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Phenolic fraction of tobacco smoke condensate potentiates benzo [a]pyrene diol epoxide-induced cell transformation: Role of protein kinase C

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1. Introduction

Tobacco smoke condensate (TSC) has been demonstrated to be tumorigenic in several animal species [1–7]. Tobacco smoke contains a variety of compounds of which at least 40 compounds have carcinogenic potential [8–10]. Polynuclear aromatic hydrocarbons (PAHs) represent major constituents of tobacco smoke and the tumorigenic potential of PAHs is well documented [9–12]. PAHs by itself are not carcinogenic, but require metabolic activation to exert their carcinogenic effects. For example benzo(a)pyrene (BP), a prototype PAH, present in TSC 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 [13]. The carcinogenicity of TSC in mouse skin and the known content of PAHs in TSC suggest that the PAHs present in the cigarette smoke do not fully account for the observed TSC carcinogenicity [5,14,15]. Therefore, it was speculated that either other carcinogens or tumor-promoting substances, or both, are present in the TSC. 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 [16,17]. In animal studies it is observed that the weakly acidic phenolic components of tobacco smoke potentiates tobacco smoke neutral components (PAHs) induced tumorigenicity [16,17]. Tobacco smoke contains more than 200 semi-volatile phenolic compounds [18]. The major phenolic components in tobacco smoke are phenols; alkyl substituted phenols e.g. cresols, ethyl/dimethyl/trimethyl phenols; di- and trihydroxybenzenes e.g. catechol, resorcinol, hydroquinone, etc. [18]. The underlying mechanism of tumor promotion by TSC phenolic fraction is unknown. The possible mechanism(s) of tumor promotion by this fraction may include any of the known biological effects of established tumor promoters.

In context of tumor promotion, it is observed that AP-1 transcription factor has a role in TPA-induced cell transformation in JB6 (P⁺) cells (48,49). It is also observed that NF-κB activity

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

is required for the maintenance of transformed phenotypes in JB6 cells (51,52). Modulation of transcription factors AP-1/NF- κ B and activation of PKC by established tumor promoter TPA and others has been well documented in different cell lines including JB6 cells [19–28]. Although they are differentially modulated depending on cell types and other criteria [19–34] down-regulation of PKC has been implicated to have a role in tumor promotion [27,28, 36]. It is observed that activation of PKC or increased intracellular PKC substrate phosphorylation is inversely related to tumor promotion in JB6 (P⁺) cells (35,50). In this study to gain an insight into the molecular mechanism by which weakly acidic phenolic fraction potentiates PAH-induced carcinogenicity, we examined its effect on activation of AP-1, NF- κ B and PKC in BPDE treated cells. Our results show that down-regulation of PKC by TSC phenolic fraction may have a role in potentiation of BPDE-induced carcinogenicity.

2. Materials and Methods

2.1. Cells and reagents

Promotion sensitive mouse epidermal JB6 Cl 41 cells were obtained from American Type Culture Collection (ATCC, VA, USA). Cl 41 cells stably transfected with firefly luciferase reporter gene driven by minimal NF- κ B or AP-1-responsive region were the gift from Dr. Nancy Colburn, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD. (+/-)-anti-BPDE was purchased from the NCI Chemical Carcinogen Reference Standard Repository. Tobacco smoke condensate was obtained from Arista Laboratories (Richmond, VA); phospho-(Ser) PKC Substrate antibody from Cell Signaling Technology (MA); PKC inhibitors staurosporine, bisindolylmaleimide, Go6976 and rottlerin from EMD Chemicals, Inc. (Gibbstown, NJ); modified Eagle's Medium (MEM) and fetal calf serum (FCS) from Invitrogen Life Technologies (CA). All other chemicals were of analytical grade.

2.2. Cell culture

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 calf serum, 2 mM L-glutamine, 10 mM sodium pyruvate and penicillin/streptomycin (50 µg/ml each). Cells were checked on a routine basis for *Mycoplasma* contamination by the Gibco Mycotect.

