Published in final edited form as:

Food Chem Toxicol. 2011 September ; 49(9): 2348–2355. doi:10.1016/j.fct.2011.06.037.

Concentration dependent effects of tobacco particulates from different types of cigarettes on expression of drug metabolizing proteins, and benzo(a)pyrene metabolism in primary normal human oral epithelial cells

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Abstract

The ability of tobacco smoke (TS) to modulate phase I and II enzymes and affect metabolism of tobacco carcinogens is likely an important factor in its carcinogenicity. For the first time several types of TS particulates (TSP) were compared in different primary cultured human oral epithelial cells (NOE) for their abilities to affect metabolism of the tobacco carcinogen, (BaP) to genotoxic products, and expression of drug metabolizing enzymes. TSP from, reference filtered (2RF4), mentholated (MS), reference unfiltered, (IR3), ultra low tar (UL), and cigarettes that primarily heat tobacco (ECL) were tested. Cells pretreated with TSP concentrations of 0.2 – 10 µg/ml generally showed increased rates of BaP metabolism; those treated with TSP concentrations above 10 µg/ml showed decreased rates. Effects of TSPs were similar when expressed on a weight basis. Weights of TSP/cigarette varied in the order: MS ≈ IR3 > 2RF4 > ECL > UL. All TSPs induced the phase I proteins, cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1), phase II proteins, NAD(P)H dehydrogenase quinone 1 (NQO1), and microsomal glutathione S-transferase 1 (MGST1), and additionally, hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2), as assessed by qRT-PCR. The pattern of gene induction at probable physiological levels favored activation over detoxification.

Keywords

tobacco smoke; benzo(a)pyrene; DNA adducts; gene array; Ah receptor; estrogen; oral epithelial cells; multiple individuals

1. Introduction

Tobacco smoke (TS) contributes to a large percentage of human cancer in many sites including the oral cavity (Hecht, 2003; IARC, 2004). TS contains many carcinogens, the

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Conflict of interest statement. The authors declare that there are no conflicts of interest.

most potent of which, in significant levels, are thought to be polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines and aromatic amines (Hecht, 2003). The mechanisms by which tobacco smoke leads to carcinogenesis in oral tissues presumably include damaging DNA (leading to mutagenesis), but other factors involved in carcinogenesis such as promotion, enzyme induction and inhibition, or saturation of detoxification enzymes may also play a role (Rubin, 2001). Two major factors in mediating carcinogenesis by procarcinogens are the metabolic activation and detoxification abilities of the target organ. Generally, (but not necessarily always) phase I activities (usually cytochrome P-450s) activate procarcinogens, and phase II activities (e.g., glutathione transferases, UDP-glucuronosyl transferases, sulfotransferases) detoxify (Guengerich, 2000; Talalay, 2000).

The environmental carcinogen, benzo(a)pyrene (BaP) is a prototypical PAH carcinogen that is found in TS and other combustion products (Hecht, 2003; Phillips and Grover, 1994). BaP leads to DNA damage in many organs and induces mutations *in vivo* in oral tissues of experimental animals (Guttenplan et al., 2004; Phillips and Grover, 1994). In a long-term feeding study it induced papillomas and carcinomas in tongue, esophagus and forestomach of mice (Culp et al., 1998).

There are currently many types of tobacco products on the market and some of these are purported to represent reduced risk products. Estimating their potential genotoxic and related effects represents an important research area with respect to risk posed by tobacco products (Johnson et al., 2009). Epidemiological studies cannot be carried out until the products have been in use for many years and finding appropriate shorter term biomarkers is still a challenging problem. *In vitro* models then could fulfill an important niche. For the oral cavity a particularly appropriate model would be one involving normal human tissue. It is likely that primary cells would be more appropriate than cell lines to study the early effects of TS, as cell lines may not be completely normal, due to their immortalization. The use of cells from multiple individuals would help ensure that results observations are not patient specific.

Carcinogenic PAH's such as BaP require metabolic activation in order to exert genotoxic effects (Baird et al., 2005; Xue and Warshawsky, 2005) and proteins important in their metabolism include cytochrome P-450 1A1 and 1B1 (Guengerich, 2004). Here, for the first time, normal oral cells from multiple individuals were employed to investigate the effects of TSPs from different types of cigarettes on the ability of the cells to metabolize BaP to genotoxic products, and to induce the expression of phase I and II proteins.

2.1. Materials and Methods

³H(G)-BaP (55 Ci/mmol) was purchased from GE Healthcare (formerly Amersham Radiochemicals) and diluted with unlabeled BaP (Sigma-Aldrich, St. Louis, MO) to 0.55 – 1.1 Ci/mmol. Nicotine and α -naphthoflavone were purchased from Sigma-Aldrich). BPDE was obtained from the Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, (Lexena, KS).

