture

B16F10, a mouse melanoma procured from National Center for Cell Science, Pune, India, was used throughout this study. Cells were grown in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS and 1% penicillin, streptomycin, and neomycin antibiotic (Gibco BRL) at 37°C in a humidified incubator with 5% CO_2. For induction studies, cells were grown to semi-confluence and harvested with 0.025% trypsin and 0.52 mM ethylenediaminetetra-acetic acid in PBS. Then 1×10^6 cells/well were plated in six well plates and cultured in DMEM for 24 h and the spent medium was then removed with further continuation of culture in DMEM having 2% heat inactivated FBS and 1% penicillin, streptomycin, and neomycin antibiotic, with or without supplementation of the test substances for a stipulated time period.

Preparation of the placental sphingolipid (PSL)

The preparation of PTLF from a vehicle-free prototype human-placental extract [14] was reported previously [19–21] and was used to get PSL through a series of chromatography (silicic-acid, sephadex LH20) and finally by preparative HPTLC. Melanogenic activity of fractions was screened by using B16F10 cell culture system during the purification steps.

Treatment of cells with PSL and other sphingolipids

Based on the usual procedure, PSL and other sphingolipids were dissolved in chloroform/methanol (2:1 *v/v*) and aliquoted for taking appropriate amounts in sterile experimental vials. Organic solvents were removed completely and then DMEM containing 2% heat inactivated FBS was added to it. With mandatory ultrasonication, a uniform dispersion was achieved suitable for sphingolipid uptake by cells in culture. These were then used to study the effect of PSL and other sphingolipids by adding to the 24 h cultured cell removing the spent medium as stated already [20,21]. The viability of these cells was determined by the MTT [3-(4, 5-dimethyl-thiazol-2-yl)]-2, 5-diphenyltetrazolium bromide assay and trypan blue dye exclusion method [19].

Measurement of melanin content

After 24 h of treatment with test substances in DMEM containing 2% heat-inactivated FBS, the cells were made free of culture medium, detached by short incubation in trypsin/ethylenediaminetetra-acetic acid, and washed in PBS to use for experimentation. Cells, after taking a small aliquot for counting, were sonicated in 200 μl 1N NaOH and kept over night to measure melanin content. Melanin of unknown samples was estimated at 405 nm using synthetic melanin (0–200 μg/ml, Sigma Chemical Co.), as a standard [21].

Tyrosinase activity

Tyrosinase activity of the cell extract was determined spectrophotomatrically and staining DOPA in gels, using L-DOPA as the substrate as described previously [20]. The protein content of the extract supernatant was estimated by the method of Lowry using BSA as the standard [27]. For spectrophotometric assay, the supernatant protein (20 μg) were incubated in duplicate for 1 h at 37°C in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% L-DOPA. The absorbance was then monitored at 450 nm in a Shimadzu Spectrophotometer (Model: UV-2401 PC, UV-VIS; Shimadzu Corporation, Kyoto, Japan) and compared with purified mushroom tyrosinase (Sigma Chemical Co.) as standard. For DOPA staining in gels, about 75 μg of supernatant protein from each extract was resolved by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). The gels were incubated in PBS having 2 mM L-DOPA and 4 mM 3-methyl-2-Benzothiazolinone hydrazone (MBTH). Dopa oxidase activity of tyrosinase was visualized in gels as bright dark bands containing dopa-melanin after interaction with enhancer MBTH. The band intensity was quantitated by scanning densitometer (Model: Image Scanner, Amarsham Pharmacia Biotech, Buckinghamsire, UK) and the Software, Image Master Total lab version 1.11.

Transfection and fluorescence spectrophotometric analysis

The expression vector pTyrP.TYRwt-GFP, a plasmid encoding wild type tyrosinase tagged with enhanced green fluorescent protein (EGFP) and having a 2,500 bp Tyrosinase promoter in place of cytomegalovirus (CMV) promoter of

the pEGFP plasmid, was a generous gift from Dr. Ruth Halaban, Yale University, School of Medicine, New Haven, CT, USA. The neomycin gene conferring resistance to geneticin (G418) in the vector was exploited to select the transfectants. B16F10 and 3T3-Swiss fibroblasts cells were stably transfected with pTyrP.TYRwt-GFP plasmid, using Lipofectamine 2000 reagent (Invitrogen life technologies, Carisbad, California, USA) as described earlier [21]. Then the 1×10^6 stable transfected cells per well were cultured for 24 h in 6-well plates with or without 10 μg/ml PSL or 10 nM α-MSH. For DAPI staining, cells were fixed using 2% paraformaldehyde and then stained with 1 μg/ml DAPI for 10 min at room temperature. The effects of PSL-induced transcriptional activation were analyzed by an inverted fluorescence microscope (Model: OLYMPUS 1×70, Olympus Optical Co. Ltd., Shibuya-ku, Tokyo, Japan).