2.3. Preparation of Phenolic Fraction from Tobacco Smoke Condensate (TSC)

The whole tobacco smoke condensate was obtained from Arista Laboratories, Richmond, VA. The weakly acidic phenolic fraction of TSC was prepared following the procedure as described elsewhere [16] and is depicted in chart 1. TSC from 1000 cigarettes (15 g TSC) was dissolved in a mixture of 2N HCl and peroxide-free ether (1:3 v/v). The aqueous acidic layer was separated and the ether layer was then washed with saturated sodium bicarbonate to remove carboxylic acids, and with portions of 3% KOH to remove any carboxylic acid remaining together with the phenols present. The phenols were recovered from the KOH washing, neutralized with 2N HCl, excess sodium bicarbonate added, and the mixture was saturated with NaCl and extracted with portions of ether. The combined ether washings containing phenols were dried over anhydrous MgSO₄ and the solvent is removed by heating in a water bath. Phenols obtained (yield 1.4 g) were stored in aliquots in small sealed dark ampoules at -25°C and is called the phenolic fraction of TSC. In order to avoid any significant changes of the phenolic samples due to storage, the stored samples of TSC phenolic fraction from each preparation was used within 3 to 4 weeks and each small ampoule once opened was used for one time.

2.4. Determination of anchorage-independent cell growth

Anchorage-independent growth of promotion-sensitive JB6 Cl41 cells on soft agar was determined by following the procedure as described by others [37]. 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 (0.5 μ M) alone or with BPDE (0.5 μ M) first for 1 hour followed by treatment with noncytotoxic concentration of TSCPhFr (10 and 30 μ g/ml) in 1.5 ml 0.33% agar EMEM (10% serum). After treatment cells in the top agar were then poured onto the prehardened 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.

2.5. NF- κ B and AP-1 activation assay

The activation of the transcription factors NF- κ B or AP-1 was determined in appropriately treated JB6 (Cl 41) cells harboring either NF- κ B or AP-1-luciferase reporter plasmids. After selection in presence of 400 μ g/ml G418 cells in mid-log growth, cells were treated with BPDE dissolved in DMSO (0.05–0.1% of the culture volume) for 1 hour in serum-free medium and further incubated in medium containing 5% serum for 16 hours (unless mentioned otherwise) in the absence of BPDE. To examine the effect of TSC phenolic fraction, cells were first treated with BPDE for 1 hour in serum free medium and then with TSC phenolic fraction in 5% serum-containing MEM. Cells were harvested 16 hours after BPDE treatment. Luciferase activity in respective cell extract was performed according to the manufacturer's instructions using Luciferase Assay kit (Promega, CA). Briefly, cells were lysed in lysis buffer (supplied in the kit), freeze-thawed and then centrifuged briefly at 14,000 rpm. Twenty μ l of the supernatant was mixed with 100 μ l of the luciferase assay reagent, and the light intensity was measured by a Luminometer with a 2 seconds measurement delay and 10 seconds measurement read. NF- κ B/AP-1 activity in the respective cell extract was expressed as light units (LU) per mg protein.

2.6. Determination of intracellular PKC substrate phosphorylation

We performed Western immunoblotting of the cell extract (lysed in SDS sample buffer 6 hours after BPDE treatment) to determine PKC substrate phosphorylation as a measure of intracellular PKC activation using Phospho-(Ser) PKC Substrate Antibody (Cell Signaling Technology). Phospho-(Ser) PKC Substrate Antibody detects endogenous levels of cellular proteins only when phosphorylated at serine residues in particular motif found in PKC substrates (surrounded by Arg or Lys at the -2 and +2 positions and a hydrophobic residue at the +1 position). The antibody does not cross-react with non-phosphorylated serine residues, with phospho-threonine in the same motif, or with phospho-serine in other motifs (Cell Signaling Technology data sheet).

