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Phenolic fraction of tobacco smoke inhibits BPDE-induced apoptosis response and potentiates cell transformation: Role of attenuation of p53 response

Jagat J. Mukherjee¹, Subodh Kumar, Ronald Gocinski, and Jacquan Williams

Environmental Toxicology and Chemistry Laboratory, Great Lakes Center, State University of New York College at Buffalo, 1300 Elmwood Avenue, Buffalo, New York 14222.

Abstract

Polynuclear aromatic hydrocarbons (PAHs) present in tobacco smoke are regarded as chemical carcinogens. Previously we observed that weakly acidic phenolic fraction of tobacco smoke condensate (TSCPhFr) which is devoid of PAHs significantly potentiates ($\pm$)-*anti*-BP-7,8-diol-9,10-epoxide (BPDE)-induced anchorage-independent cell growth of promotion sensitive JB6 cell indicating its tumor promoting potential. In the present communication we report that further fractionation of phenolic components from TSCPhFr did not show any significant potentiation of BPDE-induced cell transformation by any of the HPLC purified phenolic fractions indicating several phenolic components as a whole is needed for observed activity. Although the tumor promoting activity of weakly acidic phenolic fraction of tobacco smoke had been indicated long before, no studies have been pursued to understand the mechanism(s) underlying the tumor promoting activity of TSCPhFr. We observed that BPDE, an ultimate carcinogenic metabolite of tobacco smoke carcinogen benzo[a]pyrene, elicits apoptosis induction which is significantly inhibited by TSCPhFr. Increased cell transformation and decreased apoptosis by TSCPhFr was associated with attenuation of BPDE-induced p53 accumulation. JB6 cells transfected with p53 siRNA showed significantly less apoptosis induction by BPDE compared to control cells. In p53 impaired cells (which are observed to have faster growth rate compared to normal cells) TSCPhFr has practically negligible effect on apoptosis induction in response to BPDE. Also in p53 null HCT116 p53^{-/-} cells BPDE-induced apoptosis is unresponsive to TSCPhFr. Inhibition of BPDE-induced NF- κ B activation was also observed by us previously. Interestingly treatment of cells with NF- κ B-specific inhibitor IKK-NBD peptide showed no effect on BPDE-induced apoptosis, whereas TSCPhFr showed moderate inhibition of apoptosis in NF- κ B inhibited cells compared to control cells. Our observations indicate that attenuation of BPDE-induced p53 response has a role in apoptosis inhibition and increased cell transformation by TSCPhFr. These findings have implication with regard to the underlying mechanism of tumor promoting activity of TSCPhFr in PAH-induced carcinogenesis. Although p53 mediated NF- κ B activation has a role in apoptosis induction, the role of NF- κ B in TSCPhFr mediated potentiation of PAH-induced cell transformation is not clear from our studies.

Introduction

Polynuclear aromatic hydrocarbons (PAHs) represent a major constituent of tobacco smoke and the tumorigenic potential of PAHs is well documented (1–3). PAHs require metabolic activation to exert their carcinogenic effects. For example benzo[a]pyrene (BP) present in

¹To whom correspondence should be addressed jmmukher@buffalostate.edu.

Conflict of interest statement

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tobacco smoke is metabolically activated by the cytochrome P-450-dependent monooxygenase system preferentially to anti-BP-7,8-diol-9,10-epoxide [BPDE], a reactive electrophile which binds to cellular DNA predominantly at the N² position of deoxyguanosine (dG) and which is implicated as the ultimate carcinogenic metabolite of BP (4). The carcinogenicity of tobacco smoke condensate (TSC) in mouse skin and the known content of PAHs in TSC suggest that the PAHs present in the cigarette smoke do not account for the observed TSC carcinogenicity (5, 6). The tumor-promoting activity of tobacco smoke condensate (TSC) on PAH-initiated mouse skin has been reported (6, 7). Early attempts to assess the relative contribution of tumorigenic agents present in TSC showed three discrete fractions (neutral, acidic and nicotine-free basic fractions) none of which could account for the total tumorigenic activity of whole TSC (8). Later it was demonstrated that the weakly acidic fraction of TSC called the phenolic fraction possesses strong tumor-promoting activity comparable to that of whole TSC (9, 10). Tobacco smoke contains more than 200 semi-volatile phenolic compounds (11). The major phenolic components in tobacco smoke are phenols; alkyl substituted phenols e.g. cresols, ethyl/dimethyl/trimethyl phenols; di- and trihydroxybenzenes e.g. catechols, resorcinol, hydroxyquinone etc. (11, 12). It is not known whether single or several phenolic components present in tobacco smoke condensate phenolic fraction (TSCPhFr) is(are) required for tumor promoting activity comparable to TSCPhFr.

