Permeation and reservoir formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) across porcine esophageal tissue in the presence of ethanol and menthol

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Environmental influences may affect carcinogen absorption and residency in the tissues of the aero-digestive tract. We quantified the effect of ethanol and menthol on the rates of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) absorption using a fully validated *in vitro* diffusion system, capable of accurately and precisely quantifying tobacco carcinogen permeation and reservoir formation in porcine esophageal mucosa. Confocal microscopy was employed to visualize the location of B[a]P in the exposed membranes. Markedly different extents of permeation and reservoir formation for the tobacco carcinogens were recorded in the presence of ethanol and menthol. The water-soluble NNK permeated the membrane rapidly, while the lipophilic B[a]P did not appreciably diffuse through the tissue. Significantly different extents of reservoir formation were observed for the different carcinogens and in the presence of the different penetration-enhancer solvents. Alcohol (at 5% concentration) did not influence the permeation or reservoir formation of NNK. A mentholated donor solution (0.08%) both decreased the flux of NNK and significantly increased the tissue reservoir formation. The magnitude of the reservoir formed by B[a]P was relatively extensive (even though membrane permeation rates were negligible), being greatest in the presence of both ethanol and menthol. This suggests synergy between the two penetration-enhancer species acting on this carcinogen. Confocal microscopy studies confirmed that there was an appreciable intracellular, and specifically nuclear, association of the B[a]P species during the reservoir formation process. The aqueous solubility of the diffusing species and the presence of penetration enhancers appeared to be key factors in the absorption and cellular binding processes. The results presented support the hypothesis that the use of mentholated cigarettes, or the concomitant consumption of alcohol while smoking, may have marked effects on the fate of tobacco chemicals. This finding may help to explain elevated rates of esophageal squamous cell carcinoma in African Americans.

Abbreviations: ANOVA, analysis of variance; B[a]P, benzo[a]pyrene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HMB, 2-hydroxy-4-methoxybenzophenone.

Introduction

Cancers of the esophagus represent an important public health problem. In the USA, an estimated 14 250 new cases were diagnosed in the year 2004, and 13 300 deaths would result from the disease (1). This lethality rate rivals that of pancreatic cancer and is more than four times that of rectal cancer (1). The statistic that 90% of individuals diagnosed with an incident cancer of esophagus are dead at five years, underlines the importance of focusing on prevention of this condition (2). Unfortunately, the measures needed for primary prevention do not seem as clear-cut for populations at highest risk of this disease (e.g. African Americans) as for those populations represented in most epidemiologic studies (3,4).

Esophageal cancer incidence and mortality rates among African Americans were over three times those of Whites in the late 1980s (5,6). This large differential emerged between 1950 and 1977, when the age-adjusted esophageal cancer incidence rate approximately doubled in African Americans. Thereafter, the incidence increased slightly until leveling off in the mid-1980s (5). During the same period, the rates remained virtually unchanged in Whites. The changes in the profile of histological subtypes is of note; squamous cell carcinomas in African Americans increased, while adenocarcinomas remained relatively constant in both races (7,8). In the years since, rates of squamous cell carcinomas have remained constant in both races, albeit with African Americans remaining at a distinct disadvantage in relation to Whites, owing to the increases in rates prior to the mid-1980s. In contrast, adenocarcinoma rates have increased markedly during this latter period (from the late 1980s), rising ~3–4% per year, with the increase being confined almost entirely to Whites (7,8). Adenocarcinomas generally arise from the distal esophagus, near its junction with the stomach, and are strongly associated with gastro-esophageal reflux disease. Squamous cell carcinomas, which are more strongly associated with tobacco and alcohol, tend to arise from the body of the proximal esophagus (9–11).

Analytic epidemiologic studies conducted in the West, and almost exclusively in Whites, indicate that tobacco smoking and the consumption of alcoholic beverages, and especially the combination of the two, account for >95% of all squamous cell carcinomas of the esophagus (12). What is both puzzling and fascinating is that African Americans neither smoke nor drink more than Whites, yet their rates for this cancer are three to four times higher (5,6). In South Carolina, squamous cell esophageal cancer incidence in African Americans is over six times higher than that observed in Whites (13), even though they smoke considerably less (14).

Although overall rates of exposure to alcohol are not much different in African Americans than Whites, differences exist in the type of alcoholic beverages consumed (15–17). An even larger difference between African Americans and Whites is in the type of cigarette smoked. It is a curious historical fact that

when menthol brands were first introduced before World War II, African Americans and women evinced a preference for these brands (18). In a classic example of the interaction between marketing and product preference, menthol brands were targeted specifically at African Americans (19). From their introduction through 1955, mentholated brands represented ~3% of all cigarette sales, with African Americans and women accounting for a disproportionate share of purchases (20). From 1956 to the middle of the 1970s, menthol cigarettes increased in popularity (18,21), achieving ~29% of market share in the late 1970s. Mentholated cigarette sales began falling in the late 1980s and now account for just under 26% of total sales (21). Currently, ~75% of African American smokers use mentholated brands (versus 23% of Whites) (22). The observation that exposure to mentholated cigarettes could explain some of the discrepancy between overall tobacco exposure and rates of esophageal cancer (and other cancers of the upper aero-digestive tract) in African Americans, led to a number of studies in the late 1980s (5,23–26). The reasons for these disparities in incidence form the background to this study.

It has been established in transdermal absorption studies that potential diffusion enhancers, such as ethanol, markedly increase the diffusion of co-administered chemicals through the skin (27,28). It is highly likely that a similar penetration enhancement of cigarette-smoke carcinogens occurs in oral and esophageal tissues, and this penetration enhancement may be one mechanism to explain the strong association between cigarette smoking, heavy alcohol consumption and the development of squamous cancers (29). Similarly, menthol is a known penetration enhancer for chemicals diffusing through the skin (27). Therefore, the presence of menthol in certain brands of cigarettes, together with alcohol, may have a synergistic, penetration-enhancing effect in esophageal tissue permeation; the two chemicals collectively contributing to enhanced carcinogenicity in this tissue (especially in African Americans).