For fluorescence spectrophotometric analysis, 1×10^6 transfected cell pellets, obtained after PSL and α-MSH treatment, were suspended in 1 ml PBS and its fluorescence was analyzed in a spectrofluorimeter (Hitachi F4500, Japan) at 25°C with excitation at 484 nm and emission at 510 nm.

Western blot analysis

To determine the amount of proteins expressed, Western blot analysis was performed by the techniques described earlier [21]. Briefly, 25 μg of total protein from each cell extract supernatant was electrophoresed and transferred onto PVDF membranes (Immobilon-P, Millipore Corporation, Bedford, MA, USA) using a Transblot system (Transblot SD: Semidry transfer cell, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blots were then detected using primary antibodies, rabbit anti-tyrosinase (H-109) (1:100 dilution) (Santa Cruz Biotechnology, USA) and rabbit anti-GFP (1:2500 dilution) (Invitrogen life technologies, Carisbad, California, USA) followed with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1: 1000 dilution)(Sigma Chemical Co., St. Louis, MO, USA) and signals were visualized with chemiluminescent HRP detection reagents (Cell Signaling technology, Inc. Beverly, MA, USA). In order to confirm the results, each experiment was repeated more than three times. Loading control experiments were also carried out by Western blotting using the antibodies to β-actin. Membranes were processed for densitometric analysis using an imaging densitometer (Model: Image Scanner, Amarsham Pharmacia Biotech, Buckinghamsire, UK) and a software (Image Master Total lab version 1.11).

Reverse transcription-polymerase chain reaction (RT-PCR)

To determine the amount of m-RNA expressed, RT-PCR analysis was performed by the techniques described earlier [21]. Briefly, total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription and polymerase chain reaction were carried out with 1 μg isolated total RNA sequentially in the same tube using Qiagen one step RT-PCR kit (Qiagen). The oligonucleotide primers used for PCR are as follows: tyrosinase upstream 5′-GGC CAG CTT TCA GGC AGA GGT-3′; downstream 5′-TGG TGC TTC ATG GGC AAA ATC-3′; actin upstream 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′; downstream 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. The reaction was cycled 25× through 60 s at 94°C, 60 s at 56°C, and 60 s at 72°C. Fifty percent of reaction mixture was analyzed by electrophoresis on 1% agarose gels and stained by ethidium bromide. In order to check the reproducibility of the results, each experiment was carried out more than three times.

Statistics

Results were analysed by Student's *t*-test to determine the level of significance.

Results

Effect of PSL on melanogenesis and tyrosinase activity

To study the effect of PSL on B16F10 melanogenesis, PSL treatment at various concentrations (0.01-20 μg/ml) for 24 and 48 hrs was carried out and a significant up-regulation of melanin synthesis was observed in a dose-dependent manner at concentrations of 1–10 μg/ml. Identically, PSL treatment induced tyrosinase, the rate-limiting enzyme of melanin synthesis, remarkably, correlating with increase in melanin synthesis compared to untreated cells. Melanin synthesis was induced to 1.73-(5.46 versus 3.14 pg melanin/cell) ($P < 0.01$), 3.09-(9.71 versus 3.14 pg melanin/cell) ($P < 0.001$), and 4.77-fold (14.99 versus 3.14 pg melanin/cell) ($P < 0.001$) for 24 h treatment with the PSL at a dose of 1, 5, and, 10 μg/ml, respectively (Fig. 1A). Extending the period of treatment from 24 to 48 h and increasing the dose of PSL above 10 μg/ml, no further enhancement of this melanogenesis was observed; hence this was considered as an optimum dose with 24 h of treatment as the period of stimulation. The enzyme induction was 92.5 ($P < 0.001$) and 79.6 units/mg protein ($P < 0.001$) at 10 and 20 μg/ml of PSL doses, respectively, compared to unstimulated cells as 19.58 units/mg protein for 24 h of treatment (Fig. 1B). Extending the period of treatment to 48 h, the enzyme induction was 52.1 and 45.5 units/mg protein at similar doses compared to unstimulated cells as 12.69 units/mg protein.