PAHs are known to be DNA damaging and an important component of cellular response to DNA damage is the induction of p53 protein (12). BP and other PAHs induce p53 in animal or cell systems (13, 14). Tumor promoters are known to inhibit p53 induction in response to DNA-damage induced by BP and other DNA-damaging agents in mouse and human cells (15–17). Important downstream events of p53 response include regulation of transcription of target gene products involved in growth arrest, DNA repair and apoptosis (18–23) which represent the protective events in response to DNA damage. Impairment of DNA damage-induced p53 response can abrogate aforementioned cellular protective responses thereby favoring tumorigenesis. Besides p53, NF-κB is another transcription factor which is activated in response to DNA damage. NF-κB is known to have either anti- or pro-apoptotic function depending on the nature of apoptotic stimulus (24–26). Thus depending on the nature of external stimulus, NF-κB activation may have dual roles on DNA damage-induced cellular protective response of apoptosis.

Although the tumor promoting activity of TSCPhFr has been observed before no studies have been pursued to understand the underlying mechanism of tumor promotion by TSCPhFr. In this context we previously showed that TSCPhFr significantly increased BPDE-induced cell transformation and attenuates intracellular PKC substrate phosphorylation (27). It is also observed that inhibition of PKC delta by pharmacological inhibitor rottlerin potentiates BPDE-induced cell transformation (27) indicating abrogation of PKC delta may have a role in cell transformative function of TSCPhFr. Other studies also showed that PKCdelta suppresses neoplastic phenotype in HCT116 human colon cancer cells and homozygous knockout of p53 renders HCT116 cells unresponsive to PKC-delta mediated suppression of transformed phenotype (28) indicating p53's role in tumor suppressive function of PKC-delta. In this study we report that attenuation of p53 response has a role in anti-apoptotic function of TSCPhFr in BPDE treated cells and the role of NF-κB activation in this regard is not definitive.

Materials and methods

Cells and reagents

Promotion sensitive JB6 Cl41 mouse epidermal cell line and HCT 116 colorectal carcinoma cell line were obtained from American Type Culture Collection (ATCC, VA, USA). P53

knock out HCT 116 (HCT 116^{p53-/-}) was a kind gift from Dr. M. Saeed Sheikh (SUNY Upstate Medical University, Syracuse, USA). IKK-NBD peptide from Enzo Life Sciences, N.Y., (+/-)-anti-BPDE from the NCI Chemical Carcinogen Reference Standard Repository. Tobacco smoke condensate was obtained from Arista Laboratories (Richmond, VA); modified Eagle's Medium (MEM) and fetal bovine serum (FBS) from Invitrogen Life Technologies (CA). All other chemicals were of analytical grade.

Cell Culture and treatment

JB6 Cl 41 cells were cultured in a humid atmosphere at 37 °C and 5% CO₂ using modified Eagle's medium (MEM) containing 5% fetal bovine serum, 2 mM L-glutamine, 10 mM sodium pyruvate and penicillin/streptomycin (50 µg/ml each). HCT 116 cells were grown as monolayers in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin (50 µg/ml each). Cells were checked on a routine basis for *Mycoplasma* contamination by the Gibco Mycotect.

Unless mentioned otherwise cells were treated with BPDE dissolved in the vehicle tetrahydrofuran (concentration < 0.05% in the cell culture medium) for 1 hour. To examine the effect of TSCPhFr, cells were treated with 0.5 µM BPDE for 1 hour followed by treatment with TSCPhFr (20 µg/ml) for the entire period until harvest. Cells were harvested at different time intervals after BPDE treatment as described in each experiment.