Polycyclic aromatic hydrocarbons like benzo[*a*]pyrene (B[*a*]P) ($\log P = 6.40$) and nitrosamine compounds like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) ($\log P = 0.090$) are considered to be two of the most potent and prevalent tobacco carcinogens that cause oral and esophageal cancer (30,31). While NNK is a systemic carcinogen and B[*a*]P is believed to act locally, both appear to be carcinogenic in a dose-dependent manner. We now know that most tobacco carcinogens require metabolic activation to form DNA adducts (carcinogen metabolites bound covalently to DNA) by which they exert their carcinogenic effects. In addition, there is some evidence that high-affinity esophageal microsomes are responsible for the activation of tobacco carcinogens, yielding metabolites that interact directly with the DNA (32). In light of this knowledge, it is assumed that if menthol, ethanol or the combination of the two can increase the permeation of the carcinogens, or prolong their residency time within the squamous tissues by reservoir formation, then these diffusion aspects may potentiate esophageal cancer risk. Theoretically, greater concentrations of the carcinogens, exposed to the cellular genetic material for longer periods of time, should increase the propensity for deleterious biochemical reactions to occur within the esophageal mucosa.

To date, there is little information in the literature concerning the exact diffusion rates for tobacco-smoke carcinogens permeating esophageal mucosa. The aims of the present study were (i) to make use of the Franz diffusion cell system to accurately quantify the permeation of NNK and B[*a*]P through pig esophageal tissues, from saturated donor solutions, (ii) to investigate the effect of ethanol and menthol on these permeation rates and (iii) to investigate the mucosal route of penetration (inter- or intra-cellular diffusion) of B[*a*]P, a fluorescent chemical, using confocal microscopy. It is believed that penetration-enhancer chemicals (e.g. alcohol or menthol) partition into the biological membrane from the donor solution, and alter the biochemical composition of the membrane barrier. This subsequently decreases the chemical resistance to diffusion of the co-administered test chemical. Similarly, the presence of the penetration enhancer in the membrane may facilitate binding of the test chemical to increase the size and duration of the reservoir formed in the tissue. With this investigative protocol, we would obtain quantitative intrinsic diffusion data for the carcinogens, and explore the route of permeation through the mucosa at the cellular level. Resulting data may give valuable insight into physicochemical aspects of the process of esophageal carcinogenesis, e.g. N'-nitrosornicotine (NNN) is a known specific esophageal carcinogen that should have a similar tissue diffusion profile to NNK since their calculated $\log P$-values are similar (0.090 for NNK and −0.076 for NNN). Furthermore, this experimental regimen would allow us to show if any tissue penetration enhancement of these model carcinogens was caused by the presence of ethanol or menthol. These evaluations, in turn, would assist in providing the essential ground-work for designing a representative animal model *in vitro* system for assessing human esophageal carcinogenicity.

Human esophagus epithelium comprises non-keratinized, stratified squamous tissue with melanocytes, endocrine and Langerhans cells. The basal zone (proliferative cells) and the suprabasal zone comprise squamous cells that flatten and mature as they approach the lumen. The underlying lamina propria and the submucosa comprise of loose connective tissue, vessels, glands and ducts; and form ~70% of the epidermal thickness. Porcine esophagus is often used as a model for human tissue because its morphology, histology and biochemistry are similar to that of man (33–35). The esophageal tissues of both species consist of non-keratinized, squamous epithelial cells; in the adult pig the stratified squamous epithelium is 28–40 cell layers thick, with the outer squamous layers comprising more than one-third of the entire thickness.

Materials and methods

Materials

NNK was purchased from Toronto Research Chemical (Toronto, Canada). B[*a*]P was purchased from Fluka Chemie Gmbh (Buchs SG, Switzerland). DL-menthol 99%, polyoxyethylene 20 cetyl ether (Brij 58) and 2-hydroxy-4-methoxybenzophenone (HMB) were purchased from Acros Organic (New Jersey, USA). Ethanol (100%) was purchased from AAPER Alcohol and Chemical (Kentucky, USA). Sodium chloride, acetonitrile HPLC grade and 0.2 μm filters were purchased from Fisher Scientific (New Jersey, USA). Hamilton syringes (750 LT, 500 μl) were purchased from Hamilton (Nevada, USA). Parafilm® was purchased from Pechiney Plastic Packaging (Chicago, USA). Double-distilled, de-ionized water for HPLC was used throughout this study. Franz permeation cells were purchased from Permegear (Pennsylvania, USA).

Preparation of the esophageal tissues

Whole porcine esophagus was obtained from a local slaughterhouse at the time of animal sacrifice. The mucosal layer of the lumen was separated from the surrounding muscular layers by making a longitudinal incision through the outer muscle/connective tissue of the esophagus, incising down to the

submucosal plane. Blunt lateral dissection along this plane easily separated the luminal mucosa from the muscularis. Care was exercised to avoid any physical damage to the luminal tissue strata during the isolation and separation process. The exposed squamous tissue was irrigated with phosphate-buffered saline (PBS) and cut longitudinally along its cylindrical section to yield a sheet of tissue. The mucosa was cut into circular disks of 1.5 cm diameter for mounting between the flange surfaces of the Franz permeation cell, with the lumen side oriented toward the donor chamber. Since multiple, replicate cells were used in each study ($n = 12–20$), using esophageal tissue from different animals, it was immediately evident from the results obtained if the membrane had sustained any damage during the preparation phase. In these very infrequent cases, the recorded permeation rates would typically be markedly greater than the mean for the undamaged cells and normal statistical validation would exclude the data of these damaged membranes.

