Polymeric black tea polyphenols inhibit mouse skin chemical carcinogenesis by decreasing cell proliferation

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Abstract. *Objectives:* The aim of this study was to investigate the antitumour promoting effects and possible mechanisms of action of the most abundant polymeric black tea polyphenols (PBPs 1–5) or thearubigins, *in vivo*. *Materials and methods:* Effect of PBP pre-treatments on 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promoted skin papillomas was studied in 7,12-dimethylbenz(a)anthracene initiated mice over 40 weeks. Cell proliferation and apoptosis, in epidermis of the skin, were measured using appropriate immunohistochemical staining. Mitogen-activated protein kinase signalling studies were conducted with Western blot analysis at 10, 20, 30 and 40 weeks of promotion. *Results:* Pre-treatments with PBP fractions differentially altered latency, multiplicity and incidence of skin papillomas as compared to TPA treatments thereby exhibiting antipromoting effects. Most PBP fractions decreased TPA-induced cell proliferation by decreasing activation of signalling kinases (c-Jun N-terminal protein kinase, extracellular signal-regulated protein kinase, p38 protein kinase and Akt), transcription factors (activator protein-1 and nuclear factor kappa B) and inflammatory protein (cyclooxygenase 2). TPA-induced epidermal cell apoptosis was also decreased by pre-treatment with most PBP fractions. Higher levels of p53 and p21 in skin cells pre-treated with PBP fractions followed by TPA treatment as compared to only TPA-treated animals suggested possible activation of a cell cycle checkpoint. *Conclusions:* PBP-2 was observed to be the most potent polymeric polyphenol fraction and PBP-4 and PBP-5 showed only marginal activity, whereas PBP-1 and PBP-3 displayed intermediate efficacies. In conclusion, the protective effects of PBP fractions could be attributed to inhibition of TPA-induced cellular proliferation.

INTRODUCTION

Cancer chemoprevention can be defined as the use of natural or synthetic compounds to prevent, suppress, delay or reverse the process of carcinogenesis that is comprised of initiation, promotion and progression. In the process of initiation, normal cells acquire mutations through endogenous

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and/or exogenous agents. These initiated cells, in the process of promotion, clonally expand to give rise to neoplasia that may further metastasize during progression. In the last decade, a number of dietary compounds, which have shown success as chemopreventive agents in preclinical studies, have attained limited success in subsequent clinical trials. Such failures warrant the need to understand the mechanism of observed chemoprevention in appropriate *in vivo* model systems (Gescher *et al.* 2001; Lambert & Yang 2003; Seifried *et al.* 2003).

Tea polyphenols like (-)-epigallocatechin-3-gallate (EGCG – the most effective catechin in green tea) and theaflavins (black tea polyphenols) have reached clinical trials after their chemopreventive efficacies have been established by various preclinical studies (Chung *et al.* 2003; Moyers & Kumar 2004). However, similar studies on thearubigins (TR) or polymeric black tea polyphenols (PBPs) are limited. PBPs are the most abundant polyphenols in black tea but are structurally and chemically ill-defined (Haslam 2003; Sang *et al.* 2004). They have been reported to be heterogeneous polymers of flavano-3-ols and flavan-3-ol gallates with di- and tri-benzotropolone skeletons (Haslam 2003; Sang *et al.* 2004). In our laboratory, we have previously isolated and partially characterized five different PBP fractions (Krishnan & Maru 2006) by modifying the PBP isolation method of Brown *et al.* (1969). According to the method and solvents used for isolation, PBP-1 fraction might contain theafulvins and rest of the PBP fractions (PBPs 2–5) closely resemble n-butanol soluble acidic thearubigins (Ozawa *et al.* 1996; Catterall *et al.* 1998). These PBP fractions, which are polymeric pro-anthocyanidin in nature, have shown competence in inhibiting initiation of carcinogenesis by decreasing benzo(a)pyrene-DNA adduct formation *in vitro* and in mouse skin (Krishnan & Maru 2004, 2005; Krishnan *et al.* 2005). The objective of the present investigation was to study the effect of these PBP fractions on promotion of carcinogenesis measured as tumour latency, incidence and multiplicity using a well-defined protocol for two stage mouse skin chemical carcinogenesis. Here, a low dose of the mutagen 7,12-dimethylbenz(a)anthracene (DMBA) acts as initiator, and repetitive exposures to tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), generate skin papillomas. The mechanism of PBP-mediated antipromoting activity was investigated by studying its effects on cell proliferation, signalling cascades, cell cycle arrest proteins and apoptosis in mouse skin. Most PBP fractions could inhibit mouse skin chemical carcinogenesis by decreasing cell proliferation.

MATERIALS AND METHODS

Isolation and analyses of PBP fractions from black tea

Five polymeric black tea polyphenol fractions were isolated from a popular brand of black tea powder (Mumbai, India) in our laboratory, employing a Soxhlet extractor (Krishnan & Maru 2006). Briefly, black tea powder was serially extracted in a Soxhlet extractor (Borosil Glass Works Ltd., Mumbai, India) with chloroform, ethyl acetate and n-butanol (AR/HPLC grade, Sisco Research Laboratories Pvt. Ltd., Mumbai, India). The ethyl acetate extract yielded PBP-1 whereas the n-butanol extract contained PBP-2 and PBP-3. The tea powder residue was then brewed with distilled water, the resultant solution acidified with sulphuric acid and extracted with n-butanol, to obtain PBP-4 and PBP-5. The yields of PBP-1, -2, -3, -4 and -5 from 100 g of black tea powder were 2.68 g, 3.79 g, 1.34 g, 2.20 g and 0.32 g, respectively. The PBP fractions were confirmed to be free of other biologically active contaminants such as caffeine, EGCG, epigallocatechin, epicatechin gallate, gallic acid, gallic acid, epicatechin, catechin and theaflavin(s) using thin layer chromatography with chloroform : ethyl acetate : formic acid (6 : 4 : 1) as the solvent system. The polymeric nature of PBPs was confirmed by Fourier-transformed

infrared and nuclear magnetic resonance analyses (Krishnan & Maru 2006). However, complete chemical characterization of PBP fractions was not possible due to their matrix reactivity and highly complex polymeric nature (Haslam 2003; Sang *et al.* 2004). PBP mixture (PBP-mix) used in this study was composed of all the five PBP fractions (PBP 1–5) in proportions as present in whole black tea (2.68 : 3.79 : 1.34 : 2.20 : 0.32, respectively).