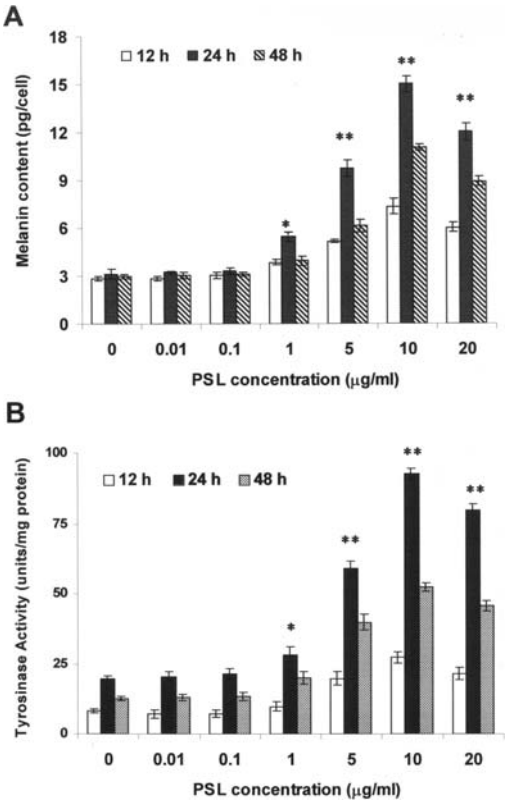

Fig. 1 Effect of PSL on melanogenesis and the tyrosinase activity in B16F10 cells. Dose and time kinetics of melanogenesis (A) and tyrosinase activity (B). Tyrosinase activity in B16F10 cells estimated by measuring Dopa oxidase activities of tyrosinase as described in the 'Materials and Methods.' Results are the means ± SE from triplicate determinations from three separate experiments. $^{*}P < 0.01$; $^{**}P < 0.001$

PSL induced melanogenesis by increasing tyrosinase expression at protein and mRNA levels

Tyrosinase is regulatory on the mammalian melanogenesis, and the effect of PSL on its expression at the protein and mRNA level in the mouse melanoma B16F10 were assessed by Western blot and RT-PCR analysis, respectively. The results shown in Fig. 2 revealed that treatment with 10 μg/ml PSL for 24 h, tyrosinase was induced by 8.31 ($P < 0.001$) and 5.96-fold ($P < 0.001$) at protein and mRNA level, respectively, taking untreated as one fold in each case. When compared with physiological regulators of melanin synthesis, α-MSH, SPC, and PTLF were found to induce tyrosinase expression by 5.47-, 2.57-, 6.35-fold and 3.81-, 2.7-, and 4.74-fold at protein and mRNA level, respectively, while C2-Cer reduced the tyrosinase expression significantly to 0.58- and 0.76-fold at protein and mRNA level, respectively (Fig. 2). Consistent to tyrosinase expression at protein and mRNA level, S1P and C2-Cer reduced the melanin synthesis by decreasing melanin to 2.17 ($P < 0.01$) and 1.84 pg melanin/cell ($P < 0.01$), respectively, while PTLF, PSL, SPC, and α-MSH, acted as melanogenic stimulators by increasing the melanin synthesis to 11.42 ($P < 0.001$), 14.99 ($P < 0.001$), 7.71 ($P < 0.001$), and 9.50 pg melanin/cell ($P < 0.001$), respectively, in B16F10 cells compared to untreated cells as 3.14 pg melanin/cell (Fig. 3).

Effect of actinomycin D and cycloheximide on PSL-induced melanogenesis, tyrosinase activity and its expression

PSL-mediated stimulation of melanogenesis in B16F10 cell was examined in the presence of actinomycin D and cycloheximide, transcriptional and translational inhibitor, respectively, to find the mechanism of enzyme induction. When B16F10 cells were incubated with optimal stimulatory dose of PSL (10 μg/ml) and 5 μg/ml actinomycin D or 10 μg/ml cycloheximide for 24 h, melanin synthesis was reduced from the induced level of 4.77-fold to 1.24- or 1.98-fold, respectively (Fig. 4A). Melanogenesis induced to 2.93-fold by α-MSH (10 nM) alone was lowered to 1.11- or 1.92- fold in presence of actinomycin D or cycloheximide, respectively (Fig. 4A). The dopa oxidase activity of tyrosinase in B16F10 cells increased to 4.6- and 3.1-folds after the stimulation with PSL and α-MSH, respectively, was decreased to almost 1.06-fold in the presence of actinomycin D or nearly 1.87-fold due to cycloheximide in both cases (Fig. 4B). Similarly, tyrosinase expression induced by PSL (8.17-fold) and α-MSH (5.47-fold) was decreased to almost 0.78-fold in the presence of actinomycin D or nearly 1.15-fold due to cycloheximide in both cases (Fig. 4C).