Microscopic examination of the histological regions presented in the epithelial specimens prepared by this technique indicate that the combined squamous mucosa, lamina propria and submucosa are ~400 μm thick, with a further 300–400 μm of loosely associated smooth muscle cells with very large intercellular spaces (see photograph). We presume that the close coherence of the muscle tissue is destroyed by the blunt dissection on separation. *In vivo*, any chemicals absorbed across the mucosal epithelium would normally be cleared to systemic circulation from the highly vascularized submucosa. The outermost muscularis layers of the organ are not expected to participate markedly in the clearance process. Therefore, the separation of the tissue at the submucosa-muscularis plane is considered appropriate.

The separated esophageal mucosa used in the *in vitro* permeation experiments reported here does not have a high-resistance, keratinous layer (unlike the stratum corneum of the skin). However, we presume that there is still appreciable resistance to diffusion presented by the luminal squamous tissue (a stratum that must inherently be physically strong to resist the physical and chemical assault from the contents of the digestive tract). The zones of porous muscle cells that remain on the submucosa after separation no longer form a uniform layer and, thus, cannot provide any significant resistivity to the permeant migration from the epithelium to the receptor fluid. This leaves a potential diffusion barrier of ~400 μm thickness in the mucosa and submucosa. It is possible to estimate an 'effective' diffusion barrier thickness from the steady-state flux data obtained in the experiments. If our assertions are correct that some stratal components of the squamous layer are acting as the rate-limiting step to diffusion, then an estimated 'effective' diffusion barrier thickness should be substantially <400 μm, and the lag time to permeant appearance in the receptor fluid should be relatively short. On the other hand, if the submucosa is hindering the passage of the permeant into the receptor fluid then the effective barrier thickness should be closer to 400 μm and the lag time should be substantially longer.

Diffusion experiments

Franz diffusion cells (Figure 1) are routinely used in transdermal drug delivery research (36). These are bi-chambered cells (donor and receptor compartments) that terminate in flat, ground-glass flanges, between which the test membrane (esophageal mucosa) is sandwiched. The units used in this study had an effective diffusion area of 1 cm^2 and a receptor compartment volume of 7.0 ml. Donor solutions of the model carcinogen to be tested were applied to the exposed squamous cell surface of the porcine membrane in the donor chamber of the cell, and this compartment was sealed from the atmosphere with Parafilm® to prevent evaporation of the solvent. The receptor solution
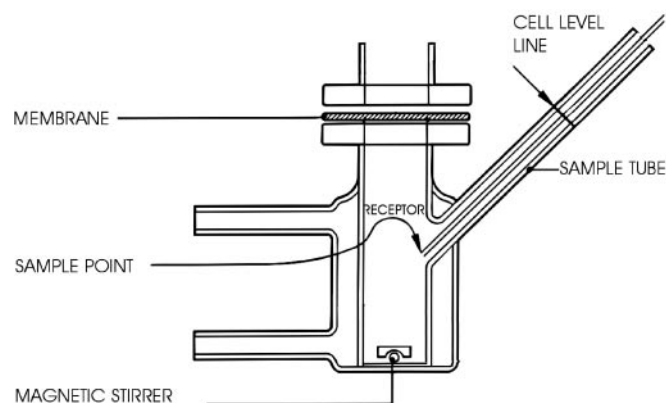

Fig. 1. Static Franz cell for testing esophageal permeation.

was maintained at 37°C and was continuously agitated by magnetic stirrer-bar. Receptor sample aliquots were removed through the cell side-arm using a Hamilton syringe, and replaced with an equivalent volume of fresh solvent.

Assay

NNK and B[*a*]P permeating into the receptor chamber were analyzed by an HPLC system validated to ICH guidelines (37). The system consisted of photoiodide array detector (Waters 2996, MA, USA), coupled to a pump and automatic injector (Waters 2695). The analytical column used was a 3.9 × 150 mm, reversed-phase Nova pack C18, 4 μm (Waters), and the column temperature was maintained at 30°C. The chromatographic conditions for NNK were as follows: the flow rate was 0.6 ml/min and the mobile phase consisted of water/acetonitrile (70/30). The wavelength of the UV detector was 229 nm and the eluent retention time was 3.1 min. The mobile phase used for B[*a*]P was water/acetonitrile (15/85), pumped at a flow rate of 1.0 ml/min, which produced a retention time of 4.5 min at a UV detector wavelength of 296 nm. HMB was used as internal standard.

Solubility testing and preparation of saturated solutions

Saturation of the donor vehicle with the test diffusion chemical is common practice in membrane diffusion studies, as this system presents the maximum thermodynamic delivery potential of the diffusant to the membrane. Delivery solvent bonding effects are, theoretically, negated with saturated donor solutions, allowing determination of the intrinsic diffusive potential of the chemical through the membrane. In sub-saturation conditions, the affinity of the donor solvent for the diffusant may be so intense that partitioning into the membrane from solution is hindered. Initial diffusion studies therefore usually involve saturated donor solutions to determine the innate potential of the chemical to diffuse through the membrane. These initial studies may then be followed by donor solutions containing diffusants at closer to physiological concentrations.