Purification of phenolic components from TSCPhFr

Weakly acidic phenolic fraction from tobacco smoke condensate (TSCPhFr) was obtained by solvent fractionation as described by us before (27). TSCPhFr thus obtained was subjected to further purification by reverse phase HPLC using Zorbax C18 column (0.5 micron, 250 mm × 4.6 mm). Conditions for separation are given in Figure 1. The effluent was monitored for UV absorption on UV detector at 254 nm suitable for measurement of phenolic compounds. Total fractions eluted were reconstituted as four discrete fractions (I, II, III, and IV) based on retention times as shown in Figure 1, dried under nitrogen, reconstituted in THF and finally stored at -86°C in dark sealed vials under Argon until used for treatment of cells.

Anchorage-independent cell growth assay

Anchorage-independent growth of promotion-sensitive JB6 Cl41 cells on soft agar was determined by following the procedure as described by others (29). Briefly, 60mm culture dishes were first plated with 7 ml of 0.5% agar EMEM containing 10% calf serum (heat inactivated) and allowed to solidify. Exponentially growing JB6 cl41 cells after trypsinization (10,000 cells) were either treated with BPDE (0.5µM) alone or with BPDE (0.5µM) first for 1 hour followed by treatment with different preparations of TSCPhFr (20 µg/ml) in 1.5 ml 0.33% agar EMEM (10% serum). After treatment cells in the top agar (0.33% agar) were then poured onto the pre-hardened base medium containing 0.5% agar. The dishes were incubated at 37°C (5% CO₂). After 14 days the number of colonies (containing at least 8 cells) in each dish was counted.

Apoptosis assay

Apoptosis assay was performed either by using Cell Death Detection ELISA^{Plus} apoptosis kit (Roche Applied Science, IN; Cat. No. 1774425) or by FITC-annexin V/propidium iodide (PI) staining. For ELISA assay we followed the procedure described by us before (30). Briefly, cells were cultured in a 96-well plate, and after appropriate treatments cells were further incubated in serum-containing medium for 24 hours and apoptosis were followed by measuring the levels of cytosolic histone-bound DNA fragments following the instructions

given in the company manual. Apoptosis in each well was determined by measuring the optical density at a wavelength of 405 nm with a reference wavelength of 492 nm in a microplate reader. The absorbance readings were used to represent the degree of apoptosis. Cells treated with 20 μ M hydrogen peroxide (a known apoptosis inducer) represent positive control for apoptosis experiment using Roche ELISA kit.

Apoptosis assay in JB6 cells was also performed by FITC-annexin V/propidium iodide (PI) staining as described by others (58). Briefly, Cell suspensions were mixed with five μ l of FITC-labeled annexin V followed by incubation at room temperature for 10 minutes, and then two μ l of PI solution (10 μ g/ml) was added to the cells. The scatter parameters of the cells (10,000 cells per experiment) were analyzed by using Becton Dickinson FACScans system. Viable population (PI positive), early apoptotic (annexin positive), late apoptotic and necrotic (both annexin and PI positive) cells were counted and the data were analyzed using WinList software (Variety Software House, Topsham, ME).

Western immunoblot of p53 protein

After appropriate treatments cells were lysed in lysis buffer consisting of 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM sodium β -glycerophosphate, 1 mM Na_3VO_4 , 10 μ g/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 5 μ g/ml leupeptin, and 100 μ g/ml aprotinin (Sigma). An equal amount of the cell extract was separated by 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P (Millipore) membrane. After transferring the proteins, the membrane was blocked in 5% skim milk powder. For p53 detection, the membrane was incubated with rabbit polyclonal antibody p53 (Pab 240) (Santa Cruz Biotechnology Inc., CA) at a concentration of 0.7 μ g antibody/ml solution. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) was used as secondary antibody. The proteins were visualized by enhanced chemiluminescence using Amersham's ECL Western Blotting Detection Reagents (Amersham Biosciences, NJ, USA).

Transient transfection of p53-targeted siRNA

In a 6-well tissue culture plate 3×10^5 JB6 Cl41 cells were seeded per well in 2 ml antibiotic-free medium supplemented with 5% fetal bovine serum and cultured for 24 hours to have 50–60% confluency. Cells were transfected with p53 siRNA (mouse) duplex (Santa Cruz Biotechnology Inc., CA) following the instructions given in company protocol. Briefly, cells were transfected with 100 pmols siRNA in presence of 5 μ l siRNA transfection reagent (Santa Cruz Biotechnology Inc., CA) for 5 hours at 37°C followed by addition of fresh growth medium (1 ml) containing 10% FBS without removing the transfection mixture and further incubation for 24 hours. Control cells were transfected with negative control siRNA-A (Santa Cruz Biotechnology Inc., CA; Cat. No. sc-37007) consisting of scrambled sequence. Medium was replaced by fresh medium and cells were harvested 24 hours after transfection for p53 Western immunoblot assay. For chemical exposure, 24 hours after transfection, cells were appropriately treated and respective response was monitored.