Animals and treatments

All animal studies were conducted after approval from the Institutional Animal Ethics Committee as per Committee for the Purpose of Control and Supervision of Experiments on Animals (Government of India) guidelines. Female S/RVCri-ba or 'bare' mice (6–8 weeks old) – hairless mutants that are highly susceptible to skin tumorigenesis by the two stage protocol were obtained from the animal colony of Advanced Centre for Treatment, Research and Education in Cancer (India) (Bhisey *et al.* 1987; Bhisey & Veturkar 1990). The animals were housed in plastic cages (six per cage), maintained under standard conditions (24 ± 2 °C, $50 \pm 10\%$ relative humidity and 12-h light:dark cycles) and provided with standard diet and plain drinking water *ad libitum*. Animal experiments are described as follows.

Treatment regime for two-stage skin carcinogenesis

Mice were initiated with a single topical dose (20 nmol) of DMBA (Sigma, St. Louis, MO, USA) in 100 μ L acetone to dorsal skin. After initiation, treated animals were randomized into 16 treatment groups each containing not less than 10 mice, for each time point. Starting from 1 week after DMBA treatment, animals were treated with TPA and PBP fractions (Table 1a). Skin tumour promotion was carried out by topical application of TPA (1.8 nmol per application in 100 μ L of acetone as vehicle; Sigma). In respective pre-treatment groups, 200 μ g of PBPs 1–5/200 μ g PBP-mix/200 μ g EGCG (Sigma) in 100 μ L of acetone was applied topically 20 min prior to TPA application. All treatments were carried out twice weekly for 10, 20, 30 and 40 weeks. Animals in all groups were observed for apparent signs of toxicity such as weight loss or mortality during the entire period of study. All mice were monitored twice weekly for appearance of skin papillomas (1 mm² size or more) for 40 weeks. Average latent period (in weeks) was calculated from the time of appearance of the first skin papilloma, in each mouse, for all treatment groups. Mice were also scored for multiplicity and incidence of skin papillomas at every week, until the end of the 40 weeks of promotion. Mice were sacrificed 6 h after respective 10, 20, 30 and 40 week time points of promotion, for further analyses.

Treatment regime for mitogen-activated protein kinase inhibitor studies

To confirm the role of mitogen-activated protein (MAP) kinases and phosphoinositide-3 (PI3) kinase in TPA-induced epidermal cell proliferation, DMBA-initiated mice were topically treated with 5 μ mol of U0126, SB203580, SP600125 and LY294002 (Sigma) twice a week for two weeks, which inhibited activation of MAP kinase kinase (MEK) 1/2 (which is responsible for activation of ERK), p38, JNK 1/2/3 and PI3 kinase, respectively, 20 min prior to 1.8 nmol of TPA.

Treatment regime for determination of ornithine decarboxylase activity

Mice were initiated with a single topical dose of DMBA (20 nmol) in 100 μ L acetone and were randomly divided into four groups of at least 10 animals each (Table 1b). One week after DMBA initiation, mice were topically treated either with 100 μ L acetone (vehicle) or PBP-mix (200 μ g of mixture of PBP-1 to PBP-5 in equal proportions, that is, each PBP fraction at 40 μ g, in 100 μ L acetone). Twenty minutes later, mice were topically treated with acetone or TPA

Table 1a. Treatment regime for two-stage skin carcinogenesis

Groups	Initiation	Pre-treatment	Promotion
1: Vehicle control	DMBA	Acetone	Acetone
2: TPA	DMBA	Acetone	TPA
3: PBP-1	DMBA	PBP-1	Acetone
4: PBP-2	DMBA	PBP-2	Acetone
5: PBP-3	DMBA	PBP-3	Acetone
6: PBP-4	DMBA	PBP-4	Acetone
7: PBP-5	DMBA	PBP-5	Acetone
8: PBP-mix	DMBA	PBP-mix	Acetone
9: EGCG	DMBA	EGCG	Acetone
10: PBP-1 + TPA	DMBA	PBP-1	TPA
11: PBP-2 + TPA	DMBA	PBP-2	TPA
12: PBP-3 + TPA	DMBA	PBP-3	TPA
13: PBP-4 + TPA	DMBA	PBP-4	TPA
14: PBP-5 + TPA	DMBA	PBP-5	TPA
15: PBP-mix + TPA	DMBA	PBP-mix	TPA
16: EGCG+TPA	DMBA	EGCG	TPA

Table 1b. Treatment regime for determination of ornithine decarboxylase activity

Groups	Initiation	Pre-treatment	Promotion
1: Vehicle control	DMBA	Acetone	Acetone
2: PBP-mix	DMBA	PBP-mix	Acetone
3: TPA	DMBA	Acetone	TPA
4: PBP-mix + TPA	DMBA	PBP-mix	TPA

(1.8 nmol) in 100 µL acetone. Animals were sacrificed 6 h after the last treatment and their skins were excised for determination of ornithine decarboxylase (ODC) activity.

Determination of ODC activity

Epidermis from the skin of sacrificed mice previously treated for analyses of ODC activity was removed employing a Watson's skin grafting knife with suitably adjusted cutting angle. ODC activity in the epidermal extract was measured by the amount of $^{14}\text{CO}_2$ liberated from the substrate, DL-[1- ^{14}C] ornithine hydrochloride (Amersham Biosciences, Buckinghamshire, UK; specific activity = 58 mCi/mmol) as previously described (O'Brien 1976).