3. Results

3.1. TSC phenolic fraction potentiates anchorage-independent growth of BPDE-treated Cl 41 cells

To examine the tumor promoting activity of TSC phenolic fraction we investigated whether TSC phenolic fraction can potentiate BPDE induced anchorage-independent growth of promotion sensitive JB6 cells (Cl 41). TSC phenolic fraction was prepared from tobacco smoke condensate as described before (Chart 1). Figure 1 indicates that BPDE by itself has some ability to cause anchorage-independent cell growth of JB6 cells on soft agar compared to untreated cells. Interestingly, we observed that TSC phenolic fraction at noncytotoxic concentration significantly potentiates BPDE-induced anchorage-independent cell growth in a dose-dependent manner. Treatment of cells with 30 μ g/ml TSC phenolic fraction caused

almost 4.3 fold potentiation of BPDE-induced anchorage-independent growth on soft agar. Regarding cytotoxicity of TSC phenolic fraction we observed that treatment of C141 cells with 60 µg/ml concentration of TSC phenolic fraction is well below the cytotoxic level (more than 95% cell survival) as determined by Trypan blue exclusion assay (data not shown). TSC phenolic fraction by itself has no effect on anchorage-independent cell growth activity. This finding in *in vitro* cell culture model supports the previous observation of tumor promoting activity of TSC phenolic fraction in mouse skin [16,17].

3.2. TSC phenolic fraction inhibits BPDE-induced NF-κB activation but not AP-1 activation

The transcription factors AP-1 and NF-κB are known to be influenced by established tumor promoter TPA [19–26]. Although AP-1 and NF-κB have roles in cell transformation, NF-κB activation status in JB6 cells was observed to have a threshold value beyond which cell death instead of increased cell transformation is observed [38]. We examined whether potentiation of BPDE-induced cell transformation by TSC phenolic fraction is associated with the modulation of activities of these two transcription factors. We used C141 cells harboring AP-1-responsive and NF-κB-responsive luciferase gene to monitor the activation of AP-1 and NF-κB respectively. BPDE treatment of cells showed induction of both AP-1 and NF-κB activity (figure 2A and 2B) in a dose-dependent manner. 0.5 µM BPDE treatment caused 8.2 fold and 48 fold induction of AP-1 and NF-κB activity respectively compared to untreated cells. Interestingly, treatment of cells with TSC phenolic fraction did not have any effect on BPDE-induced AP-1 activation (figure 2A), whereas BPDE-induced NF-κB activation was found to be significantly inhibited by TSC phenolic fraction in a dose-dependent manner (figure B, 44% and 83% inhibition by 10 µg/ml and 30 µg/ml TSC phenolic fraction respectively). TSC phenolic fraction by itself has no effect on either AP-1 or NF-κB activation. To our knowledge this is the first report that TSC phenolic fraction inhibits NF-κB activation in chemical carcinogen-damaged cells.

3.3. TSC phenolic fraction inhibits intracellular PKC substrate phosphorylation and PKC inhibition attenuates BPDE-induced NF-κB activation

Increased hyperplasia associated with down-regulation of PKC activity in mouse skin has been observed with established tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and it has been suggested that down-regulation of PKC may be a permissive event for epidermal hyperplasia and tumor promotion [27,28]. Although the role of PKC in tumor promotion is not straightforward without complication there are suggestions that its down-regulation may be important in tumor promotion [35,36]. PKC is also known to have a role in the activation of NF-κB (39–41). In this study we examined whether TSC phenolic fraction as a tumor promoter has ability to interfere with intracellular PKC activation. PKC activation is monitored by determining intracellular PKC substrate phosphorylation as described in “Methods”. Figure 3 demonstrates that in C141 cells there is high basal level of PKC substrate phosphorylation. Cells treated with either BPDE or TSC phenolic fraction did not show any significant change of PKC substrate phosphorylation. Interestingly, cells treated with 30 µg/ml TSC phenolic fraction significantly reduced PKC substrate phosphorylation in BPDE treated cells. These findings indicate that attenuation of BPDE-induced NF-κB activation by TSC phenolic fraction is associated with PKC down-regulation.

Inhibition of PKC substrate phosphorylation by TSCPhFr is not due to its ability to activate intracellular phosphatase and sequester intracellular PKC substrates. Previously we observed that PKC activation by phorbol ester TPA (10 ng/ml) as measured by PKC substrate phosphorylation is not affected at all by TSCPhFr (unpublished data). If TSCPhFr has ability to activate phosphatase and sequester intracellular PKC substrates then it is expected that TSCPhFr would also inhibit phorbol ester-induced PKC substrate phosphorylation, but it does not.