Effect of PSL on tyrosinase promoter activity

Induction of melanogenesis in B16F10 melanoma is characterized by the induction of tyrosinase and may be mediated through the enhancement of tyrosinase promoter activity. We, therefore, studied the effect of PSL on this parameter. The plasmid containing a 2.5-kilobase pair fragment of the tyrosinase promoter followed by human tyrosinase wild type gene and the enhanced green-fluorescent protein (EGFP) coding sequence as a reporter gene, named as pTyrP.TYRwt-GFP (a generous gift from Dr. Ruth Halaban), was investigated after transfection into B16F10 mouse melanoma and NIH3T3 fibroblasts. Stable transfectants were selected by using G418 and incubated with or without the 10 μg/ml PSL and 10 nM α-MSH. When PSL was added to the culture, GFP expression was remarkably higher than α-MSH (Fig. 5A). Fluorometric analysis also revealed a significant upregulation of tyrosinase promoter activity by PSL (4.7-fold) ($P < 0.001$) and α-MSH (3.8-fold) ($P < 0.001$) compared to untreated cells as one fold (Fig. 5B). Furthermore, Western blot analysis confirms the PSL-induced tyrosinase promoter activity by increasing 6.57-fold TYR-GFP expression compared to untreated cells

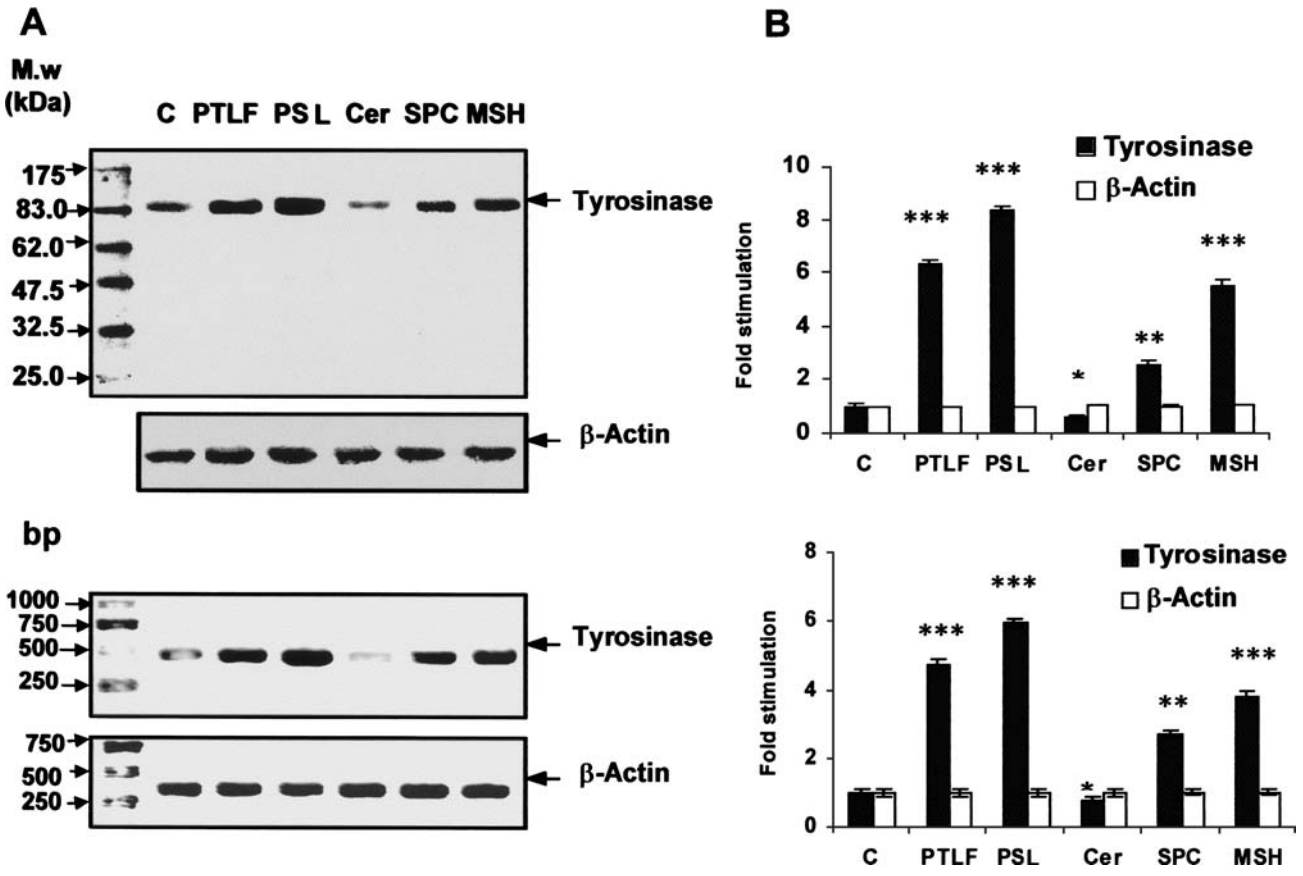

Fig. 2 Effect of PSL on tyrosinase expression at protein and mRNA levels in B16F10 cells for 24 h *in vitro*. (A) Cells were treated with or without 100 μg/ml PTLF, 10 μg/ml PSL, 10 μM C2-Cer, 10 μM SPC and 10 nM α-MSH, then analyzed by Western blotting using anti-tyrosinase and anti-β-actin as a loading control (upper panel) and tyrosinase mRNA was detected by RT-PCR analysis and actin used as an internal control for comparable loading (lower panel) (B) Densitometric scanning of band intensities obtained from three separate experiments of tyrosinase protein and mRNA. The cumulative (control value taken as one-fold in each case) data are presented as means ± SE. $^{*}P < 0.02$; $^{**}P < 0.01$, $^{***}P < 0.001$

as one-fold (Fig. 5C). However, PSL-induced upregulation of tyrosinase promoter activity was not observed in the NIH3T3 fibroblasts stable transfectants (Fig. 5D).

Discussion