Results

Phenolic components of TSCPhFr as a whole is required for potentiation of anchorage-independent growth of BPDE treated JB6 Cl41 cells

We previously observed that weakly acidic TSCPhFr obtained from tobacco smoke condensate (TSC) potentiates anchorage-independent growth on soft agar (indication of cell transformation) of promotion sensitive mouse epidermal JB6 Cl41 cells (27). In an effort to identify the active phenolic component(s) responsible for potentiation of BPDE-induced cell transformation, we further separated crude TSCPhFr by HPLC using C18 reverse phase

column (Fig. 1). Since the peaks are not base line separated we collected the eluted peaks as four discrete fractions designated as fraction I (12.0–17.5 min), fraction II (17.5–24.5 min), fraction III (24.5–25.5 min) and fraction IV (25.5–33.0 min). The respective fractions were collected under argon to inhibit any oxidative change of the phenolic components. We observed that unfractionated crude TSCPhFr at 20 µg/ml concentration significantly potentiates BPDE-induced anchorage-independent cell growth on soft agar (3 fold), whereas none of the four fractions at similar concentration showed any significant potentiating effect on BPDE-induced cell transformation (Fig. 2). Combination of four fractions (I to IV) also show significant potentiation of BPDE-induced cell growth on soft agar (2.6 fold) although to a lesser extent compared to unfractionated TSCPhFr. It is interesting that the extent of potentiation by combined four HPLC fractions is much higher than the additive effect of individual HPLC fraction (1.8 fold when individual effects are added). This may suggest that the increased potentiating effect of combined four fractions may involve synergistic interactions. We also examined the effect of several binary and ternary combinations of respective fractions on anchorage-independent cell growth and it was observed that none of the combinations show any significant potentiation of BPDE-induced cell growth on soft agar (data not shown). This finding indicates that several phenolic components present in crude TSCPhFr and distributed over four HPLC separated fractions as a whole mediates the observed effect of TSCPhFr.

TSCPhFr inhibits BPDE-induced apoptosis response

In order to understand the mechanism of potentiation of *in vitro* cell transformation ability of BPDE by TSCPhFr, we examined whether TSCPhFr interferes with PAH-induced cellular protective response of apoptosis since our previous findings showed that BPDE treatment of different cell lines is associated with induction of apoptosis (30). We determined BPDE-induced apoptosis response in JB6 Cl41 cells in presence of unfractionated crude TSCPhFr or HPLC purified four phenolic fractions individually. Apoptosis determined by using ELISA assay kit (figure 3A) show that BPDE (0.5 µM) elicits induction of apoptosis in Cl41 cells and unfractionated TSCPhFr (20 µg/ml) significantly inhibits BPDE-induced apoptosis response by 72%. None of the four HPLC purified fractions (each 20 µg/ml) showed any significant modulation of BPDE-induced apoptosis indicating several phenolic components present in TSCPhFr are required for inhibition of BPDE-induced apoptosis. Flow cytometry experiment with FITC-annexin V/PI staining also showed BPDE-induced apoptosis response in JB6 cells and the interference of this apoptosis response by TSCPhFr (figure 3B). It is evident that TSCPhFr (20 µg/ml) significantly decreases BPDE-induced early apoptotic cell population (13.54% of total) by 87%, whereas the effect on necrotic and late apoptotic cell population together (7.55% of total) is marginal with 9% increase. The marginal increase may be either due to the additional necrotic effect of TSCPhFr or due to conversion of respective proportion of early apoptotic cells (9%) to necrotic phase. Even we observed a marginal increase in necrotic cell population by TSCPhFr, aforementioned findings clearly indicate that TSCPhFr has ability to inhibit BPDE-induced apoptosis response to a significant extent.