Immunoblotting and immunohistochemical staining

Skin of the animals sacrificed at different time points was excised and part of it was fixed in 10% buffered formalin, which was further processed for routine histology and for immunohistochemical staining using primary antibodies against phosho-c-Jun N-terminal kinase (p-JNK), phosho-extracellular signal-regulated kinase (p-ERK), phosho-p38 (p-p38), cyclooxygenase 2 (COX-2), c-jun, c-fos and nuclear factor kappa B (NF-κB) p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunohistochemical staining was carried out according to guidelines for the Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA) using 1 : 100 dilution of primary antibody. The rest of the skin was snap frozen at -80°C and the epidermal layer was manually extracted using Watson's skin grafting knife. This was then fractionated into either epidermal

lysate or nuclear extract, using previously described cell fractionation protocols (Afaq *et al.* 2004). Protein concentrations were determined (Lowry *et al.* 1951), respective lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and samples were transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 h in 5% milk in TBS-Tween 20 and were incubated at 4 °C overnight with respective antibodies at a dilution of 1 : 1000. The appropriate horseradish peroxidase-conjugated secondary antibody was incubated with the samples for 1 h at room temperature followed by three 20-min washes of TBS-Tween 20, after which the membrane was exposed to luminol reagent (Santa Cruz Biotechnology). Immune complexes were detected by autoradiography. Cyclin D₁, p-JNK, p-ERK, p-p38, p-Akt, COX-2, p21, Bcl2 and Bax were assayed by immunoblotting in epidermal lysates where β -tubulin and β -actin were used as a loading controls, whereas c-jun, c-fos, NF- κ B p65 and p53 proteins were detected in nuclear extracts where histone H1 was used as loading control.

Measurement of cell proliferation and apoptosis

Formalin-fixed, paraffin wax-embedded 5 μ m thick sections from at least four animals per group were assayed for proliferating cell nuclear antigen (PCNA) staining, for determination of PCNA labelling index (Hall *et al.* 1990). Sections were incubated with monoclonal antimouse PCNA (PC10, 1 : 50 dilution, BD Pharmingen, San Diego, CA, USA). Detection was performed with Vectastain ABC system (Vector Laboratories), which contained 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. Apoptosis of epidermal cells was assayed as per the guidelines for the *in situ* terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assay kit (Chemicon International Inc., Temecula, CA, USA), where cells undergoing apoptosis were detected as stained nuclei. The PCNA labelling index and apoptotic index were calculated and each was expressed as number of positively stained cells $\times$ 100/total number of cells, in each treatment group.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using NF- κ B (5'-AGTTGAGGGGACTT-TCCCAGGC-3') and activator protein-1 (AP-1) (5'-AGCTTCGCTTGATGACTCAGCCGG-3') oligonucleotide probes (Sigma) labelled with [γ -³²P] ATP (BRIT, Hyderabad, India) by T4 polynucleotide kinase and purified on a sepharose G-25 column. The binding reaction was carried out in a total volume of 25 μ L containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 4% (v/v) glycerol, 10 μ g of nuclear extracts, and 20 000 c.p.m. of labelled probe. After 50 min incubation at room temperature, 2 μ L of 0.1% bromophenol blue was added, and samples were electrophoresed through a preran 5% non-denaturing polyacrylamide gel at 150 V, in a cold room for 2 h. Finally, the gel was dried and exposed to X-ray film.

Statistical analysis

Statistical analyses were performed using SPSS 14.0 for Windows software (SPSS Inc., Chicago, IL, USA). Data are presented as mean $\pm$ SE. Means of all data were compared by ANOVA with *post hoc* testing. $P < 0.05$ was considered statistically significant.

RESULTS

PBP pre-treatments inhibited DMBA-initiated and TPA-promoted skin carcinogenesis

Based on net body weight gain and histopathological evaluation of tissues, neither toxicity nor mortality was observed in animals from any of the treatment groups during the experimental period.

The antipromoting effects of PBP fractions were analysed by assaying modulation in latency period, incidence and multiplicity of DMBA-initiated and TPA-promoted skin papillomas (Table 2). Mice that were not treated with TPA showed no papillomas for the duration of experiment. Pre-treatments with individual PBPs 1–4, or PBP-mix, 20 min prior to TPA treatment brought about a significant increase in the mean number of weeks of onset (latency period) of TPA-induced skin papillomas. However, pre-treatments with PBP-5 did not alter the latency period of TPA-induced skin papillomas. TPA-promoted animals showed time-dependent increase in multiplicity (average number of papillomas per mouse), whereas pre-treatments with individual PBPs 1–5, PBP-mix and EGCG prior to TPA treatments significantly decreased multiplicity at 20, 30 and 40 weeks when compared to TPA-treated animals at respective time points. When incidence (percentage of mice with papillomas) of skin papillomas in different experimental groups was compared, pre-treatments with PBP-2 showed significant decrease in incidence at all time points. However, PBP-1, -3 and -4 showed decrease in incidence only at initial time point of 10 weeks but a similar decrease was not observed at 20, 30 and 40 weeks. Here, as well, PBP-5 remained inactive at decreasing incidence, at all time points. Pre-treatment with the most potent green tea polyphenol, EGCG, prior to TPA treatment, also resulted in a significant decrease in skin tumorigenesis as compared to only TPA-promoted mice.

PBP-2 appeared to be the most potent PBP fraction as pre-treatments with PBP-2 not only enhanced the latency period but also decreased multiplicity and incidence of TPA-promoted papillomas. PBP-5 appeared to be least effective as it decreased multiplicity without altering the latency period and incidence of TPA-induced papillomas, whereas other PBP fractions showed intermediate activity (Table 2).