Saturated solutions of NNK and B[*a*]P were used individually for the donor phase in this series of experiments. Normal saline (NS) was used as the solvent for NNK, which has a high aqueous solubility. B[*a*]P is a highly lipophilic compound that has very low solubility in water, thus a cosolvent was added to the NS to slightly increase the solubility of this carcinogen. The cosolvent used for B[*a*]P was 0.5% Polyethylene glycol 20 oleyl ether (Brij). This cosolvent has been validated not to disrupt the resistive integrity of membranes at low concentrations (38). Saturated solutions of each chemical were prepared by sonicating excess solute in the appropriate solvent for 15 min and then stirring the suspensions for 48 h at 37°C. Aliquots were taken from the stirred solution every 6 h for 48 h, centrifuged and filtered through a 0.2 μm filter. The saturation concentrations of NNK and B[*a*]P in the filtrate were measured by HPLC after appropriate sample dilution. The apparent solubility of NNK in saline was found to be 25 mg/ml, and the solubility of B[*a*]P in 5% Brij–NS was found to be 30 μg/ml. A saturated solution of menthol in saline was also prepared by the same method described above at a loading of 0.8 mg/ml. An array of saturated donor solutions in NS were prepared for this test protocol as follows: NNK, NNK in 5% ethanol, NNK in 0.08% menthol, NNK in 0.08% menthol–5% ethanol; BAP in 0.5% Brij, BAP in 5% ethanol–0.5% Brij, BAP in 0.08% menthol–0.5% Brij, BAP in 0.08% menthol–5% ethanol–0.5% Brij. The combination of ethanol and menthol is important to test, as individuals who both smoke and drink tend to engage in these behaviors together (39,40). The concentrations of ethanol and menthol were chosen to parallel typical *in vivo* exposure to these agents: the typical alcohol content of malt liquor beverages and the amount of menthol delivered from a typical mentholated cigarette (41,42).

Permeation study

At time zero, 0.5 ml of the appropriate saturated test solution was placed in the donor chamber of each assembled Franz Cell. Thereafter, receptor sample aliquots of 200 μl were removed at 15-min intervals for the first hour, and at 30-min intervals for the following 5 h, using a 250 μl Hamilton syringe. The NNK and B[*a*]P sample aliquots were transferred to HPLC vials containing appropriate volumes of diluting solvent and internal standard, and were analyzed for carcinogen concentration. This analysis provided the mass permeating per unit area versus time data, which were used to calculate the steady-state diffusion rate. In all cases, the Franz cell receptor volume was replenished with the specific receptor fluid (saline for NNK and 0.5% Brij–NS for B[*a*]P). The saturated solution remaining in the donor chamber after 6 h was removed in order to conduct a mass balance analysis. The donor surface of each membrane was washed for complete solute recovery and dilution purposes, with 6000 μl of saline for NNK and 10 000 μl 0.5% Brij–NS for B[*a*]P. The wash and donor solutions for each cell were combined and analyzed by HPLC. This mass balance analysis would give a good estimate of how much carcinogen is bound as a reservoir within the esophageal tissues after 6 h of experimentation.

Data treatment

The flux ($\underline{J}$) of carcinogen ($mg/cm^2 \cdot h$) permeating the esophageal membrane in the Franz cell is given by the mass (mg) of permeant in the receptor solvent [as determined at each sampling time (t) by HPLC analysis] and the membrane diffusion area (cm^2) for each cell. This flux data may be expressed by Fick's second law of diffusion (Equation 1), which takes into account the donor concentration of permeant (C), its partition coefficient between the donor solution and the membrane (K), the diffusion coefficient of the permeant in the squamous tissue (D), and the thickness of the membrane (L).

$$J = CKD/L = CK_p. \tag{1}$$

We are concerned primarily with the linear, steady-state portion of the permeation plots, where an intrinsic, equilibrium state of carcinogen diffusion is occurring under the test constraints implemented in the study. Since only J and C are known, a simplified version of the Fick's law equation [$J = CK_p$, where K_p (cm/h) is the permeability coefficient] is commonly used to describe this permeation data.

A different equation is often used in permeation research to estimate membrane diffusivity or the effective barrier thickness:

$$D = L^2/6\, t_{LAG}, \tag{2}$$

where t_{LAG} is the diffusion lag time obtained from the intersection of the linear, steady-state permeation plot with the time axis. A comparison of the steady-state flux rates for the different experimental parameters employed will show the influence of the environmental conditions on the absorption of the carcinogens through the esophageal tissue. Furthermore, an estimation of the effective membrane thickness will indicate the fraction of the prepared tissue that is acting as a diffusional barrier.

Statistical analysis

All data were analyzed using SAS (version 8.2e) (SAS Institute, Cary, NC). The effects of the two treatments (ethanol and menthol) on NNK and B[a]P mass balance were assessed using analysis of variance (ANOVA). Mean values of NNK penetration for replicate cells were compared over time using repeated measures ANOVA. Pairwise comparisons of the least-squares means were used to identify mean values that were significantly different ($P < 0.05$). The P-values were adjusted for multiplicity using Tukey's method. The number of samples (n) represents the number of cells monitored in each test group.

Confocal microscopy

The esophageal membranes that were exposed to B[a]P in the typical permeation experiments described above were examined by confocal microscopy at the end of the 6-h permeation experiment. The purpose of this procedure was to identify the transverse route of diffusion of the fluorescent carcinogen through the mucosal tissue. Membranes were preserved overnight in 4% paraformaldehyde at 4°C, prior to embedding in OCT compound and cryo-sectioning at a thickness of 20 μm. This allowed a full-depth image of the mucosal layer (luminal surface to basal membrane) to be captured. Sections were rinsed in PBS and stained in a 1:1000 dilution of Sytox green (to stain cell nuclei) and a 1:200 dilution of rhodamine phalloidin (to stain F-actin). B[a]P fluoresces in the low UV range and a 405 blue diode laser was used for excitation of the specimen sections. This procedure generated a fluorescent signal of sufficient intensity to image the presence of B[a]P in the treated samples. Images were collected on a Zeiss LSM 510 META confocal scanning laser microscope with a 63× oil immersion objective. All images in the blue (B[a]P) channel were collected with identical laser, iris, gain and offset parameters.

Results and discussion

Effect of menthol on permeation and reservoir formation of NNK

The repeated measures ANOVA suggests a possible difference in the time profiles for the four treatment groups ($P = 0.07$). The permeation data for NNK in saline, or when delivered from a 0.08% menthol-in-saline solution are depicted in Figure 2 and summarized in Table I. There is a markedly lower permeation rate for this carcinogen in the presence of menthol; the mean rate being significantly different at each sampling time, and the linear, steady-state flux profiles being significantly different by confidence interval (CI) analysis. Clearly, the permeation of NNK through the esophageal membrane in the presence of menthol was significantly slower than the permeation from the control saline delivery solvent. However, there were significant differences in the size of the NNK reservoir formed from the different delivery vehicles. The magnitude of NNK reservoir formed in the esophageal mucosa on exposure was significantly greater ($P = 0.02$) for the mentholated solution (4.405 mg, $n = 12$) compared with the control (3.666 mg, $n = 20$).