Total particulate matter from five types of cigarette smoke was dissolved in acetone (see below). Marlboro Menthol Smooth (MS) and Carlton, an ultra-low tar (UL) were purchased on the open market. Reference cigarettes, IR3, containing a full flavored (high tar) reference blend of non-filter cigarette tobacco and 2RF4, a full flavored filtered (medium tar) reference blend that approximates the average filter tobacco, were obtained from the Tobacco and Health Research Institute, (Lexington, KY). The extract from Eclipse, a cigarette that primarily heats, rather than burns tobacco, was kindly supplied in DMSO by Dr. Wanda Fields, (RJ Reynolds Tobacco Co., Winston-Salem, NC). The cigarette smoke

was generated with an automated cigarette smoking machine (CH Technologies, New Jersey). An automatically regulated piston pump produced a two second puff of 35 ml volume (a standard used in US smoke exposure studies). The smoke from one pack of cigarettes was impinged onto a Cambridge filter and particulates were extracted from the filters in acetone and diluted as necessary. The filters were weighed before and after particulates were extracted. The weights of TSP/cigarette (in mg) are IR3-17.1, 2RF4-11.2, UL-2.0, MS-20.7 and ECL, 7.3. Concentrated stock solutions of TSPs were kept at -80°C and aliquots were diluted in DMSO to prepare working stocks and stored at -20°C until used.

2.1 Patients

Tissue samples were collected from a random unbiased sampling of patients who received treatment in the Oral Surgery clinic at the Dental School. In accordance with the IRB protocol for this study no patient information was provided and no selection was utilized. From the time the sample was procured to the time it was cultured and used, at least two weeks elapsed, so that any original induction of drug metabolizing enzymes which might have been present in the original tissue would be long gone. On the average only about 10% of the patients at the oral surgery clinic are smokers. Patient samples were identified by the year and patient number for that year (e.g., 90-08 means, i.e. 90th specimen in 2008).

2.2. Cell culture

NOE - Gingival mucosa from third molar extractions was put into culture as described (Xu et al., 1996). Tissue was brought to the laboratory on ice in DMEM/Ham's F12, 5% FCS, 200 $\mu\text{g}/\text{ml}$ gentamicin, 5 $\mu\text{g}/\text{ml}$ amphotericin and maintained in a refrigerator overnight. Tissue was then minced into small 1–3 mm sized pieces and epithelial cells allowed to grow out using explant outgrowth techniques in AmnioMax C-100 media (Invitrogen). At 60–80% confluency, NOE were harvested and all experiments were performed on first passaged cells grown in Keratinocyte Growth Medium (Lonza). Tissues or cells from different patients were not pooled.

2.3. Cell treatments

For experimental use, based on individual patient sample yield, equal numbers of NOE were seeded in Keratinocyte Growth Medium and transferred to Keratinocyte Basal Medium the next day. Where DNA adducts were measured, $5 - 10 \times 10^5$ cells/dish were plated. For media assays $2 - 4 \times 10^5$ cells per assay condition were plated. In general equal numbers of cells were plated on two dishes to allow duplicate determinations for each assay condition. The following day cells were exposed to TSP or solvent control (0.1% DMSO) and harvested for RNA isolation 16 h later, or used for BaP metabolism. Unless otherwise indicated, for BaP metabolism, ^3H -BaP (0.2 – 0.5 μM final concentration) was added to the culture medium 16 h after the TSP addition. The rate of metabolism of BaP increased with pretreatment time of cells with TSP for about six hr and then remained almost constant for at least ten hours (see Results for time response). Aliquots of media were centrifuged to remove debris and unattached cells, and quantified for levels of BaP metabolites by liquid scintillation counting and/or HPLC, 3– 6 h after the addition of BaP.

Both total radioactivity and BaP-tetrols were monitored. BaP-tetrols were detected by HPLC using a Restek Pinnacle II, 3 μm , 150×2.1 mm C18 high performance liquid chromatography column. Tetrols were eluted isocratically in a mobile phase of 21 or 23% acetonitrile containing 10 mmol/L ammonium acetate (pH 6.0) at a flow rate of 0.3 mL/min. The eluate was analyzed using the fluorescence detector set at 344 nm excitation and 400 nm emission. A Shimadzu high performance liquid chromatography system consisting of an LC-20AD solvent delivery system, a SIL-10Ai autoinjector, an SPD-20AVUV-VIS

detector, and an RF-10AxL fluorescence detector was used for analysis. Quantitation of the adducts was achieved by comparison with standards of the B[a]P tetrol isomers. These were generated by incubating anti-BPDE in water at room temperature for 30 min (Shugart et al., 1983) leading to one major product (designated BaP tetrol 1–1) and one minor adduct, (BaP tetrol 1–2) (Alexandrov et al., 1992; Rojas et al., 1994).