Thus potentiation of BPDE-induced cell transformation as well as attenuation of BPDE-induced apoptosis response by HPLC purified fractions of TSCPhFr is impaired when compared to unfractionated TSCPhFr. These findings indicate that apoptosis inhibition by TSCPhFr possibly has a role in potentiation of BPDE-induced anchorage-independent cell growth and this may represent one of the mechanisms by which TSCPhFr potentiates BPDE-induced cell transformation.

Inhibition of BPDE-induced apoptosis response by TSCPhFr is associated with attenuation of p53 response

For further mechanistic insights with respect to inhibition of BPDE-induced apoptosis response by TSCPhFr, we examined its effect on signaling pathway relevant to DNA damage-induced apoptosis induction and cell transformation. The transcription factors p53 and NF- κ B are known to be the important regulators of apoptosis and cell growth in response to cellular stimuli (24–26, 31–35). Previously we observed that TSCPhFr inhibits BPDE-induced NF- κ B activation in JB6 Cl41 cells harboring NF- κ B reporter plasmid (27). Here we examined the effect of TSCPhFr on p53 response in BPDE treated cells. It is observed that BPDE (0.5 μ M) treatment of cells caused a significant accumulation of p53 protein, whereas in presence of TSCPhFr (20 μ g/ml) BPDE-induced p53 accumulation is attenuated by 73% (determined by measuring the intensity of p53 band in Western immunoblot using Alpha Inotech software) (Fig. 4). In this context it is also observed that none of the HPLC purified phenolic fractions is effective in attenuation of BPDE-induced p53 accumulation indicating several phenolic components present in crude TSCPhFr as a whole are required for the observed inhibitory effect on p53 response.

Inhibition of BPDE-induced apoptosis by TSCPhFr is greatly interfered by p53 impairment

Attenuation of BPDE-induced p53 response by TSCPhFr prompted us to examine whether it has any role in apoptosis inhibition in BPDE treated cells. JB6 Cl41 cells were transiently transfected with p53 siRNA (Santa Cruz Biotechnology, CA) to down-regulate p53 expression following the procedure as described in Materials and Methods. Down-regulation of p53 expression was confirmed by Western immunoblotting of p53 protein (Fig. 5A) which shows that p53 accumulation by BPDE is significantly inhibited in p53 siRNA transfected cells. In p53 siRNA transfected cells BPDE-induced accumulation of p21 protein, a direct transactivation target of p53, is also significantly inhibited (Figure 5A, lower panel). These observations suggest that in p53 siRNA transfected cells down-regulation of p53 expression is associated with abrogation of its function. BPDE-induced apoptosis response was found to be less in p53-siRNA transfected cells (3.5 fold increase) compared to untransfected cells (6 fold increase) (Fig. 5B). Interestingly inhibition of BPDE-induced apoptosis by TSCPhFr is marginal (27%) in p53-siRNA transfected cells compared to 72% in untransfected cells as shown in figure 3. We further examined the effect of TSCPhFr in colorectal cancer cells p53-WT HCT116 and p53-null HCT116 (p53^{-/-}). BPDE treatment of HCT116 (p53^{-/-}) cells was unable to induce accumulation of p53 protein as opposed to p53-WT HCT116 cells (Fig.5C). In HCT116 p53-null cells BPDE-induced apoptosis is 75% less compared to HCT116 p53-WT cells (Fig.5D). TSCPhFr inhibited BPDE-induced apoptosis by 62% in p53-WT cells whereas the ability of TSCPhFr to inhibit BPDE-induced apoptosis is almost lost in p53-null cells (Fig.5D). These findings indicate that attenuation of p53 response play a role in inhibition of BPDE-induced apoptosis by TSCPhFr.

Variable effects of NF- κ B inhibition on BPDE-induced apoptosis in presence and absence of TSCPhFr