Effect of ethanol and ethanol–menthol on permeation and reservoir formation of NNK

Permeation data for NNK when delivered from a 5% ethanol-in-saline, or from a combined saline vehicle containing 0.08% menthol and 5% ethanol are shown in Figure 3. The combined delivery solvent was tested to investigate possible synergistic effects of menthol and ethanol on carcinogen delivery. There was no significant difference in the magnitude of the mean flux of NNK when 5% ethanol was compared with the control
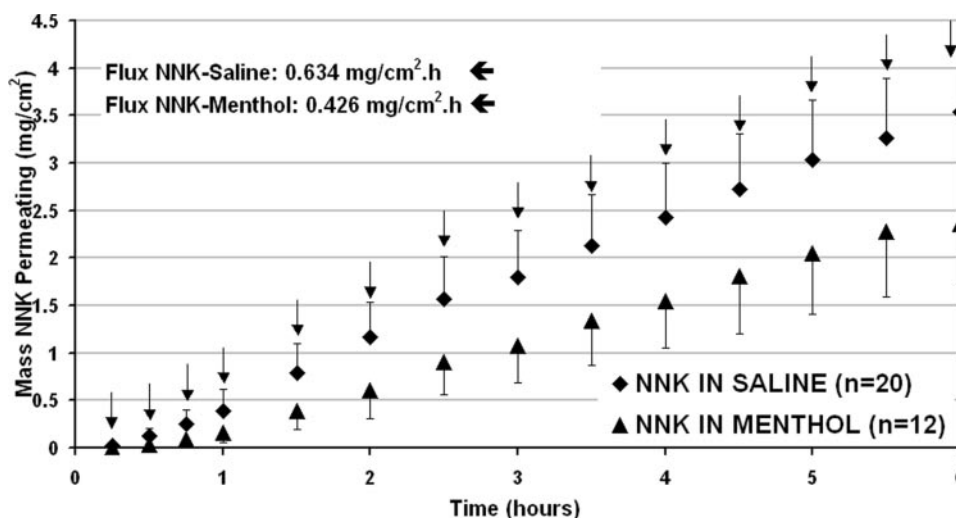

Fig. 2. Mean (±SD) NNK permeation through esophageal mucosa at each sample time, from saline donor vehicles with and without 0.8% menthol (arrows indicate significant differences in data pairs). Steady-state flux values have been calculated from all data in the linear segment of the plot (typically data after the 30-min sample).

saline delivery vehicle, or when delivery was compared from the combined ethanol/menthol vehicle and control.

Similarly, the difference in the reservoir formed in the esophageal mucosa was not significantly different ($P = 0.22$) for the ethanol donor solution when compared with the control. The average reservoir formation ($n = 12$) was calculated to be 3.19 mg for the ethanol delivery solution (3.67 mg for the control solution). However, when both ethanol and menthol are present, the magnitude of the reservoir size decreases to 3.004 mg ($n = 12$), significantly different ($P = 0.044$) when compared with the control (3.666 mg, $n = 20$). Therefore, a significant interaction effect between menthol and ethanol ($P = 0.02$) was observed in the NNK reservoir formation.

Estimation of effective barrier thickness

We can use Equation 2 to estimate the effective diffusional barrier thickness of the exposed tissue. If we assume a general diffusion coefficient for small organic molecules in biological tissue of 1 of 10^{-10} m^2/s, and we obtain the linear intercept for the NNK/saline flux data with the time axis at ~15 min, then the calculated thickness of the effective diffusional barrier layer is 73 μm. Although this is only an estimate because we do not know the exact diffusion coefficient for NNK in this prepared membrane, this thickness is substantially less than the average 400 μm dimension of the total mucosa–submucosa obtained from micrographs. This suggests that there is a smaller stratal component of the overall tissue that is acting as the rate-controlling system. This value for hydrated esophageal squamous tissue is in general congruity with the thickness of the stratum corneum (the highly keratinized, relatively desiccated, principal diffusional barrier of the skin), which normally measures between 10 and 30 μm. The arithmetical estimation of the effective diffusional barrier at 73 μm implies that there is a relatively small histological component of this composite tissue (presumably the luminal mucosa) acting as the predominant barrier to diffusion. The relatively thick layer of the submucosa, therefore, cannot be dominating the kinetics of the permeation process.

Permeation studies with B[*a*]P

The permeation profile was completely different when B[*a*]P was evaluated as the diffusant, using the same delivery vehicles as described above. B[*a*]P is highly lipophilic and has very limited aqueous solubility, thus high permeation rates were not expected through the, essentially, aqueous tissue. We observed no quantifiable amounts of B[*a*]P permeating through the porcine esophageal mucosa from any of the four delivery solvents over 6 h of experimentation, compared with an average flux of 0.634 mg/cm^2·h for NNK under the control test conditions. However, mass balance analysis at 6 h showed that markedly different extents of B[*a*]P reservoir formation occurred dependent on the delivery vehicle in contact with the mucosa (Figure 4 and Table I). The smallest reservoir was formed in the presence of ethanol (1.025 ± 0.374 μg), which was significantly less than the reservoir formed by the saline control (3.594 ± 0.342 μg, $P < 0.0001$). Reservoir masses were similar for saline and saline/menthol (3.557 ± 0.357 μg). The B[*a*]P reservoir was greatest for the saline/ethanol/menthol delivery vehicle (4.940 ± 0.342 μg), which was significantly higher than that from the saline ($P = 0.0386$) or saline/menthol ($P = 0.0374$) vehicles. Furthermore, a significant interaction effect ($P < 0.0001$) between menthol and ethanol was observed in the mass balance analysis, resulting in a significant increase in the reservoir magnitude compared to the control. Reservoir formation with B[*a*]P therefore appears to be highly delivery-solvent dependent; further