2.4. DNA adducts

DNA was isolated and then hydrolyzed in 0.1M HCl at 90°C for 2 h. This treatment releases B[a]P tetrols from the N2-BPDE adducts (Alexandrov et al., 1992; Rojas et al., 1994). After acid hydrolysis, the samples were cooled to room temperature and applied to the HPLC as described above.

2.5. PCR arrays

The effects of TSP on gene expression was determined using the Human Drug Metabolism, RT2 Profiler™ PCR Array (PAHS-002, SuperArray, Frederick, MD) in a 96 well format. This array assays by qPCR of RNA a number of phase I and phase II genes, in addition to other drug-metabolizing genes. RNA was isolated using an RNAeasy Kit, (Qiagen, Valencia, CA). RNA quality was checked with a NanoDrop ND-1000 UV spectrophotometer and an Agilent 2100 Bioanalyzer. RNA was converted to cDNA with an RT² First Strand kit from the SA Biosciences. cDNA (2ul), RT² SYBR® Green qPCR Master Mix (SA biosciences) (12.5 ul) and water (10.5 ul) were added to each well. Real time PCR arrays were carried out using a Bio-Rad Opticon 2 Real-Time PCR System. ΔCt values (treated vs. controls) were obtained relative to the average of five housekeeping genes, (*B2M, HPRT1, RPL13A, GAPDH, ACTB*) present in the arrays. Real time PCR results were analyzed using software provided by the manufacturer. Where sufficient RNA was available (patients 90 and 98) arrays were performed in duplicate and the average value and the deviation from the average is given. In cases where selected genes (rather than gene arrays) were analyzed, primer pairs were purchased from Sigma-Genosys, (St. Louis, MO). CYP1B1 was not included in the PCR array and was only analyzed by this method. The Ct values for the individual genes were normalized to the β-actin gene.

3. Results and Discussion

To our knowledge this study is the first study to report the effects of TSPs on gene expression and BaP metabolism in primary oral epithelial cells from normal individuals, and the first to use such cells from multiple individuals. There have however been several studies on the effects of tobacco products on gene expression in oral cancer cells (Nagaraj et al., 2006; Gumus, 2008; Boyle, 2010).

3.1. Effects of TSP on metabolism of BaP

All of the NOEs tested metabolized BaP to genotoxic products. This is consistent with previous reports showing that oral and keratinocyte cell lines metabolize BaP to BPDE-DNA adducts and water soluble metabolites (presumably BaP-tetrols) (Hughes et al., 2008; Nair et al., 2009; Rinaldi et al., 2002) and that human keratinocytes isolated from oral squamous cell tumors and human oral tissue metabolize the BaP metabolite, BaP-7,8-dihydrodiol to BaP-tetrols (Rinaldi et al., 2002). Here metabolism of BaP to genotoxic products was evaluated by the ability of the NOE to convert BaP to: 1) BaP-tetrols (the decomposition product of (±)-*anti*-dihydrodiol 9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene – the major carcinogenic metabolite of BaP) released into the culture medium, 2) total radioactivity of metabolites of ³H-BaP in the culture medium and 3) BaP-DNA adducts in NOE. Monitoring metabolism of BaP by radioactivity released into the culture medium has the advantage that it is very sensitive relative to detection of BaP tetrol,