We previously observed that BPDE induces NF- κ B activation in JB6 cells and TSCPhFr significantly inhibits BPDE-induced NF- κ B activation (27). NF- κ B is known to have either anti- or pro-apoptotic function depending on the nature of apoptotic stimulus (24–26). Here we examined whether NF- κ B inhibition by TSCPhFr has any role in apoptosis inhibition. We measured BPDE-induced apoptosis response in co-presence of IKK-NBD peptide, a NF- κ B-specific inhibitor (Enzo Life Sciences, NY) and TSCPhFr (Figure 6). IKK-NBD peptide at 100 μ M concentration was found to inhibit BPDE-induced NF- κ B activation significantly (>80%) in NF- κ B reporter JB6 cells (data not shown). We observed that treatment of JB6 cells with IKK-NBD peptide alone induces apoptosis response indicating NF- κ B inhibition is pro-apoptotic. This indicates that NF- κ B inhibition as we observed previously (27) may

not have any role in anti-apoptotic function of TSCPhFr. We also observed that the extent of cellular apoptosis response in presence of BPDE and NBD peptide together is mostly the same as the sum of the individual response by BPDE and NBD peptide. This indicates that NF- κ B inhibition does not interfere with apoptosis induction by BPDE. Interestingly, in presence of NBD peptide inhibition of BPDE-induced apoptosis by TSCPhFr is much less (40%, figure 6) compared to the inhibition in absence of NBD peptide (72%, see figure 3). Different apoptosis responses, induced by NF- κ B inhibition in presence and absence of BPDE and TSCPhFr as observed, do not clearly suggest any definitive role of NF- κ B in TSCPhFr-mediated inhibition of BPDE-induced apoptosis.

Discussion

We previously showed that the weakly acidic phenolic fraction of tobacco smoke condensate (TSCPhFr) potentiates BPDE-induced anchorage-independent cell growth (27). This observation has implication in its ability to function as a tumor promoter by potentiating PAH-induced cell transformation. In this context, our attempt to purify the phenolic component(s) responsible for potentiation of anchorage-independent cell growth shows that several phenolic components present in TSCPhFr as a whole is needed for observed effect of potentiation. The mechanism by which TSCPhFr potentiates PAH-induced cell transformation is not known. In the present investigation, we pursued studies to obtain an insight into the mechanism by which TSCPhFr potentiates cell transformation. It is known that BPDE-induced DNA damage elicits cellular protective response of apoptosis (36–42) to eliminate the damaged cells, and it is expected that any interference with this protective apoptotic response will favor survival of damaged cells leading to permanent mutational fixation of the damage and cell cycle de-regulation. We examined whether TSCPhFr has any modulating effect on BPDE-induced apoptosis response. Our data show that potentiation of BPDE-induced anchorage-independent cell growth by TSCPhFr is associated with significant inhibition of BPDE-induced apoptosis response indicating apoptosis inhibition may have a role in potentiation of anchorage-independent cell growth. This view is supported by our further observation that inability of HPLC fractions purified from TSCPhFr to potentiate anchorage-independent cell growth is associated with inability to inhibit apoptosis response in BPDE treated cells. One can argue that inhibition of BPDE-induced apoptosis response by TSCPhFr may not reflect the actual interference with apoptosis; rather this may be due to the conversion of apoptotic cells to necrotic phase which is not identifiable by the ELISA method used in apoptosis assay. Our alternative apoptosis assay by Flow Cytometry with FITC-annexin V/PI staining clearly show that BPDE-induced cell death is due to mostly apoptosis and some necrosis, and TSCPhFr decreases BPDE-induced early apoptotic cell population by 87% with a marginal effect on late apoptotic and necrotic cell populations. Thus our findings clearly demonstrate that TSCPhFr has ability to interfere with BPDE-induced apoptosis response.

DNA damage-induced p53 response is known to play a pivotal role in induction of cellular protective response of apoptosis (31, 33, 43–46). We and others observed that BPDE treatment of cells induces p53 accumulation (47–49). In the present study we show that TSCPhFr attenuates BPDE-induced p53 accumulation as well as inhibits apoptosis response indicating p53 attenuation may have a role in apoptosis inhibition. This view is confirmed by our observation that p53 impairment in JB6 cells or in colorectal cancer cells (HCT 116) not only inhibits BPDE-induced apoptosis but also impairs ability of TSCPhFr to inhibit BPDE-induced apoptosis. In this context, it is worth mention that BPDE is still able to induce apoptosis in p53 impaired cells particularly in p53 null-HCT 116 cells. So the observed BPDE-induced apoptosis response involves both p53-dependent and p53-independent signaling pathways. However, impairment of anti-apoptotic function of TSCPhFr in BPDE treated p53 deficient cells suggests the role of p53 attenuation in this

regard. One can argue that anti-oxidative function of phenols present in TSCPhFr may reduce BPDE resulting in decreased BPDE-DNA adduct formation, which may be responsible for observed decreased apoptosis response by TSCPhFr. In our experimental protocol cells were first treated with BPDE for 1 hour followed by TSCPhFr. BPDE, which is the ultimate carcinogenic metabolite of the PAH benzopyrene (BP) is highly unstable in aqueous medium and instantaneously binds to DNA. So, BPDE-DNA adduct formation will be complete within 1 hour of pre-treatment of cells with BPDE. Consequently, the anti-oxidative function of TSCPhFr is practically unable to reduce BPDE because it is no more available as free reducible molecule.