A prototype hydroalcoholic human placental extract [14–16] induced melanogenesis significantly in age-onset black mouse C57BL/6J having graying of hair with an elongated telogenic hair growth cycle and B16F10 mouse melanoma [17]. With respect to Glycosphingolipid(s) in the extract, sphingolipids were identified with a benzidine and premulin spray showing four distinct TLC spots [18]. Total lipids isolated from the vehicle-free extract named PTLF (placental total lipid fraction) having these sphingolipids stimulated melanogenesis in the B16F10 mouse melanoma [19–21]. In an attempt to identify the specific sphingolipid present in PTLF and responsible for the effect on melanogenesis, we have purified PSL from PTLF by sequential silicic-acid column chromatography, sephadex-LH20 column chromatography, and finally isolated as a single spot by preparative HPTLC. A comparative TLC analysis with SPC, S1P, and C2-Cer reveled that PSL sphingolipid spot did not match with these melanogenic regulators (Fig. 6). The in-depth chemical analysis of PSL is in progress and will be recorded in future. We report here that PSL-induced melanogenesis occurs by upregulating the tyrosinase activity and its expression at translational and transcriptional level through promoter activation.

Dose and time kinetics of melanogenesis and tyrosinase activity reveled that 10 μg/ml of PSL is optimum for maximum melanin synthesis and enzymatic activation with 24 h of incubation (Fig. 1). The induced dopa oxidase activity of tyrosinase was correlated well with enhanced melanin production by PSL. Therefore, increased tyrosinase activity may be found responsible for the increased melanin content of PSL-treated cells. Western blot analysis and RT-PCR analysis revealed an increase in the expression of tyrosinase at protein and mRNA level, respectively, in PSL-treated B16F10 cells

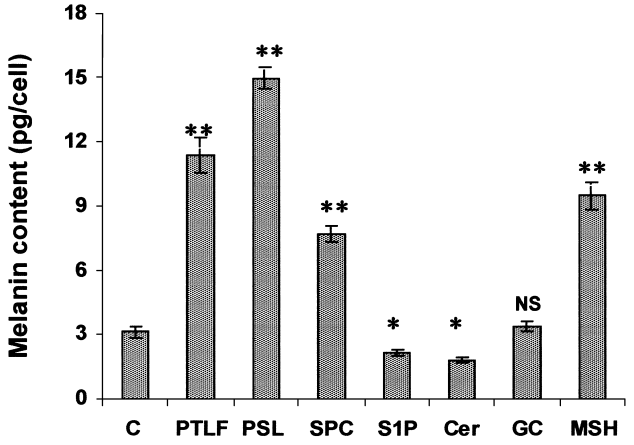

Fig. 3 B16F10 cells were incubated for 24 h *in vitro* with or without 100 μg/ml PTLF, 10 μg/ml PSL, 10 μM C2-Cer, 10 μM SPC and 10 nM α-MSH and melanin content was determined as described in the 'Materials and Methods.' Results are the mean ± SE from triplicate determinations. $^{*}P < 0.01$, $^{**}P < 0.001$