as tritiums are released from each position in BaP that is involved the generation of tetrols, and also metabolism of BaP at alternative positions. However, because tritium might be released by other metabolic pathways involving BaP, it was necessary to determine whether radioactivity in the culture medium correlated with tetrols released into the medium and with levels of BaP-DNA adducts. Typical chromatograms of acid hydrolyzed DNA from NOE treated with BaP, and of culture medium in the presence or absence of TSP are shown in Figure 1. Levels of both DNA adducts, and BaP-tetrols in culture medium correlated well with levels of ^3H -BaP metabolites in the culture medium (Figure 2). The latter assay was then predominantly used because its high sensitivity allowed the use of fewer cells. In addition to the BaP-tetrol peak smaller peaks were also observed in the culture medium. These may have resulted from spontaneously depurinated BaP adducts that can be formed from the BaP radical cation or BPDE (Todorovic et al., 1997). Such adducts would also contribute to tritium released into the cell medium. A time response for the effect of pretreatment time on BaP metabolism was carried out with (Figure 3). This was to determine an appropriate pretreatment time before addition of BaP. The enhancement of metabolism of BaP rapidly increased with TSP pretreatment time up to about 4 – 6 hours and then slowly plateaued. Similar results were reported for several human oral cell lines (Nagaraj et al., 2006). When TSP was added simultaneously with BaP, metabolism of BaP was actually reduced. When 5 $\mu\text{g/ml}$ IR3 TSP was added simultaneously with BaP metabolism of BaP decreased from 3.6 ± 0.6 to 1.9 ± 0.3 fmol/ml medium (data represents the average from two different patients). This observation suggests that TSP's contain inhibitors of BaP metabolism and in the absence of induction of BaP-metabolizing proteins (which requires several hours) inhibition of BaP metabolism predominates. Presumably, after induction of BaP-metabolizing proteins (probably CYP 1A1/1B1 – see below) the increased metabolic capacity of the cells more than compensates for partial inhibition of metabolism of BaP, and the rate of metabolism increases. At high concentrations of TSP induction of CYP1A1/1B1 begins to level off (see below) and inhibition (which should be directly proportional to TSP concentration) becomes relatively more important.

At the lower concentrations tested all of the TSPs appreciably enhanced the ability of NOE to metabolize BaP to genotoxic products (Figure 4). Strong enhancing effects of TSP on metabolism of BaP to water soluble metabolites were observed at the lowest level tested (1.2 $\mu\text{g/ml}$). Due to limitations in cell number, it was not possible to test the effects of all of the TSPs in each NOE, but each TSP was tested in cells from at least three patients and most in 4 or more cases. Although inter-individual differences in the rates of metabolism of BaP were observed, major inter-individual differences between the *relative* effects of TSPs on metabolism of BaP were not observed (Figure 4). In general, the enhancing effects of TSPs on BaP metabolism increased up to a concentration of TSP in the culture medium of about 2 – 5 $\mu\text{g/ml}$ - above which the rates gradually declined (Figure 4). For one TSP (MS), enhancement of BaP metabolism appeared to peak at a somewhat higher value (12.5 $\mu\text{g/ml}$) but there was a large deviation between samples at this concentration. Nicotine, a common major component of TSP, had no effect on the rate of BaP metabolism at concentrations from 1 – 50 μM . This concentration range includes the concentrations that would be present in the medium as a result of nicotine in the TSP (Hoffmann et al., 1997; Hoffmann, 2001). α -naphthoflavone (10 μM) an inhibitor of AhR activation (Wilhelmsson et al., 1994), almost completely prevented the enhancement in BaP metabolism by all of the TSPs (results not shown).

3.2. Modulation of gene expression by TSP

The effects of TSP on induction of selected human drug-metabolizing genes were examined in focused real-time PCR arrays. To maximize positive calls, in initial experiments higher concentrations of TSPs were tested (Table 1) and subsequently concentration responses for

the major modulated genes were carried out (Table 2). Of the 84 genes included in the array, 23 were upregulated or downregulated by at least two-fold in NOE from one or more patients and/or by one or more TSPs. All of the tobacco smoke extracts led to enhanced levels of several genes including CYP1A1, NQO1, MGST1 and HSD17B2 (Table 1). CYP1B1 was not included in the arrays supplied by the manufacturer and was assayed separately by qPCR. It too was induced by TSP (Table 2). Although some genes were consistently induced by all of the TSPs, others were patient specific (Table 1). There were relatively small differences in induction at concentrations of 80 and 5 ug/ml (Table 1).

The TSPs consistently induced the phase I proteins, CYP1A1 and 1B1 and also NQO 1, which is a sentinel for phase II enzymes (Dinkova-Kostova et al., 2007; Zhang et al., 1992). Agents that induce CYP1A1 and 1B1 also induce certain phase II enzymes as a consequence of the activation of the Ah receptor (Nebert et al., 2000). In addition, NQO 1 and GPX2 (also overexpressed in most TSP-treated NOE) are induced as a consequence of the activation of the antioxidative response element (Kwak et al., 2001). Possibly significant is the observation that the induction of CYP1A1 and 1B1 was observable at low concentrations, where induction of NQO 1 was not detected. This would suggest that even if phase II enzymes partially protected against BaP genotoxicity resulting from TSP exposure, their relative effectiveness would be reduced at low (and probably physiological) levels. MGST1, a glutathione-S-transferase, is presumably one of the phase II enzymes upregulated as a result of activation of the AhR or antioxidative response element (Knight et al., 2008). Also upregulated by TSP in all NOE examined thus far, was hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2). HSD17B is important in the production of estradiol from precursors. It has been reported that tobacco smoke activates estrogen receptor as well as the AhR (Meek and Finch, 1999) and estradiol induces at least one form of HSD17B (Rotinen et al., 2009). These observations may indicate that a TSP component may possess structural features similar to estrogen receptor binders. Taken together these results may be relevant to observations that women are more susceptible than men to the carcinogenic effects of tobacco in the oral cavity (Muscat et al., 1996) and a recent report associating hormone replacement therapy with increased risk of mortality from lung cancer (Chlebowski et al., 2009).