PBP pre-treatments inhibited DMBA-initiated and TPA-promoted skin carcinogenesis by inhibiting TPA-induced cell proliferation

To understand the protective effects of PBP fractions on DMBA-initiated and TPA-promoted skin papillomas, TPA-induced cell proliferation in the epidermis was studied employing cell proliferation markers, that is, PCNA and cyclin D₁, at various time points. At all exposure intervals studied, TPA-promoted mouse skin displayed significantly higher PCNA labelling indices as compared to animals treated with acetone only (Fig. 1a). Pre-treatments with PBPs 1–4, PBP-mix and EGCG significantly decreased TPA-induced PCNA labelling index at all the time points studied. However, PBP-5 was able to decrease TPA-induced proliferation only at 10 weeks. To further confirm this, another proliferation-related protein, cyclin D₁, was assayed, in epidermal extracts of treated mice, by immunoblotting. Cyclin D₁ levels were significantly increased in TPA-treated mice as compared to vehicle control animals. Pre-treatments with PBP-1, -2, -3, PBP-mix and EGCG significantly decreased cyclin D₁ levels at all time points studied. However, pre-treatments with PBP-4 decreased TPA-induced cyclin D₁ levels only at the earlier time point of 10 weeks, whereas pre-treatments with PBP-5 did not show significant alteration when compared to the TPA treatment group (Fig. 1b,c).

Considering both, PCNA labelling index and levels of cyclin D₁, pre-treatments with PBP-2 were found to be the most effective in decreasing TPA-induced cell proliferation.

TPA-induced cell proliferation is governed by MAP kinase signalling

Pre-treatments with all the four MAP kinase inhibitors (U0126, SB203580, SP600125 and LY294002) significantly lowered the PCNA labelling index as compared to animals treated with TPA alone where the pre-treatments with U0126 were most effective (Fig. 2a–c). Similarly, pre-treatments with U0126 were most effective in decreasing cyclin D₁ induction by TPA, nuclear translocation of NF- κ B p65 and hence in blocking NF- κ B DNA binding activity

Table 2. Effects of pre-treatments with individual PBPs 1–5, PBP-mix and EGCG on latency period, multiplicity and incidence of DMBA-initiated and TPA-promoted mouse skin carcinogenesis

Group No. (as per Table 1a)	Treatment	Latency (in weeks)	Multiplicity Time points (in weeks)				Incidence (%) Time points (in weeks)			
			10	20	30	40	10	20	30	40
2	Acetone + TPA	11 ± 0.28	0.41 ± 0.001	4.00 ± 0.01	8.24 ± 0.09	12.00 ± 0.1	13	100	100	100
10	PBP1 + TPA	17 ± 1.72	0.05 ± 0.002*	0.91 ± 0.05*	1.90 ± 0.01	4.20 ± 0.04*	6	58*	76	100
11	PBP2 + TPA	14 ± 1.58*	0.00*	0.35 ± 0.001*	1.00 ± 0.05*	2.90 ± 0.05	0	31*	63*	69*
12	PBP3 + TPA	18 ± 1.01*	0.00*	0.59 ± 0.002*	2.50 ± 0.04*	4.21 ± 0.04*	0	38*	100	100
13	PBP4 + TPA	15 ± 1.44*	0.05 ± 0.001	1.42 ± 0.01	3.90 ± 0.04*	5.59 ± 0.04*	6	70	100	100
14	PBP5 + TPA	11 ± 1.21	0.39 ± 0.003	2.00 ± 0.05	4.10 ± 0.05*	7.01 ± 0.1	13	95	100	100
15	PBPmix + TPA	20 ± 1.58*	0.00*	0.81 ± 0.02	3.20 ± 0.02*	4.90 ± 0.09	0	100	100	100
16	EGCG + TPA	19 ± 1.92	0.00*	1.19 ± 0.004*	3.80 ± 0.02	5.20 ± 0.09	0	72	100	100

Mean ± SE; *n* = 10 mice per group.
**P* < 0.01 compared to group no. 2 – acetone + TPA-treated, ANOVA with Tukey’s test for latency and multiplicity and Fisher’s exact test for incidence.

Figure 1. Effects of PBP pre-treatments on DMBA-initiated and TPA-promoted cellular proliferation in skin epidermis in animals treated as per Table 1a. (a) Percentage of cells undergoing proliferation in skin epidermis was evaluated as PCNA labelling index. Results are expressed as number of the positively stained cells $\times 100$ /total number of cells in each treatment group and analysed using ANOVA with Tukey's test. Columns, mean of values from 10 fields per animal at $\times 400$ magnification (observed under light microscope) of four mice per treatment group; bar, SE; $*P < 0.01$ less than corresponding value of acetone- and TPA-treated animals (group 2 in Table 1a). (b) Epidermal levels of cyclin D₁ in treated animals were analysed by immunoblotting from epidermal lysates. Results have been shown as representative blots from different treatment groups at all four time points assayed (10, 20, 30 and 40 weeks) with respective loading controls. (c) Relative protein levels of cyclin D₁ presented after normalizing with corresponding levels of β -tubulin. Results were analysed using ANOVA with Bonferroni's test. Columns, mean of values from four animals per treatment group; bar, SE; $*P < 0.01$ less than corresponding value of acetone- and TPA-treated animals (group 2 in Table 1a).

Figure 2. Effects of MAP kinase inhibitors (U0126, SB203580, SP600125 and LY294002) on TPA-induced cell proliferation and signalling molecules. (a) Percentage of cells undergoing proliferation in skin epidermis was evaluated as PCNA labelling index. Results are expressed as number of positively stained cells $\times 100$ /total number of cells in each treatment group and analysed using ANOVA with Tukey's test. Columns, mean of values from 10 fields per animal at $\times 400$ magnification (observed under light microscope) of four mice per treatment group; bar, SE; $*P < 0.01$ less than corresponding value of TPA-treated animals. (b) Results have been shown as representative blots of proteins cyclin D₁, COX-2, p21 and p-Akt analysed by immunoblotting from skin epidermal lysate; whereas c-jun, c-fos, NF- κ B p65 and p53 were analysed by immunoblotting from nuclear lysates obtained from skin epidermis. (c) Representative blots for EMSA carried out by incubating nuclear lysates with radiolabelled AP-1 and NF- κ B oligonucleotides.