Table I. Mean flux and reservoir formation of NNK and B[*a*]P in porcine esophageal mucosa

Donor solutions tested ($n = 12$)	Carcinogen flux (mg/cm^2·h)	Average reservoir formation in membrane
NNK in saline (control) ($n = 20$)	0.634	3.666 mg
NNK in 5% ethanol	0.595	3.187 mg ($P = 0.22$)
NNK in 0.08% menthol	0.426[a]	4.405 mg ($P = 0.02$)[a]
NNK in 5% ethanol and 0.08% menthol	0.596	3.004 mg ($P = 0.04$)[a]
B[*a*]P in saline (control)	[b]	3.594 μg
B[*a*]P in 5% ethanol	[b]	1.025 μg ($P < 0.0001$)[a]
B[*a*]P in 0.08% menthol	[b]	3.557 μg ($P < 0.90$)
B[*a*]P in 5% ethanol and 0.08% menthol	[b]	4.940 μg ($P = 0.04$)[a]

[a]Statistically significant when compared with control for each carcinogen.
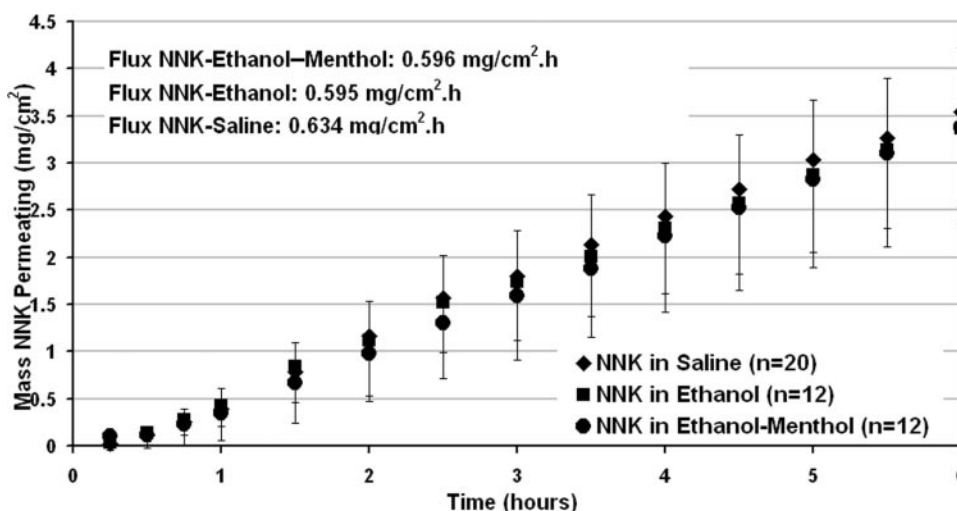[b]No quantifiable permeation of B[*a*]P.

Fig. 3. Mean (±SD) NNK permeation through esophageal mucosa at each sample time, from saline donor vehicles containing 5% ethanol, or 5% ethanol–0.08% menthol. Steady-state flux values for all data in the linear segment of the plot are listed.
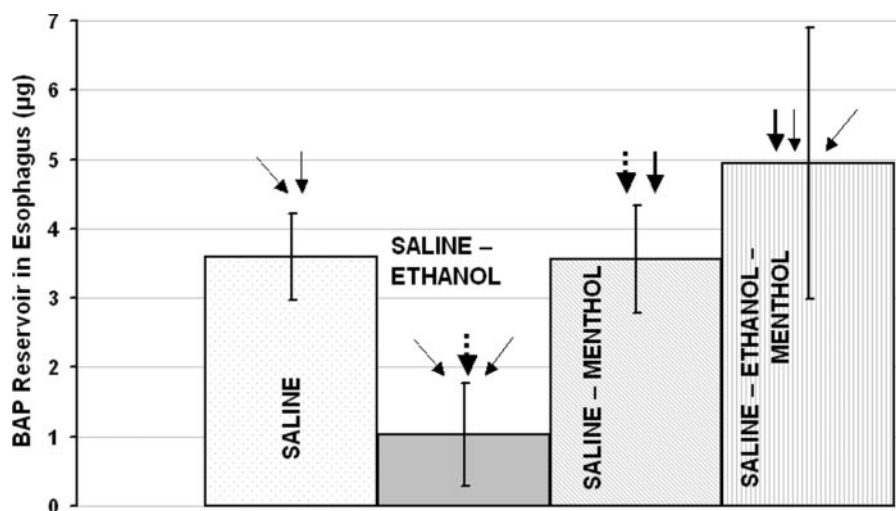
Fig. 4. Mean reservoir formation of B[*a*]P in porcine esophageal mucosa ($\pm$SD) for different delivery vehicles after 6-h exposure (arrow pairs indicate statistically significant differences) ($n = 12$ for each vehicle tested).

investigations of this concept may help to explain the local (rather than systemic) carcinogenic potential of B[*a*]P.

Confocal microscopy studies with B[*a*]P

An important aspect in determining the carcinogenic potential of these tobacco-based chemicals is to elucidate their route of permeation (inter- or intracellular) through the squamous tissue. The presence of a BAP reservoir within the esophageal mucosa is confirmed by the micrographs shown in Figure 5; where the control (Figure 5a) shows only the cellular auto-fluorescence (red), while the B[*a*]P fluorescence (green) is visible in the carcinogen-exposed tissue (Figure 5b). The nuclei, smooth muscle cells and B[*a*]P fluorescence in the exposed membrane could be visualized after appropriate histological staining (Figure 5c). The distribution of B[*a*]P fluorescence was homogeneous throughout the tissue; however, there appeared to be a concentration of the carcinogen in the vicinity of the basement membrane, adjacent to the smooth muscle tissue. Localization of the carcinogens in the proximity of these germinal cells is, theoretically, potentially more damaging than exposure of the cells nearer the organ lumen. Three-dimensional analysis of the image depicted in Figure 5d shows that B[*a*]P was localized inside the cells of the tissue and was associated closely with the cell nuclei.