Concentration response behavior—The observation that metabolism of BaP increases and then decreases with increasing concentration of TSP, suggests that there are at least two different mechanisms by which TSP affects the metabolism of BaP. Induction of CYP1A1/1B1 and enhancement of metabolism of BaP occurs at the lower concentrations of TSP (Tables 1,2, Figures 1,2,4), but levels of CYP1A1/1B1 appear to plateau at higher concentrations (Table 1 and 2). However, as described above, inhibition (likely competitive) of the metabolism of BaP by TSP also occurs and this would not be expected to level off at higher concentrations, unless metabolism was almost totally inhibited. Presumably when TSP and BaP are simultaneously added, the relatively short time of treatment (3 h) before analysis of BaP metabolites precludes appreciable induction of BaP-metabolizing proteins (Figure 3), and inhibition of constitutive activity predominates. At the longer pretreatment times routinely used in the assays, the rapid concentration-dependent increase in CYP1A1/1B1 levels at low TSP concentrations results in increased rates of metabolism of BaP even in the presence of inhibitors. However, when levels of CYP 1A1/1B1 plateaued at higher TSP concentrations (Tables 1 and 2), inhibition of BaP metabolism by the increasing concentration of TSP inhibitors likely became predominant. The higher concentrations were not noticeably toxic, as the appearance of the TSP-treated cells was no different than that of the untreated controls, suggesting that toxicity was not contributing the decline in BaP metabolism at high concentrations of TSP.

The involvement of CYP1A1/1B1 in the activation of BaP was further indicated by the observation that the TSPs failed to enhance metabolism of BaP when cells were pretreated with α -naphthoflavone before the addition of TSP (results not shown). α -naphthoflavone is an inhibitor of AhR activation (Wilhelmsson et al., 1994) which prevents induction of genes such as CYP1A1/1B1 that are modulated by the AhR.

3.3. Concentration considerations

An important consideration is the relationship between the applied concentration in the culture medium and the serum and tissue levels of TSP components *in vivo*. One method of approximating this relationship is to take the level of nicotine and its metabolites in the blood of smokers as an approximate physiological level. Using this as an approximate physiological concentration, TSP concentration could then be adjusted to bracket this level in the culture medium. In a recent study by Hecht and coworkers (Church et al., 2009) mean levels of the major nicotine metabolite, cotinine, in smokers' serum were about 220 ng/ml. As cotinine represents about 2/3 of the nicotine and its metabolites in smokers serum (Stepanov et al., 2007) and a typical nicotine level in tobacco smoke particulates from filtered cigarettes is about 10% by weight (Hoffmann et al., 2001), then 3 ug/ml of TSP would approximately correspond to the mean serum level of nicotine + metabolites in smokers' plasma ($3 \text{ ug/ml} \times 10\% = 300 \text{ ng/ml}$). This is of course only a first approximation, but it represents an intermediate concentration in this study, so that both higher and lower concentrations were tested, and it seems likely that some of these concentrations correspond to physiological levels of at least some of the components of TSP. The 3 ug/ml concentration is generally much lower than TSP concentrations utilized in previous reports in cultured cells (Fields et al., 2005; Foy et al., 2004; Fukano et al., 2006; Nagaraj et al., 2006; Putnam et al., 2002; Rickert et al., 2007). A recent study by Boyle et al. reported that levels of CYP1A1/1B1 in oral tissue were about 2–4 fold higher in smokers than nonsmokers (Boyle et al., 2010b). Extrapolating from Table 2 we estimate the level of TSP that would result in a similar enhancement in NOE would be in the range of 3 – 5 ug/ml. Hence, with respect to the ability of TSP to induce CYP1A1/1B1 in cultured human cells, and induction of these proteins in oral tissue from smokers, there is good correspondence, indicating the lower concentration range in the current study is relevant to cellular levels in oral tissue in smokers.