Our previous study showed that attenuation of PKC activity (particularly PKC delta) is involved in potentiation of BPDE-induced anchorage-independent cell growth by TSCPhFr (27). It is also observed by others that homozygous knockout of p53 renders HCT116 cell line unresponsive to tumor suppression by PKCdelta overexpression indicating p53 might be involved in the suppression of the neoplastic phenotype induced by PKCdelta (28). Other studies also indicate that PKC-delta potentiates p53-dependent apoptosis in response to genotoxic stress (59) and PKC-delta is a potential upstream regulator of p53 both at transcriptional and post-translational levels (60). Thus our previous finding of down-regulation of PKC-delta by TSCPhFr (27) constitutes a logical ground of the expectation that down-regulation of BPDE-induced p53 response may play a role in apoptosis inhibition associated with increased cell transformation by TSCPhFr as implicated in our present report. Thus our present finding of the role of p53 in this regard is the validation of our previous findings with respect to the expected role of PKC-delta in p53-dependent regulation of apoptosis and cell transformation.

Regarding further downstream effect of p53 signaling, it is reported that the transcription factor NF- κ B may either be positively or negatively regulated by p53 status (50–52). Our previous observation showed that BPDE treatment of cells elicits NF- κ B activation and TSCPhFr significantly inhibits BPDE induced NF- κ B activation (27). So, our present observation of p53 induction by BPDE is an associated phenomenon with NF- κ B activation indicating the possibility of positive correlation between p53 and NF- κ B responses. Ability of BPDE to induce NF- κ B activation has also been reported by others (53). NF- κ B has been reported to have both pro- and anti-apoptotic role depending on the cellular stimuli (24–26). In an attempt to understand the role of NF- κ B in our present study, we observed that NF- κ B inhibition by NBD peptide partially impairs the ability of TSCPhFr to inhibit BPDE-induced apoptosis indicating NF- κ B inhibition may have a role in anti-apoptotic and cell transformative function of TSCPhFr. Interestingly, we also observed that NF- κ B inhibition by NBD-peptide is pro-apoptotic by itself and BPDE-induced apoptosis is not interfered by NF- κ B inhibition. Induction of apoptosis by treatment of cells with NF- κ B inhibitors including NBD-peptide has been observed by others (54–57) implying anti-apoptotic function of NF- κ B. These observations raised the doubt about any possible role of NF- κ B inhibition in anti-apoptotic and cell transformative function of TSCPhFr. Thus based on differential apoptosis responses by NF- κ B inhibition under different scenario, we concluded that NF- κ B inhibition may have a role in anti-apoptotic function of TSCPhFr although not definitive.

In conclusion, the data obtained from these studies indicate that several phenolic components present in weakly acidic phenolic fraction of tobacco smoke (TSCPhFr) collectively has ability to potentiate BPDE-induced tumorigenicity by inhibiting apoptosis response. Attenuation of BPDE-induced p53 response by TSCPhFr may have a role in this regard, whereas the role of NF- κ B inhibition is not definitive. Further studies are needed in this regard.

Abbreviations

BP	benzo[a]pyrene
BPDE	(+/-)- <i>anti</i> -benzo[a]pyrene-7,8-diol-9,10-epoxide
PAH	polynuclear aromatic hydrocarbon
TSCPhFr	phenolic fraction of tobacco smoke condensate
NF-κB	nuclear factor-kappa B
IKK-NBD	IKK-NEMO binding domain

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Figure 1.