Assessment of flux values

To our knowledge, there are no reported *in vivo* measurements of NNK or B[*a*]P absorption through the esophageal mucosa, however, there are reported *in vivo* measurements of absorption through the tracheal mucosa of the dog. NNK is rapidly absorbed through canine trachea (43), whereas B[*a*]P is absorbed at a much slower rate (44). Although it is reassuring that the rank order of absorption is the same in both tissues (NNK $\gg$ B[*a*]P), the flux in canine trachea appears to be much larger than the absorption we have recorded for the esophagus in this research. This is not unexpected if one compares the histology of the two tissue systems. The lining of the trachea is composed of ciliated pseudostratified columnar epithelium with goblet cells and a wide basement membrane. This epithelium appears stratified, however every cell rests on the basement membrane, so the epithelium is technically a 'simple monolayer' of cells. Furthermore, the relative

distance of the tracheal lumen to the sub-epithelial blood vessels is markedly shorter than the corresponding distance in the more histologically complex esophagus. The epithelium of the trachea must fulfill a physiological function of gaseous exchange. In addition, the tracheal epithelium does not have to be as mechanically strong as that of the esophagus since there are no physical or abrasive challenges to the trachea from the luminal environment. One would therefore expect a much greater permeation of chemicals through the trachea than through the esophagus, as corroborated by these literature reports.

Discussion

Epidemiologic studies suggest that combined use of alcohol and tobacco has a synergistic effect on the etiology of oral and esophageal cancer, but the mechanism of this carcinogenesis is still unclear. We know that ethanol is an effective permeability enhancer for drug delivery through the skin, thus we expected to see a difference in the permeation of NNK from the ethanolic donor solution and the NS control. This difference was not observed in our study; however, our results are congruent with those reported by Du *et al*. (45) who studied the effect of different ethanol concentrations on the permeation of NNN through porcine oral mucosa. These authors report no permeation enhancement effect of NNN from 5% ethanolic solution, but they observed a significant enhancement effect from 25 to 30% ethanolic donor solutions. In contrast, less NNK permeated through the esophageal membrane from the mentholated donor solution than from the control; however, the reservoir formed in the presence of menthol is significantly greater than that formed with saline or with any delivery vehicle containing ethanol. Therefore, there is a significantly greater amount of NNK bound within the esophageal mucosa in the presence of menthol. The exact reasons for these results are unclear at present; possibly menthol is modifying the donor solvent properties, increasing the affinity of the donor solvent system for the carcinogen (reduced tendency for NNK molecules to partition into the mucosal matrix). Alternatively (or additionally), penetration of the terpene into the mucosal tissue may be affecting the lipid biochemistry of the
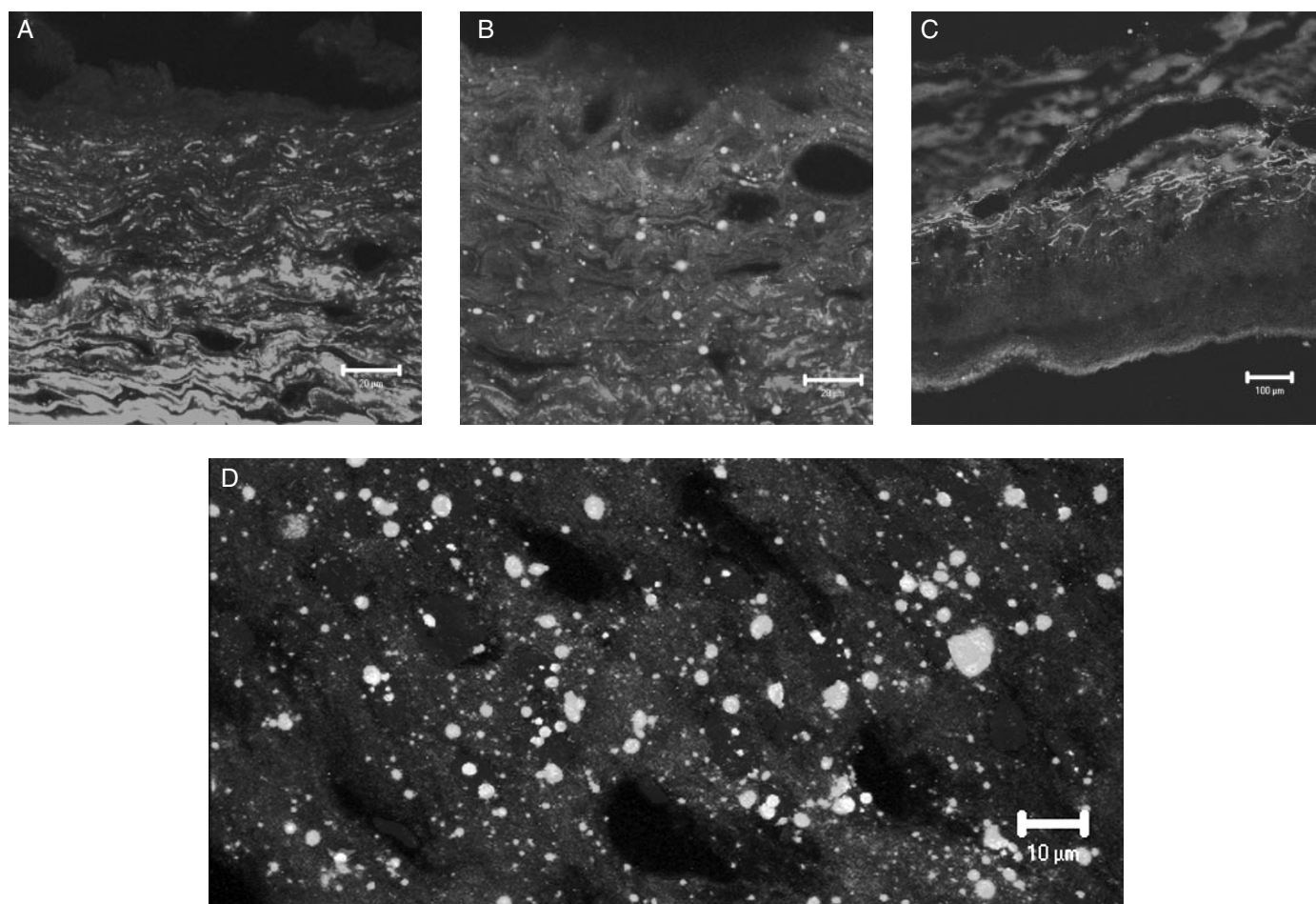
Fig. 5. Confocal microscopy images of esophageal squamous tissue: (**a**) control (untreated esophagus) showing (red) autofluorescence; (**b**) B[*a*]P-exposed membrane (at 6 h) showing the foci of fluorescence (green) due to carcinogen reservoir formation; (**c**) Histological staining, showing B[*a*]P presence (green) in esophageal tissue, blue fluorescence indicates nuclei, and red demarcates smooth muscle cells below the basal mucosal layer; (**d**) Perinuclear localization of the B[*a*]P (green), closely associating with squamous cell nuclei (blue). See on the Supplementary Data.