The TSP components responsible for the induction of CYP1A1/1B1 are speculative. Tobacco smoke contains PAH's, and many of these are strong activators of the Ah receptor (Ding et al., 1133; Kasai et al., 2006). Whatever the nature of the inducers, they are effective at very low concentrations. For instance, a concentration of TSP of 1.2 ug/ml of most TSPs resulted in a several fold enhancement in metabolism of BaP (Figure 4). Based on the known constituents of tobacco smoke a "ballpark" estimate of the average molecular weight of TSP components might be about 250 daltons (Hoffmann et al., 1997), and hence their concentration at 1.2 ug/ml TSP would be about 5 nM. However TSP is a complex mixture consisting of a myriad components and structures, most of which would not have the structural features necessary for AhR activation. So it is likely that combined concentrations of actual inducers in this mix are in the pmol range, suggesting they are very powerful AhR activators. It might be thought that PAH's in tobacco smoke could account for the induction of CYP1A1/1B1, but in tobacco smoke from a typical US cigarette only about 1 ug of total PAH's are formed. Hence, for a typical filter cigarette generating 10mg of TSP and subject to the above approximations, the concentration of PAH's in a 1.2 ug/ml culture medium would be 0.1 nM ($1/10^4 \times 1\mu\text{M}$). This is orders of magnitude below the range generally necessary to activate the AhR (Tsuchiya et al., 2003) indicating that PAH's alone cannot account for the activation of the AhR.

Conclusions

The results of this study demonstrate that exposure of NOE to TSP from several different types of cigarettes appreciably enhances the rate of metabolism of BaP by NOE to genotoxic products, but only at low concentrations of TSP, and the concentration of TSP used in a cell culture assay is critical. The low-concentration enhancement was similar for all of the cigarette types when normalized to the weight of TSP, but those cigarettes generating the highest amounts of TSP were most potent on a per cigarette basis. All of the TSPs were effective at inducing certain phase I and II proteins. Expression of these proteins is often dependent on the activation of the AhR and/or the antioxidative response element. Sustained activation of the AhR has been suggested to be sufficient to induce cancer (Bock and Kohle, 2006). Also, the upregulation of HSD17B2 by all TSPs is of interest. This study also indicates that primary NOE represent an appropriate *in vitro* model system to evaluate the effects of different tobacco products on metabolism of procarcinogens, and on gene expression. Furthermore, as has been recently demonstrated the pattern of gene expression in oral tissue from tobacco-smokers is similar to that in bronchial tissue from smokers (Boyle et al., 2010a) suggesting that NOE cells represent a useful surrogate for other respiratory and aerodigestive tissues.

highlights

- Tobacco smoke particulates (TSP) from different cigarettes exerted similar effects on primary human epithelial cells
- Pretreatment of the cells with low levels of TSPs greatly enhanced their genotoxic metabolism of benzo(a)pyrene
- Pretreatment of the cells with all TSPs greatly induced CYP1A1 and 1B1, and less so, NAD(P)H dehydrogenase quinone 1 (NQO1)
- All TSPs induced hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2), which plays a role in estrogen synthesis

Abbreviations

AhR	aryl hydrocarbon receptor
BaP	benzo(a)pyrene
BPDE	(±)- <i>anti</i> -dihydrodiol 9,10-epoxy-7,8,9,10-tetrahydrobenzo[<i>a</i>]pyrene
ECL	Eclipse
MS	mentholated smooth
NOE	normal oral epithelial cells
PAH	polycyclic aromatic hydrocarbon
Pt	patient
2RF4	Reference filtered cigarette
IR3	reference unfiltered cigarette
TS	tobacco smoke
TSP	tobacco particulate matter
UL	ultra-light

Acknowledgments

This work was supported by NIH grant # CA118397.

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Figure 1.

Figure 1A. Effect of tobacco particulate matter on DNA adduct levels in NOE exposed to BaP. Lower trace, no TSP; middle trace, 0.4 ug/ml 2RF4; upper trace, 10 fmol BP-tetrol standard (at $\frac{1}{2}$ sensitivity). Cells were incubated for 18 h with or without TSP and then for 6 h with 0.25 μ M BaP. DNA from cells was heated at 90° C for 2 h, and 4 μ g was applied to the HPLC as described in the Materials and Methods. 7.4 fmol of BaP-tetrol were formed in the presence of TSP and 3.4 fmol in its absence. NOE from patient 1– 10.

Figure 1B. Effect of tobacco particulate matter on levels of BaP-tetrol released into the culture medium of NOE exposed to BaP. Lower trace, no TSP; middle trace, 0.4 ug/ml 2RF4; upper trace, 10 fmol BP-tetrol standard (at $\frac{1}{2}$ sensitivity). Cells were incubated for 18

h with or without TSP and then for 6 h with 0.25 μ M BaP. Ten μ l of medium was applied to the HPLC using conditions described in Materials and Methods. NOE from patient 78-09.

Figure 2.