(Fig. 2), suggesting a direct association between increased tyrosinase expression and the induction of its activity by PSL. PSL, is a novel sphingolipid, acting as a melanogenic stimulator in B16F10 cells and, in recent years, few sphingolipid metabolites SPC, C2-Cer and S1P have been found to be serving as melanogenic regulators in melanocytes by upregulation/downregulation of the principle melanogenic enzyme, tyrosinase [22–24]. Thus we have included these sphingolipids along with a well-known physiological regulator of melanin synthesis, α-MSH as control at the level of melanogenesis and tyrosinase expression. Western blot and RT-PCR analysis with tyrosinase and melanin assay reflected their differences in the extent of tyrosinase expression at protein and of mRNA level (Fig. 2), and melanogenesis (Fig. 3). PSL like α-MSH [26], SPC [22], and PTLF [20] stimulated melanogenesis by upregulating the tyrosinase expression at protein and mRNA level in B16F10 cells while S1P and C2-Cer inhibited the melanogenesis by down-regulating the tyrosinase expression in B16F10 cells as observed by others in melanocytes [23,24].

The enhancement of tyrosinase mRNA may occur by a post-transcriptional mechanism such as stabilization of tyrosinase messenger or by stimulation of transcriptional activity of tyrosinase promoter. PSL mediated induction of melanin synthesis, tyrosinase activity, and tyrosinase expression were inhibited by actinomycin D and cycloheximide. These results suggest that PSL may regulate tyrosinase gene at both transcriptional and translational level (Fig. 4). Furthermore, the fluorescence microscopy, fluorometric analysis, and Western blot analysis showed that PSL stimulates transcriptional activity of tyrosinase promoter as evidenced by increased wild-type tyrosinase-EGFP expression in stably transfected B16F10 mouse melanoma (Fig. 5A–C). However, similar experiments performed in NIH3T3 fibroblast cells, showed that PSL treatment was unable to stimulate the transcriptional activity of tyrosinase promoter (Fig. 5D). It indicates that PSL induced a cell-type specific signaling mechanism in melanoma cells and that B16F10 cell-type specific signaling mechanism is missing in NIH3T3 fibroblast cells. These results indicate that a regulatory element of tyrosinase promoter is involved in the PSL-induced transcriptional activation of human tyrosinase promoter. PTLF induces melanogenesis in B16F10 cells through the activation of stress-responsive p38 MAPK upregulating the expression of tyrosinase [21]. Stress signaling when mediated via p38 kinase phosphorylation results a phosphorylation on ser 307 of the basic helix-loop-helix leucine zipper (bHLH-LZ) Microphthalmia-associated transcription factor