(Fig. 2b,c). Pre-treatments with LY294002 showed decreased activation of Akt and also restored p53 levels that were depleted by TPA treatment. Furthermore, pre-treatments with LY294002 also induced p21 as compared to TPA treatment alone. Pre-treatments with SB203580 and SP600125 were most effective in blocking c-fos and c-jun nuclear translocation, respectively, and AP-1 DNA binding activity (Fig. 2b,c). In order to further investigate the possible involvement of MAP kinases in the signalling pathway mediating COX-2 induction, we examined the effect of MAP kinase inhibitors on TPA-induced COX-2 expression. U0126, SB203580, SP600125 and LY294002 could significantly decrease TPA-induced COX-2 levels (Fig. 2b). These data suggest that MAP kinases play a central role in TPA-induced intracellular signalling cascades leading to activation of NF- κ B and AP-1 regulated genes that mediate cell proliferation in mouse skin.

PBP pre-treatments alter TPA-induced cell proliferation by inhibiting expression and activation of cell signalling and pro-inflammatory proteins

To delineate the mechanism of observed antiproliferative effects exhibited by PBPs against TPA tumour promotion, molecular events governing TPA-induced cell proliferation were studied. Activation of MAP kinases in epidermal cells of animals pre-treated with PBPs followed by TPA administration, was studied by assaying levels of phosphorylated JNK, ERK and p38, using immunoblotting in epidermal lysates at all four time points (Fig. 3a–e). As compared to vehicle control, TPA treatments elevated phosphorylated JNK, ERK and p38 in the epidermis at all time points. Pre-treatments with PBP-1, -2, -3, PBP-mix and EGCG prior to TPA significantly decreased activation of MAP kinases at all time points. Although pre-treatments with PBP-4 and PBP-5 showed significant inhibition of TPA-induced activation of JNK and ERK at 10, 20 and 30 weeks, the inhibition was not observed by 40 weeks. Pre-treatments with PBP-1, -2, -3, PBP-mix and EGCG significantly decreased TPA-induced activation of p38 at all time points, whereas similar pre-treatments with PBP-4 and PBP-5 were ineffective.

PBP-2 pre-treatments were found to be the most effective in decreasing activation of TPA-induced JNK, while EGCG and PBP-mix pre-treatments were most efficient in decreasing activation of TPA-induced ERK at all time points. Similarly, PBP-3 pre-treatments were most effective in decreasing TPA-induced activation of p38. These observations were confirmed by immunohistochemical staining of phosphorylated forms of all three MAP kinases in epidermis at 40 weeks where increased nuclear staining was also observed, indicating activation and nuclear translocation of these MAP kinases (Fig. 4).

Activation of transcription factors such as c-jun, c-fos and NF- κ B p65 was determined by assaying their nuclear localization, by immunoblotting these proteins in nuclear extracts from epidermis at all time points as well as by immunohistochemical staining of epidermis at 40 weeks (Figs 5a–e and 4). As compared to vehicle control, nuclear accumulation of c-jun, c-fos and NF- κ B p65 significantly increased in TPA-treated animals at all the time points studied. Pre-treatments with PBP-1, -2, -3, PBP-mix and EGCG significantly decreased TPA-induced nuclear accumulation of transcription factors when compared to TPA treatments at all the time points evaluated, while pre-treatments with PBP-4 and PBP-5 showed only marginal effects. Pre-treatments with PBP-2 appeared to be the most effective in decreasing TPA-induced nuclear accumulation of c-jun and NF- κ B p65 at all time points. Similarly, pre-treatments with PBP-3 were most effective in decreasing TPA-induced nuclear accumulation of c-fos at all time points. Immunohistochemical staining of all the transcription factors in epidermis confirmed the above observations (Fig. 4). Pre-treatments with PBP-1, -2, -3, PBP-mix and EGCG also decreased AP-1 and NF- κ B DNA binding activities at 40 weeks as compared to TPA only treatment whereas pre-treatments with PBP-4 and PBP-5 did not show any significant difference (Fig. 5f).

Figure 3. TPA-responsive cell signalling kinases (JNK, ERK, p38 and Akt); inflammatory protein (COX-2) and cell cycle check point protein (p21) were analysed by immunoblotting in epidermal lysates of animals treated as per Table 1a. (a–d) Results have been shown as representative blots of proteins p-JNK, p-ERK, p-p38, p-Akt, COX-2 and p21 at 10, 20, 30 and 40 weeks time points, respectively.

Cyclooxygenase 2, a marker of inflammation, was also assayed by immunoblotting at all time points and by immunohistochemical staining at 40 weeks in the tissue samples (Fig. 3a–e). TPA significantly induced COX-2 expression at all four time points. At 10, 20 and 30 weeks, this induction was significantly inhibited by pre-treatments with PBP-1, -2, -3, PBP-mix and EGCG. However, PBP-4 and PBP-5 were not effective. At 40 weeks, all the PBP fractions, except PBP-2, showed marginal effect on TPA-induced COX-2. This was confirmed by immunohistochemical staining of COX-2 (Fig. 4).

Figure 3. (e) Relative protein levels of p-JNK, p-ERK, p-p38, p-Akt, COX-2 and p21 are presented after normalizing with corresponding levels of β -tubulin. Results were analysed using ANOVA with Bonferroni's test. Columns, mean of values from four animals per treatment group; bar, SE; * $P < 0.05$ less than corresponding value of acetone- and TPA-treated animals (group 2 in Table 1a).