Normalized relationships between BaP-DNA adduct levels, BaP tetrols in culture medium and total ^3H -BaP metabolites in culture medium from NOE: Effect of TSP. At 12.5 ug/ml 2RF4: BaP adduct level, 5.2 adducts/ 10^7 nucleotides; BaP tetrol level, 1.1 fmol/ml medium; ^3H level, 4.1 fmol/ml medium. Cells were pretreated with 12 ug/ml 2RF4 for 18 h and then for 6 h with 0.5 μM ^3H -BaP (one Ci/mmol). NOE from patient 69-09.

Figure 3.

Effect of pretreatment time with TSP on metabolism of BaP by NOE. Cells were pretreated for 0, 3, 6, 18 h and 0.2 μ M BaP (one Ci/mmol) was then added and incubation was continued for three hours. NOE from patients, 35-10 and 36-10 were treated with TSP from ECL or UL, as shown in legend. In each case the background metabolism (19 – 24 pmol/ml medium) was subtracted from the values in the figure. Duplicate plates were treated for each condition and the average value and the difference from this average (error bars) are plotted on each curve.

Figure 4.

Concentration-dependent responses for effects of different TSPs metabolism of BaP by NOE. Cells were pretreated with TSPs for 18 h before addition of 0.2µM BaP (one Ci/mmol). The points of the curves represent the average values for three or more patients and each patient assay was performed in duplicate. The numbers of patients for used for each TSP were: Reference unfiltered cigarette (2RF4), 6; Ultra-light (UL), 5; mentholated smooth (MS), 3; reference unfiltered cigarette (IR3), 3; Eclipse (Ecl), 4. The inter-patient variability the SD at each point was relatively large (10 – 40%) so for clarity in presentation of the figure, the SDs are not shown.

Table 1

Effects of different TSPs on gene expression in different patients^{1,2,3}

	Patient # and Tobacco Type																	
	PT98 UL		PT98 IR3		PT98 2RF4		PT98 MS		PT90 UL		PT90 IR3		PT 90 2RF4	PT90 MS		PT14 ECL	PT 79 Low dose ⁴	PT 79 High dose ⁴
Gene ⁵	fold change	±	fold change	±	fold change	±	fold change	±	fold change	±	fold change	±	fold change	fold change	±	fold change	Low fold change	High fold change
Cytochrome P450, family 1, subfamily A, polypeptide 1	1202	172	2590	305	3658	693	2753	287	5492	343	8306	851	5078	4991	655	203	1253	1584
Hydroxysteroid (17-beta) dehydrogenase 2	6.32	0.60	7.59	0.14	6.67	1.48	8.26	1.26	5.95	1.13	9.29	1.15	8.63	6.61	0.91	5.96	6.37	5.43
Microsomal glutathione S-transferase 1	2.22	0.18	2.66	0.34	2.41	0.48	2.62	0.44	4.14	0.32	4.99	0.45	3.01	4.47	0.29	2.7	4.26	6.41
NAD(P)H dehydrogenase, quinone 1	13.01	1.79	13.82	1.20	9.23	0.46	9.04	0.83	19.74	2.23	21.33	6.05	13.18	15.56	0.51	2.96	19.86	46.21
ATP-binding cassette, sub-family A, polypeptide 1	1.43	0.03	1.43	0.09	1.33	0.09	1.22	0.07	3.58	0.23	4.58	0.00	1.93	2.32	0.08	1.88	1.70	1.64
Alcohol dehydrogenase 4 (class II), polypeptide	-2.32	0.60	-1.02	0.31	-1.50	0.76	-1.62	0.50	2.08	0.42	-2.12	0.28	-1.89	-1.06	0.42	-4.04	-1.42	1.197
Alcohol dehydrogenase 6 (class V)	-2.35	0.17	-2.17	0.10	-1.38	0.03	-1.93	0.03	1.24	0.09	1.07	0.06	1.17	1.60	0.01	1.87	-1.07	1.03
Aminolevulinatase, delta-, dehydratase	1.22	0.08	1.26	0.10	1.46	0.13	1.17	0.20	1.46	0.38	1.10	0.04	-1.16	1.45	0.11	34.03	-1.18	1.11
Aryl hydrocarbon receptor nuclear translocator	1.00	0.09	1.11	0.06	1.22	0.52	-1.07	0.04	1.71	0.34	2.08	0.22	1.32	1.93	0.15	3.59	1.51	1.24
Aryl arsenite transporter, ATP-binding, homolog 1 (bacterial)	-1.01	0.05	-1.02	0.20	1.08	0.21	-1.14	0.10	-1.48	0.35	-1.41	1.04	-1.15	-1.73	0.58	2.67	1.68	1.88
Cytochrome P450, family 2, subfamily F, polypeptide 1	-1.30	0.66	1.26	0.51	1.18	0.22	-1.61	0.06	2.25	0.39	2.61	1.38	-1.07	1.75	1.83	3.15	-2.60	-1.66
Epoxide hydrolase 1, microsomal (xenobiotic)	1.49	0.14	2.00	0.13	1.78	0.18	1.49	0.19	5.49	0.57	7.41	0.18	4.23	3.94	0.29	1.09	2.25	-1.27
Fatty acid amide hydrolase	1.03	0.18	1.20	0.14	1.24	0.19	1.20	0.07	2.12	0.24	2.97	0.48	1.92	1.45	0.07	-1.47	1.60	-1.64
Glucokinase (hexokinase 4) regulator	1.68	0.25	1.97	0.03	2.47	0.15	1.17	0.05	1.73	0.35	2.07	0.14	-1.01	1.39	0.36	4.18	1.95	3.76
Glucose phosphate isomerase	1.72	0.06	1.48	0.03	1.45	0.01	1.28	0.02	1.46	0.12	1.47	0.27	1.08	1.40	0.06	2.58	1.41	2.30
Glutathione peroxidase 2 (gastrointestinal)	9.62	0.15	12.41	0.29	7.96	0.62	6.88	0.26	19.20	0.95	23.34	3.73	13.83	13.04	0.29	1.16	5.51	11.47