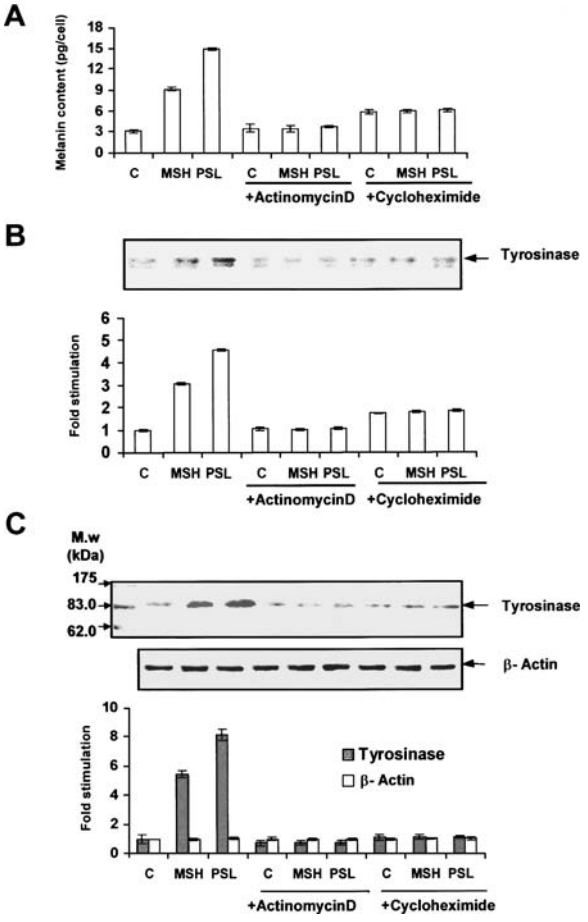

Fig. 4 Effect of Actinomycin D and Cycloheximide on PTLF enhanced melanogenesis (A), tyrosinase activity as estimated by dopa staining in gel (B) and tyrosinase protein expression (C) in B16F10 cells for 24 h *in vitro*. Cells were incubated with PSL (10 μg/ml) and α-MSH (10 nM) in presence or absence of actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) for 24 h. Densitometric scanning of band intensities obtained from three separate experiments of tyrosinase activity (B) and its expression (C). The cumulative (control value taken as one fold in each case) data are presented as means ± SE