ODC activity

Since in TPA-promoted animals increased activation of MAP kinases was observed, alterations in ODC activity were also assayed in animals treated with TPA alone as compared to animals pre-treated with PBP-mix. Mice treated with TPA alone showed significant increase in ODC activity as compared to vehicle control animals. However, pre-treatment with PBP-mix significantly reduced TPA-induced activation of ODC activity as compared to animals treated with TPA alone (Fig. 6).

BPB pre-treatments inhibit TPA-induced activation of Akt and decrease p53 levels, followed by induction of p21

Previous studies using PI3 kinase inhibitor LY294002 have suggested that TPA-induced activation of Akt caused decrease in levels of p53 and p21 (Fig. 2b). Hence, to study the effect of BPB fractions on TPA-induced activation of Akt and p53 levels, immunoblotting of p-Akt (Ser473) and p53 was carried out in epidermal extracts and nuclear lysates, respectively, at all time points. As compared to the vehicle control, TPA treatments induced significant activation of Akt, whereas pre-treatments with PBP-1, -2, -3, PBP-mix and EGCG showed significant decrease in Akt activation as compared to TPA alone at all time points. PBP-4 and PBP-5 showed marginal or no effect in altering Akt activation at all time points (Fig. 3a–e). Furthermore, as compared to the vehicle-treated group, TPA treatment caused decreased accumulation of p53 in the nucleus. Pre-treatment with PBP-1, -2 and -3 followed by TPA showed significantly higher levels of p53 in the nucleus as compared to TPA-treated animals at all time points. Pre-treatment with

Figure 4. Representative pictures of immunohistochemical staining of proteins p-JNK, p-ERK, p-p38, COX-2, c-jun, c-fos and NF-κB p65 in sections from skin epidermis of mice (treated as per Table 1a) at 40 weeks time point from at least five field of three animals analysed per group at $\times 400$ magnification. For each protein, staining of vehicle control-treated group, Acetone + TPA-treated group, pre-treatment group that is most effective in altering TPA-induced alteration as compared to vehicle control and EGCG + TPA-treated group as positive control have been presented (treatments as per Table 1a).

Figure 5. Nuclear accumulation of TPA-responsive transcription factors (c-jun, c-fos and NF- κ B p65) and p53 was studied by immunoblotting from nuclear lysate obtained from epidermis of animals treated as per Table 1a. (a–d) Results have been shown as representative blots of proteins c-Jun, c-fos, NF- κ B p65 and p53 at 10, 20, 30 and 40 weeks time points, respectively.

PBP-mix and EGCG followed by TPA resulted in higher levels of p53 as compared to TPA treatments only at 30 and 40 weeks. PBP-4 and PBP-5 pre-treatment followed by TPA, showed p53 levels similar to TPA treatment group (Fig. 5a–e). Since differential levels of p53 were obtained on PBP pre-treatment as compared to TPA alone, p21 protein levels were also examined by immunoblotting in epidermal lysates at all time points (Fig. 3a–e). Compared to the TPA treatment group, pre-treatment with PBP-1, -2, -3, PBP-mix and EGCG showed increased levels of p21 in mouse epidermis at all the four time points. PBP-4 and PBP-5 did not seem to induce p21 at any of the time points studied.

Pre-treatment with PBP-1, -2 and -3 appeared to be the most effective in inhibiting activation of Akt and maintaining higher levels of p53 and cell cycle checkpoint protein, p21 in mouse epidermis.

PBP pre-treatment inhibits TPA-induced apoptosis in the epidermis

To examine the effect of PBP fractions on apoptosis, apoptosis was studied employing an *in situ* TUNEL assay kit, at all the time points. Apoptosis in epidermal cells was assayed and is presented as the apoptotic index (Fig. 7a). Animals pre-treated with PBP fractions alone did not display any significant difference in apoptosis as compared to vehicle-treated mice, at all time

Figure 5. (e) Relative protein levels of c-jun, c-fos, NF-κB p65 and p53 have been presented after normalizing with corresponding levels of histone H1. Results were analysed using ANOVA with Bonferroni's test. Columns, mean of values from four animals per treatment group; bar, SE; * $P < 0.05$ less than corresponding value of acetone- and TPA-treated animals (group 2 in Table 1a). (f) Representative blots for EMSA carried out by incubating epidermal nuclear lysates of animals treated as per Table 1a at 40 weeks with radiolabelled AP-1 and NF-κB oligonucleotides. Lanes 1: control; 2: TPA; 3: PBP-1 + TPA; 4: PBP-2 + TPA; 5: PBP-3 + TPA; 6: PBP-4 + TPA; 7: PBP-5 + TPA; 8: PBP-mix + TPA; 9: EGCG + TPA.

Figure 6. Ornithine decarboxylase (ODC) activity in epidermal lysate was analysed in animals treated as per Table 1b. Results expressed as nmoles of ¹⁴CO₂ released in 60 min per mg protein were analysed using ANOVA with Tukey's test. Columns, mean of values from five animals per group; bar, SE; **P* < 0.05 less than corresponding value of acetone (vehicle)-treated animals (group 1 in Table 1b); ***P* < 0.05 less than corresponding value of acetone- and TPA-treated animals (group 3 in Table 1b).

points. It may be noted that vehicle control cells showed basal levels of apoptosis that significantly increased after TPA treatment. Similar levels of apoptosis were also observed in animals receiving PBP-5 pre-treatment at all time points. Interestingly, animals pre-treated with PBP-2 followed by TPA treatment showed apoptosis profiles similar to controls indicating no increased levels of apoptosis. PBP-1, -3, -4, PBP-mix and EGCG pre-treatments showed higher levels of apoptotic cells as compared to vehicle control and significantly lower levels as compared to only TPA-treated animals. Apoptosis in these cells was also tested by assaying levels of anti- and pro-apoptotic molecules such as Bcl2 and Bax, respectively, by immunoblotting in epidermal extracts (Fig. 7b). Bcl2, an anti-apoptotic protein, was decreased in epidermis of TPA-treated animals as compared to the vehicle only control group, whereas the rest of the treatment groups did not show any significant decrease in Bcl2 levels at 10 weeks. But at 20, 30 and 40 weeks, Bcl2 levels were significantly lower in TPA-treated as well as PBP-4 and PBP-5 pre-treatments groups. PBP-1, -2, -3, PBP-mix and EGCG pre-treatments followed by TPA showed marginal difference compared to the vehicle only controls at later time points. Bax, a pro-apoptotic protein, significantly increased in TPA-treated as well as PBP-4 and PBP-5 pre-treated animals as