Next we examined whether PKC inhibition has any role in the attenuation of NF- κ B activation by TSC phenolic fraction. It is observed that BPDE-induced NF- κ B activation is differentially attenuated by different inhibitors of PKC (figure 4). The extent of NF- κ B attenuation is least (27%) with conventional PKC inhibitor Go6976 (highly specific for PKC α , β I, β II and γ isoforms) and moderate with non-specific PKC inhibitors staurosporine (39%) and bisindolylmaleimide (47%), whereas highest attenuation of NF- κ B activation (62%) is observed with Rottlerin known to be highly specific for PKC δ . This suggests that PKC inhibition can cause attenuation of DNA damage-induced NF- κ B activation. Thus our observation of the attenuation of NF- κ B activation associated with inhibition of intracellular PKC activity by TSC phenolic fraction in BPDE treated cells indicates a possible link between the two.

3.4. PKC inhibition potentiates BPDE-induced anchorage-independent cell growth on soft agar

Since PKC down-regulation is implicated in tumor promotion [27,28,36] we further examined whether inhibition of PKC activity by TSC phenolic fraction has any correlation with its ability to potentiate the anchorage-independent growth of BPDE treated C141 cells on soft agar. It is evident from figure 5 that not only the carcinogen BPDE but also the PKC inhibitors Go6976 and rottlerin have varying ability to initiate anchorage-independent growth of C141 cells. The number of colonies on soft agar increased 21.9 fold, 11.4 fold and 31.5 fold with BPDE, Go6976 and rottlerin respectively. But we observed potentiation of anchorage-independent growth of BPDE treated cells by PKC inhibitors (with respect to the sum of the individual effect). Cells treated with Go6976 and rottlerin showed 1.8 fold and 3.1 fold potentiation of BPDE-induced cell growth respectively.

4. Discussion

Our present study demonstrates that phenolic fraction (weakly acidic components) of TSC potentiates BPDE-induced anchorage-independent cell growth (an *in vitro* measure of cell transformation). This potentiating effect is associated with attenuation of NF- κ B activation and PKC down-regulation. It is also evident from our findings that PKC inhibition can attenuate BPDE-induced NF- κ B activation and potentiate anchorage-independent cell growth on soft agar. Although the role of PKC in tumor promotion is not straightforward without complication there are suggestions that its “down-regulation” may be important in tumor promotion [27, 28,35,36,50]. The data presented in our study indicate that down-regulation of PKC may have a role in potentiation of BPDE-induced tumorigenicity by TSC phenolic fraction.

Based on our present findings it is not clear whether attenuation of NF- κ B activity is an associated phenomenon or has direct correspondence with potentiation of BPDE-induced anchorage-independent cell growth. We observed that treatment of cells with different concentrations of B6-Amino-4-(4-phenoxyphenyl ethylamino) quinazoline (APQ) (Calbiochem), an inhibitor highly specific to NF- κ B, either inhibits or potentiates BPDE-induced anchorage-independent cell growth (data not shown). In this regard we address some relevant issues pertaining to our findings and inference. First, if attenuation of NF- κ B by TSC phenolic fraction (as we observed) has any correspondence with potentiation of anchorage-independent cell growth, then the role of NF- κ B activation by BPDE in BPDE-induced cell transformation is questionable. Second, if PKC down-regulation attenuates NF- κ B as we observed, then BPDE-induced NF- κ B activation is expected to be associated with up-regulation of PKC. But we observed that BPDE has practically no effect on PKC. In response to the first issue it is explainable by the facts that either NF- κ B activation by BPDE is an associated phenomenon having no relevance to the cell transformation process or the predictive assignment of the role of NF- κ B toward cell transformation based on its activation status is