Phenolic components of TSCPhFr were resolved on a Zorbax C-18 column eluted with 30% methanol-water for 0–5 minutes, 30–50% methanol-water for 15 minutes and 50–100% methanol-water for 30 minutes with a flow rate of 1 ml/min. The column effluent was monitored at 254 nm wavelength for the presence of phenolic components. Pooled fractions are designated as fraction I (12.0–17.5 min), fraction II (17.5–24.5 min), fraction III (24.5–25.5 min) and fraction IV (25.5–33.0 min). Other details are given text.

Figure 2.

Effect of TSC phenolic fractions on BPDE-induced anchorage-independent growth of JB6 Cl41 cells. Cells were either treated with 0.5 μ M BPDE (1 hour) or BPDE (1 hour) followed by TSC phenolic fraction (20 μ g/ml) in 0.33% top agar medium as described in the “Materials and methods”. Each bar shows the average of triplicate experiments and the error bars indicate SD. (*) indicates statistical analysis of significance with paired t-Test ($p < 0.05$).

Figure 3.

Effect of TSC phenolic fractions on apoptosis response to BPDE. Cells were either treated with 0.5 μ M BPDE (1 hour) or BPDE (1 hour) followed by respective TSC phenolic fraction (20 μ g/ml) and harvested 24 hours after BPDE treatment to determine apoptosis response. (A) Apoptosis was monitored using a cell death enzyme-linked immunosorbent assay kit (Roche Applied Science, IN) as described in the text. Each bar shows the average of triplicate experiments and the error bars indicate SD. (*) indicates statistical analysis of significance with paired t-Test ($p < 0.05$). (B) Apoptosis was measured by staining with FITC-annexin V and PI using FACScan flow cytometer as described in the text. Cells were either untreated (i) or treated with 0.5 μ M BPDE (ii) or 0.5 μ M BPDE plus 20 μ g/ml TSCPhFr (iii) followed by harvesting cells 24 hours after treatment to determine apoptosis response. The quantitative measure of different cell populations e.g. viable (lower left quadrant R3), early apoptotic ((lower right quadrant R4), late apoptotic or necrotic (upper right quadrant R2) and necrotic ((upper left quadrant R1) are also shown in the insert of the figure.

Figure 4.

Effect of TSC phenolic fractions on p53 accumulation in BPDE treated JB6 Cl41 cells. Cells were either treated with 0.5 μM BPDE or BPDE plus respective TSC phenolic fraction (20 μg/ml). Cells were harvested 20 hours after BPDE treatment and p53 level in the cell extract was detected by Western immunoblotting using anti-p53 antibody. Other experimental details are described in the text.

Figure 5. p53 inhibition impairs the ability of TSCPhFr to inhibit BPDE-induced apoptosis response. Treatment of cells with BPDE and TSCPhFr has been described in the text. (A) Western immunoblot of p53 protein (upper panel) and the p53 transactivation product p21 protein (lower panel) in response to BPDE in JB6 control cells (transfected with scrambled siRNA) and cells transfected with p53 targeting siRNA duplex. For detection, p53 and p21 proteins were immunoblotted with p53 (Pab 240) and p21 (C-19) primary antibodies (Santa Cruz Biotechnology Inc., CA) respectively. (B) Apoptosis response by BPDE in untransfected and p53 siRNA transfected JB6 C141 cells in presence and absence of TSCPhFr. p53 siRNA transfection and apoptosis assay are described in the text. Each bar shows the average of triplicate experiments and the error bars indicate SD. (C) Western blot analysis of p53 protein expression in response to BPDE in p53-WT HCT 116 and p53-null HCT 116(p53^{-/-}) cells. (D) Apoptosis response by BPDE in p53-WT HCT 116 and p53-null HCT 116(p53^{-/-}) cells in presence and absence of TSCPhFr. Each bar shows the average of triplicate experiments and the error bars indicate SD.

Figure 6.

NF- κ B inhibition induces apoptosis by itself but partially attenuates the ability of TSCPhFr to inhibit BPDE-induced apoptosis response. For NF- κ B inhibition either untreated or BPDE treated JB6 cells (1 hour) were further treated with NF- κ B-specific inhibitor IKK-NBD peptide (100 μ M) for 24 hours. To examine the effect of TSCPhFr, BPDE treated JB6 cells were further treated with IKK-NBD peptide (100 μ M) and TSCPhFr (20 μ g/ml) for 24 hours. Apoptosis response was monitored 24 hours after BPDE treatment. Each bar shows the average of triplicate experiments and the error bars indicate SD.