Figure 7. Effects of PBP pre-treatments on TPA-induced apoptosis in skin epidermis of animals treated as per Table 1a. (a) Percentage of cells undergoing apoptosis in mouse skin epidermis were analysed as apoptotic index employing *in situ* apoptosis detection kit and results are expressed as number of the positively stained cells × 100/total number of cells in each treatment group. Results were analysed using ANOVA with Bonferroni's test. Columns, mean of values from 10 fields per animal at ×400 magnification (observed under light microscope) of four animals per treatment group; bar, SE; **P* < 0.01 less than corresponding value of acetone- and TPA-treated animals (group 2 in Table 1a). (b) Pro-apoptotic protein-Bax and anti-apoptotic protein-Bcl2 were assayed in epidermal lysates of animals treated as per Table 1a by immunoblotting. Results have been shown as representative blots of Bcl2 and Bax at 10, 20, 30 and 40 weeks time points of four animals per group. (c) Bax:Bcl2 ratio was calculated after normalizing each with respective β-actin levels. Results were analysed using ANOVA with Bonferroni's test. Columns, mean of values of ratio of Bax:Bcl2 from four animals per treatment group; bars, SE; **P* < 0.05 less than corresponding value of acetone- and TPA-treated animals (group 2 in Table 1a).

compared to vehicle control at all time points. However, PBP-2 pre-treatment followed by TPA did not induce Bax levels significantly at any of the four time points. Bax was significantly induced by PBP-1, -3, PBP-mix and EGCG pre-treatment followed by TPA treatment at 30 and 40 weeks as compared to the vehicle control group. The Bax:Bcl2 ratio was significantly higher in TPA-treated animals compared to vehicle controls, indicating higher apoptosis at all time points (Fig. 7c). PBP-2 pre-treatment did not increase the Bax:Bcl2 ratio significantly compared to the vehicle-treated group indicating only marginal apoptosis at all time points. Furthermore, pre-treatment with PBP-1, -3, PBP-mix and EGCG showed higher Bax:Bcl2 ratio compared to vehicle-treated samples but was significantly lower than TPA-treated animals at all time points. PBP-4 showed a significantly lower Bax:Bcl2 ratio indicating lower apoptosis, compared to TPA-treated groups at 10 and 20 weeks, whereas PBP-5 showed a decreased Bax:Bcl2 ratio only at 10 weeks. PBP-4 and PBP-5 did not show any significant alterations in the Bax:Bcl2 ratio as compared to TPA-treated animals at 30 and 40 weeks (Fig. 7c).

As seen by the apoptotic index and Bax:Bcl2 ratio, PBP-2 appeared to be the most potent in inhibiting TPA-induced apoptosis, whereas PBP-5 showed marginal activity only.

DISCUSSION

The mouse skin model of two-stage chemical carcinogenesis provides an experimental framework to study basic mechanisms associated with promotion of carcinogenesis and has proved useful in identifying various chemopreventive agents and their mode of action (DiGiovanni 1991). Using this model, the antipromoting efficacies of PBP fractions (PBPs 1–5) were investigated. Similar chemopreventive attributes employing EGCG, a well-established and very potent green tea polyphenol, were also studied, to validate our experimental system. Whole black tea and black tea extracts have emerged as potent antipromoting agents against ultraviolet and chemical carcinogenesis models (Katiyar & Mukhtar 1997; Javed *et al.* 1998; Yang *et al.* 2002). However, these studies involve the use of black tea extract that is essentially rich in theaflavins and free catechins and hence devoid of thearubigins or PBP fractions (Katiyar & Mukhtar 1997; Javed *et al.* 1998). Furthermore, mechanisms of observed protection have either been delineated *in vitro* or with limited parameters *in vivo* (Katiyar & Mukhtar 1997; Javed *et al.* 1998; Yang *et al.* 2002). Hence, this is the first report on delineating the mechanism(s) of observed antipromoting effects of PBPs 1–5 fractions or thearubigins *in vivo*.

It is evident from present study that PBPs 1–5 fractions exhibit differences between themselves in inhibiting TPA-induced skin carcinogenesis. PBP-1, -2, -3, PBP-mix and EGCG show strong antipromoting effects compared to PBP-4 and PBP-5. DMBA-initiated and TPA-promoted skin carcinogenesis is a well-established model in which, DMBA application causes H-ras mutation that is consistently activated by continual application of TPA, which further activates various signalling cascades (Quintanilla *et al.* 1991). Mechanisms of TPA-induced cell proliferation are well established in JB6 cell lines as well as in epidermis (Bode & Dong 2000; Dhar *et al.* 2002). The primary events that aid cell proliferation are activation of MAP kinases such as ERK, JNK and p38 (Ding *et al.* 2004; Afaq *et al.* 2005; Bourcier *et al.* 2006). Downstream effector molecules of these are transcription factors like c-jun, c-fos and NF- κ B which are also established TPA-responsive markers in skin carcinogenesis (Lu *et al.* 1994; Young *et al.* 1999; Hsu *et al.* 2000; Dhar *et al.* 2002). Upon activation, these transcription factors transactivate various downstream cell proliferation genes like *c-myc*, *cyclin D1*, *cox-2* and more, causing TPA-induced hyper-proliferation of epidermal cells, which is an important event governing DMBA-initiated

and TPA-promoted skin tumorigenesis (Lu *et al.* 1994; Young *et al.* 1999; Bode & Dong 2000; Hsu *et al.* 2000; Dhar *et al.* 2002; Ding *et al.* 2004; Afaq *et al.* 2005; Bourcier *et al.* 2006). In the present study, using different MAP kinase and PI3 kinase inhibitors, we have confirmed that MAP kinases, especially ERK and p38, play a crucial role in TPA-induced cell proliferation in mouse skin. Hence, our data suggest that TPA-induced epidermal cell proliferation might be inhibited by most PBP fractions by decreasing activation of these signalling kinases and transcription factors. Furthermore, studies on chemically induced skin carcinogenesis have revealed that induction of ODC activity is a necessary step in skin, mediated by MAP kinase activation (Afaq *et al.* 2005; Feith *et al.* 2005). Thus, its inhibition by PBP pre-treatment might also be *via* decreasing ODC activity.