complicated. The validity of the later explanation resides in the following observation by others. It is observed that TNF- α induced JB6 cell transformation efficiency is increased in a dose-dependent manner up to a maximum associated with a threshold value of NF- κ B activation beyond which TNF- α -induced cell death is noticed [38]. It needs further investigation to examine whether the increased level of NF- κ B activation above the threshold value has any correlation with cell death. Increase of NF- κ B activity up to a certain limit (depends on cell type) in response to cellular insults may have positive correlation with cell transformation, whereas beyond that limit further increase of NF- κ B activity may be associated with a completely opposite end point as cell death. If BPDE-induced NF- κ B activation status in JB6 cells is beyond the aforementioned threshold limit which is indicative of induction of cell death, then attenuation of NF- κ B will inhibit apoptotic cell death thereby favoring cell transformation process. As a partial support of this view we previously observed that BPDE indeed induces apoptotic cell death in JB6 cells [42]. In further support of our view it is also observed that NF- κ B has dual roles towards apoptotic cell death function because as a transcription factor it can protect or contribute to apoptosis [43]. Taken together this indicates that NF- κ B activation may be beneficial or detrimental in context with cell transformation. Regarding the second issue although PKC is observed to have a role in the activation of NF- κ B [39–41] it is not the only regulatory determinant of NF- κ B activation. NF- κ B can be activated by other signaling molecules without any involvement of PKC [44–47]. So, it is quite possible that BPDE may induce NF- κ B activity without up-regulating PKC. Once NF- κ B is activated by BPDE in a PKC-independent manner, any further attempt to inhibit constitutive PKC activity (as we observed with TSC phenolic fraction in presence of BPDE) may negatively regulate NF- κ B activation.

In conclusion, data obtained from our present study indicate that inhibition of PKC activity by TSC phenolic fraction may have a role in potentiation of tumorigenicity by BPDE, an ultimate carcinogenic metabolite of the PAH benzo(a)pyrene. Further studies are needed to confirm NF- κ B's role in this regard.

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Figure 1.

Effect of TSC phenolic fraction on anchorage-independent growth of BPDE treated JB6 Cl41 cells. Experimental procedure is described in the text. Each bar shows the average of three independent experiments and the error bars indicate SD. (*) indicates statistical analysis of significance with paired t-Test ($p < 0.05$).

Figure 2.

Effect of TSC phenolic fraction on activation of AP-1 and NF- κ B in JB6 (P⁺) Cl 41 cells harboring AP1-luciferase and NF- κ B-luciferase reporter plasmids. Activation of AP-1 (A) and NF- κ B (B) is measured as described in “Methods”. Cells were either untreated; treated with TSC phenolic fraction (10 μ g/ml); with BPDE (0.2 μ M, 0.5 μ M); with 0.5 μ M BPDE followed by TSC phenolic fraction (10 μ g/ml or 30 μ g/ml). Each bar indicates the mean $\pm$ SD of three parallel experiments. (*) indicates statistical analysis of significance with paired t-Test ($p < 0.05$).

Figure 3.

Inhibition of PKC substrate phosphorylation by TSC phenolic fraction. Cells were either untreated or treated with 0.5 μ M BPDE for 1 hr. followed by noncytotoxic concentration of TSC phenolic fraction for 6 hours and PKC substrate phosphorylation in the cell extract was determined by Western immunoblotting using phospho-(Ser) PKC Substrate antibody which is specific for PKC substrates phosphorylated at Ser residues within a particular motif.

Figure 4.

Effect of PKC inhibitors on BPDE-induced NF-κB activation in JB6 Cl 41 cells harboring NF-κB-luciferase reporter plasmid. Bar graph shows NF-κB activation in cells that were untreated (A); treated with 0.5 μM BPDE (B); treated with 0.5 μM BPDE plus respective PKC inhibitors staurosporine, 7 nM (C); bisindolylmaleimide, 100 nM (D); Go6976, 0.5 μM (E) and rottlerin, 30 μM (F). Each bar indicates the mean $\pm$ SD of three parallel experiments. (*) indicates statistical analysis of significance with paired t-Test ($p < 0.05$).

Figure 5. Effect of PKC inhibitors on anchorage-independent growth of JB6 cl41 cells. Experimental procedure is described in the text. Each bar shows the average of three independent experiments and the error bar indicates SD.

Chart 1.

Preparation of weakly acidic phenolic fraction from tobacco smoke condensate