esophageal tissues, increasing the transient bonding interactions of the chemical matrix with the diffusing NNK molecules (reducing flux and potentiating the reservoir).

The donor phase combination of 5% ethanol and 0.08% menthol in NS did not evince any clear synergism between the two potential penetration enhancers. Even though the NNK flux data for the combined solvent was not significantly different from that of the control, there was a statistically significant difference in the magnitude of the diffusant reservoir formed in the mucosa. This may be an artifact of the intrinsic biological variability between esophageal membrane samples, rather than a true difference in diffusive performance; additional experimentation will confirm this hypothesis. These data therefore show that menthol alone at 0.08% concentration appears to retard NNK permeation through the esophageal mucosa (but appears to potentiate squamous reservoir formation), and its effects appears to be negated in the presence of ethanol.

The slow permeation data for B[*a*]P was expected because of the lipophilicity of this chemical, however appreciable, intracellular reservoir formation in the squamous tissue resulted after exposure. Partitioning of the chemicals into the cells of this mitotic layer is more potentially damaging than binding in the inter-cellular matrix. The results observed here would suggest that there is appreciable intracellular partitioning

occurring during the diffusion equilibrium, and that permeation may follow a trans-cellular (rather than intercellular) pathway. These observations may help to explain why B[*a*]P is a local-acting carcinogen rather than a systemically active agent, and supports the evidence that B[*a*]P exerts its carcinogenicity by binding covalently to DNA.

Conclusions

We have observed markedly different extents of permeation and reservoir formation for the tobacco carcinogens applied to porcine esophageal mucosa in the presence of ethanol and menthol. At this stage, we do not know the exact reasons for these different diffusion profiles; however, the aqueous solubility of the diffusing species and the presence of penetration enhancers appear to be key factors. The water-soluble NNK permeates the membrane at high flux rates, while the lipophilic B[*a*]P does not diffuse through the tissue to any appreciable extent. Furthermore, we have observed significantly different extents of reservoir formation, both for the different carcinogen species and in the presence of different solvents within one chemical entity. While alcohol (at this concentration) does not appear to influence the permeation or reservoir formation of NNK, the presence of menthol in the donor solvent both

decreases the flux of NNK and increases the extent of tissue reservoir formation. In spite of this observation, we believe that an increase in the concentration of ethanol may modify these permeation results, based on previous observations by other researchers (45).

The magnitude of the reservoir formed in the tissue by B[*a*]P is relatively extensive, even though membrane permeation rates are negligible. B[*a*]P reservoir formation in the squamous cells is greatest in the presence of both ethanol and menthol. This may suggest some synergism between the two penetration enhancer species acting on this carcinogen (because alcohol alone decreases the magnitude of the reservoir formed, and menthol alone does not appear to influence reservoir size when compared with the control). In addition, confocal microscopy studies have confirmed that there is an appreciable nuclear association of the B[*a*]P species during the reservoir formation process. These results underscore the dramatic influence of environmental solvent properties on the potential for carcinogen permeation and reservoir formation. In addition, these data would support the theory that B[*a*]P is a locally acting carcinogen, rather than a systemically active agent.

We have fully validated this *in vitro* test system and have shown the test protocol capable of accurately and precisely quantifying carcinogen permeation and reservoir formation in porcine esophageal mucosa. The results presented here support the theory that the use of mentholated cigarettes, or the concomitant consumption of alcohol while smoking them, may have marked effects on the absorption and squamous membrane fate of tobacco chemicals. Continued experimentation in this field will yield a clearer picture of the influence of environmental chemicals on the exposure of buccal–esophageal tissues to tobacco-based carcinogens. This is the first report of a series of experiments that will examine other tobacco-borne carcinogens and the influence of a variety of environmental absorption modifiers. Furthermore, future validation of this porcine test model as an acceptable substitute for human tissue would give additional credence to these results, and would greatly facilitate the array of testing required for long-term, tobacco-related cancer chemo-prevention strategies. We are especially concerned with the broad array of environmental influences that may affect carcinogen absorption and residency in the tissues of the aero-digestive tract. The type of tobacco product used (e.g. smoked, smokeless, mentholated) (48), type of alcoholic beverage consumed (e.g. malt liquor versus spirit) and the influence of salivary composition on this absorption process are key areas for future investigation.

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