In the present study, p21, a critical regulator of the cell cycle and cell fate in epidermis, was also induced by PBP pre-treatment, possibly by restoring TPA-mediated decreased levels of p53, indicating possible activation of cell cycle arrest (Katiyar *et al.* 1997; Weinberg & Denning 2002; Park & Surh 2004). Since abrogation of PI3 kinase and hence Akt, by chemical inhibition appears to be instrumental in maintaining levels of p53 and increasing levels of p21, PBP fractions might be doing the same by decreasing Akt activation. At this point, it is noteworthy that PBP-4 and PBP-5 display protective effects only during the earlier time points (10 and 20 weeks) of the experiment, whereas the rest of the PBP fractions continued to show significant chemopreventive efficacies, even at later time points (30 and 40 weeks). This suggests the need for prolonged chemoprevention studies instead of short-term bioassays to ascertain true chemopreventive potential of any test compound. An increase in apoptosis was suspected in PBP-pre-treated animals as compared to only TPA-treated animals, due to relatively higher levels of p53. However, apoptosis studies indicated quite the contrary, suggesting that PBP fractions as well as EGCG inhibited TPA-induced apoptosis. Thus, the effects of PBP fractions on apoptosis in epidermis might not be instrumental in PBP-mediated decrease in DMBA-TPA-induced skin carcinogenesis. This further suggests that PBP fractions may inhibit chemical carcinogenesis primarily by decreasing TPA-induced cell proliferation. The present study also poses important queries on whether TPA-induced apoptosis is p53-independent in skin and the probable mechanism(s) of inhibition of TPA-induced apoptosis, by PBP fractions. The role of MAP kinases like JNK and p38 is implicated in induction of apoptosis and hence can be speculated to be instrumental in TPA-induced apoptosis and further studies focus on delineating the mechanism (Chan *et al.* 2000; Deacon *et al.* 2003).

The matrix reactivity and highly complex polymeric nature of the PBP fractions are a major hindrance in completely characterizing these fractions (Haslam 2003; Sang *et al.* 2004). The present findings indicate that the most abundant polyphenols in black tea (PBPs) possess chemopreventive properties and hence substantiates the need to chemically characterize these fractions. The monomeric (EGCG), oligomeric (theaflavins) and polymeric (PBPs or thearubigins) tea polyphenols exhibit similar anti-initiating properties such as decrease in carcinogen-DNA adduct formation and antipromotion effects such as decrease in tumour incidence and multiplicity, in multistage chemical skin carcinogenesis (Huang *et al.* 1992; Krishnan & Maru 2004). Furthermore, mechanistically as well, the effects of PBPs on decreasing the activation of ODC, cell-signalling kinases (JNK, ERK and p38), transcription factor (AP-1) and inflammatory proteins (COX-2) are very similar to that of EGCG and theaflavins (Katiyar & Mukhtar 1997; Kundu *et al.* 2003). Although, monomeric, oligomeric and polymeric tea polyphenols display similarities in their mode of action, this study and number of other *in vitro* as well as *in vivo* findings have suggested that the tea polyphenols display varying degrees of cancer chemopreventive potential. However, in absence of information about molecular structure and weight of PBP fractions, it is difficult to compare their relative efficacies within themselves and with other monomeric

and oligomeric tea polyphenols. The observed differential activities can be attributed to probable differences in structure, solubility and bioavailability that can influence their mode of action.

In the present study, it is interesting to note that topical pre-treatment with PBPs 1–3 inhibited most of the parameters induced by TPA. On the basis of this, we speculate that PBPs might decrease the effects of TPA at the cell surface, either by quenching reactive oxygen species generated by TPA or through interacting with cell surface proteins. However, this hypothesis needs to be tested in an appropriate system. Thus, in the present study, the epidermis was pre-treated topically with PBP fractions that may result in decreased uptake of TPA and thereby cause a decrease in skin carcinogenesis. Furthermore, in the present model system, the epidermal cells are likely to be exposed to higher doses of PBP fractions for longer duration compared to dose and duration of exposure to target cells of internal organs after oral administration. The oral route of exposure to PBP fractions would simulate conditions as experienced by tea drinkers. Hence, considering the probable differences in absorption, metabolism and bioavailability of PBP fractions between topical and oral administration, antipromoting efficacies of PBP fractions established in this model system need to be confirmed by oral administration as the route of exposure.

Thus, by using a mouse skin carcinogenesis model, we have attempted to decipher molecular mechanisms of chemopreventive efficacies exhibited by PBP fractions. Present findings suggest that these possess strong antipromoting efficacies and the observed protective effects of PBP fractions can be attributed to inhibition of TPA-induced cell proliferation. As we have previously reported, and in this study also, PBP-2 has emerged as the most potent polyphenolic fraction (Krishnan & Maru 2004, 2005; Krishnan *et al.* 2005). Similar mechanistic studies in different model systems along with appropriate bioavailability information on PBP fractions and other black tea flavanols will aid in elucidating appropriate cellular targets for meaningful evaluation of chemopreventive potential of black tea in humans.

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