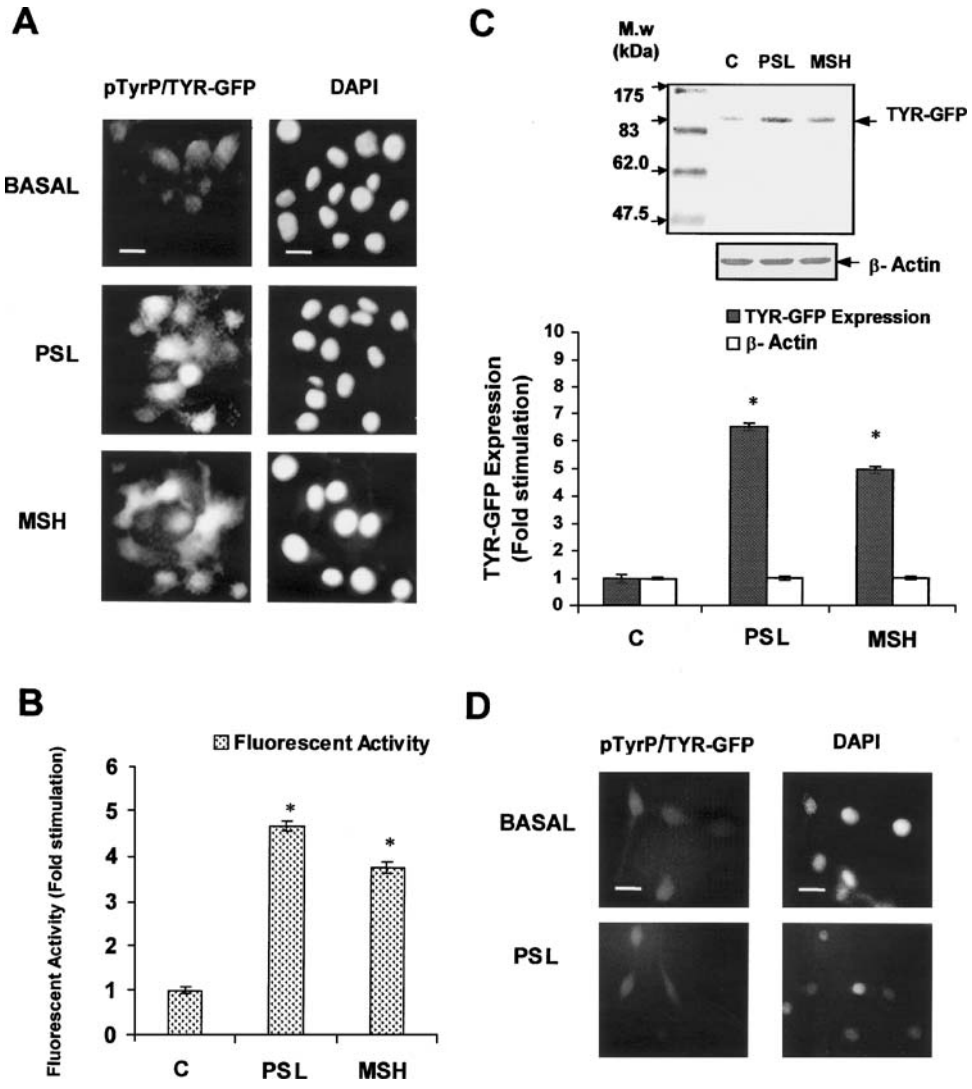

Fig. 5 Effects of PSL on tyrosinase promoter activity in B16F10 and NIH3T3 fibroblast cells. Cells were stably transfected with a plasmid pTyrP.TYRwt-GFP, containing a 2.5-kilobase pair fragment of the tyrosinase promoter cloned upstream of the wild-type tyrosinase and enhanced green fluorescent protein (EGFP) coding-sequence as a reporter gene. Stably transfected cells were incubated with or without 10 μg/ml PSL and 10 nM α-MSH for 24 h. (A) The TYRwt-GFP proteins (left panel) and nuclei of the transfected B16F10 cells by DAPI staining (right panel) were photographed under inverted fluorescence microscope. All photographs are under equal magnification, and a bar represents 25 μm. (B) Fluorescence intensity of the transfected B16F10 cells was analyzed by Fluorescence spectrophotometry at 25°C with excitation at 484 nm and emission at 510 nm. (C) Expression of TYRwt-GFP proteins was analyzed by Western blotting using anti-GFP and anti-β-actin as a loading control in transfected B16F10 cells (upper panel) and Densitometric scanning of band intensities obtained from three separate experiments (lower panel). The cumulative data (control value taken as one-fold in each case) are presented as means ± SE. $^{*}P < 0.001$. (D) The TYRwt-GFP proteins (left panel) and nuclei of the transfected NIH3T3 fibroblast cells by DAPI staining (right panel) were photographed under inverted fluorescence microscope. All photographs are under equal magnification and bar represents 25 μm

(Mitf), responsible for transcription of genes, involved in differentiation, proliferation, and survival of melanocytes [28,29]. Microphthalmia-associated transcription factor is expressed in B16F10 cells but not in NIH3T3 fibroblast cells [30]. It is likely, therefore, that Mitf is a downstream regulator of PSL-mediated melanogenesis in B16F10 cells where Mitf can bind and activate the tyrosinase promoter. The signaling mechanism and the identification of transcription factor involved in PSL-induced melanogenesis are in progress.

Here we conclude that PSL, a novel sphingolipid isolated from human placental extract, is a potent natural melanogenic stimulator of B16F10 mouse melanoma that up-regulates the tyrosinase activity and its expression at translational and transcriptional level through promoter activation. Thus PSL may induce therapeutic benefit for depigmentary disorders such as vitiligo.

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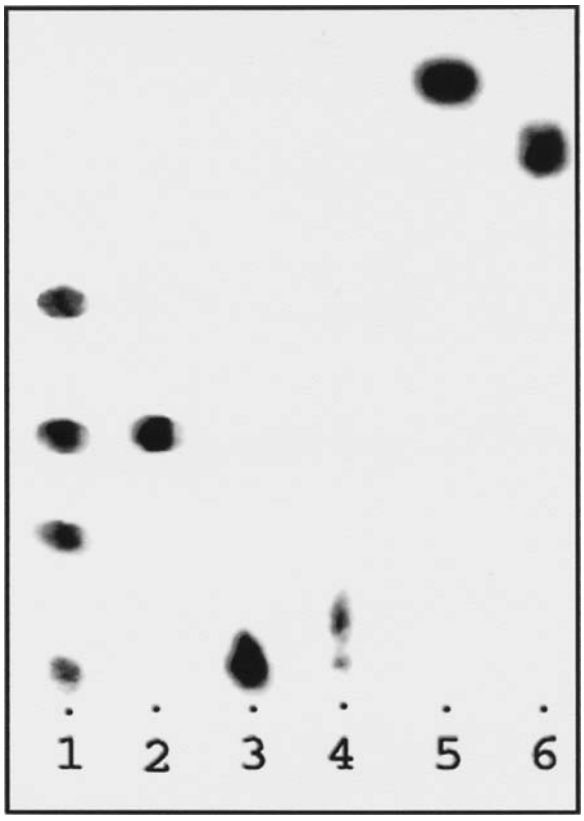

Fig. 6 TLC of sphingolipids was analyzed using solvent system $CHCl_3$/MeOH/H_20 (14:6:1 *v/v/v*) and detected by spraying the plate with primuline and made visible and photographed by illuminating under UV at 365 nm. Lane1. PTLF; lane 2. PSL; lane 3. SPC; lane 4. S1P; lane 5. C2-Cer; lane 6. GC (glucosylceramide)

the expression vector pTyrP.TYRwt-GFP. We also thank our Director, Prof. Siddhartha Roy, for his patronization and keen interest in this work. Financial assistance from the CSIR (Council for Scientific and Industrial Research), ICMR (Indian Council of Medical Research), and the DBT (Department of Biotechnology), New Delhi, are gratefully acknowledged.

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