	Patient # and Tobacco Type																		
	PT98 UL		PT98 IR3		PT98 2RF4		PT98 MS		PT90 UL		PT90 IR3		PT 90 2RF4	PT90 MS		PT14 UL	PT 14 ECL	PT 79 Low dose ⁴	PT 79 High dose ⁴
	fold change	±	fold change	±	fold change	±	fold change	±	fold change	±	fold change	±	fold change	fold change	fold change	fold change	fold change	fold change	fold change
Gene ⁵																			
Glutathione reductase	1.91	0.40	2.37	0.67	1.95	0.45	1.13	0.31	4.25	0.77	4.03	0.36	1.93	2.25	0.04	1.04	1.16	1.86	2.41
Glutathione S-transferase alpha 4	1.46	0.01	1.75	0.02	1.95	0.00	1.97	0.04	2.31	0.17	1.74	0.09	1.69	1.97	0.24	-1.96	-4.49	1.44	1.62
Glutathione S-transferase mu 2 (muscle)	1.65	0.05	2.28	0.06	2.18	0.09	1.20	0.10	2.13	0.29	2.98	0.16	-1.46	1.69	0.03	2.83	2.55	-1.84	-1.06
Glutathione S-transferase mu 3 (brain)	2.82	0.39	3.34	0.75	3.40	0.11	2.14	0.41	2.70	0.59	3.12	0.89	2.60	2.30	0.30	-1.39	-1.26	1.25	-1.02
Metallothionein 2A	3.08	0.21	2.64	0.13	1.95	0.17	2.33	0.04	2.41	0.38	2.38	0.48	1.08	1.53	0.22	1.08	1.39	4.12	4.41
Paraoxonase 2	1.15	0.04	1.13	0.02	1.08	0.01	-1.03	0.07	1.30	0.05	1.18	0.02	1.25	1.40	0.17	2.8	2.96	1.46	1.51
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1	1.28	0.13	1.17	0.02	1.19	0.01	-1.16	0.11	1.54	0.25	1.78	0.11	1.09	1.25	0.03	2.83	2.69	1.68	1.31

¹ Where there was sufficient RNA the samples were repeated and the mean +/- the deviation from the mean is given.

² Genes changed by more than 2 fold are bolded.

³ The concentration of tsp in the culture medium was 80 ug/ml unless otherwise indicated

⁴ Low concentration tsp in culture medium was 5 ug/ml, high concentration was 80 ug/ml

⁵ The first four rows in the table represent genes consistently modulated by two fold or more.

Table 2Effect of concentration of 2RF4 on expression of selected drug metabolism genes in NOE^I

µg/ml 2RF4	Fold increase		
	CYP1A1	CYP1B1	NQO1
40.0	72.1 (± 5.3)	49.9 (± 6.4)	50.2 (± 7.5)
10.6	39.5 (± 4.1)	31.5 (± 2.1)	5.5 (± 1.6)
4.0	17.0 (± 1.8)	10.3 (± 0.8)	0.8 (± 1.2)
1.0	1.6 (± 1.0)	0.7 (± 1.2)	0.4 (± 1.8)
0.0	1.0	1.0	1.0

^I NOE from Patient 90-08