Abstract. Many arylamines (including carcinogens) and hydrazine drugs are acetylated by the cytosolic N-acetyltransferase (NAT). NAT plays an important role in the first step of arylamine metabolism and has been found in immortalized human cell lines, human and laboratory animal tissues. Human colon tumor cell line (colo 205) has also been shown to acetylate arylamine and possess NAT activity. The purpose of this study was to determine whether or not N-ethylphenyl acetamide (EPA) could affect cell viability, DNA synthesis and N-acetylation of 2-aminofluorene (AF) in colo 205 cells. Acetylated and nonacetylated AF were determined by using high performance liquid chromatography. EPA displayed a dose-dependent inhibition of cytosolic and intact cells' NAT activity, inhibition of viability and DNA synthesis. Time-course experiments showed that N-acetylation of AF measured from intact colo 205 cells was inhibited by EPA for up to 48 h.

The induction of human cancers by arylamine carcinogens is a multi-step reaction, the first step involving N-acetylation, followed by cytochrome P450 metabolism (1-3). Arylamine N-acetyltransferase (NAT), a ubiquitous enzyme found in the cytosol fraction of the liver and other organs or tissues, participates in the biochemical metabolism of arylamines (4-7). NAT is an important enzyme in the biotransformation of various xenobiotics with a primary aromatic or hydrazine structure, which may play an important role in the etiology of colorectal, breast and bladder cancer (8). In humans there are two functional human NAT genes (NAT1 and NAT2), that have been identified and sequenced, encoding for two different human liver arylamine NATs (9, 10). Individuals are classified as either rapid or slow acetylators (based on NAT activity) and susceptibility to arylamine carcinogens has been associated with acetylator phenotypes. The rapid acetylator has been linked to an increased risk of colorectal cancer (11, 12) while the slow acetylator has been linked to increased drug toxicity from sulphonamide (13) and isoniazide (4), and increased susceptibility to occupational bladder cancer (14). Therefore, the genetic variations in NAT activities within target organs or tissues may indicate different risks among human populations.

Phenylacetate (PA) is a naturally occurring plasma component capable of conjugating glutamine to yield phenylacetyl-glutamine (PAG), which is subsequently excreted in the urine (15). Samid et al. pointed out that phenylacetate is both effective in inducing tumor cell maturation and free of cytotoxic and carcinogenic effects (16). Phenylacetate, a normal mammalian metabolite, is also a potent differentiating agent, but its clinical use is limited by its objectionable odor per se (17). Therefore, in our laboratory we produced N-ethylphenylacetamide (EPA) from PA (Figure 1a and b) to detect whether EPA can affect NAT activity. The reasons for this study were that: i) NAT activity was found in human colon tumor cells (colo 205) (18); ii) there is no available information which addresses the effects of EPA on NAT activity in human colon tumor cells.

Materials and Methods

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sulfonylfluoride (PMSF). Tris-HCl, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), acetyl-coenzyme A and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640, fetal bovine serum, penicillin-streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). All of the chemicals used were reagent grade. EPA was obtained from the Department of Pharmaceutical Chemistry (China Medical College, Taichung, Taiwan).

Preparation of N-ethylphenylacetamide. Phenylacetyl chloride (14.5 g, 0.1 mol) reacted with ethylamine (70% in water, 17 mL, 0.3 mol), as described in an earlier report (19), to give yellowish crystals (13.8 g, 85%), mp 72-74ÆC; IR (KBR) cm⁻¹: 3360 (-NH), 1630 (C=O); 1H NMR (300 MHz, CDCl₃): 1.02 (3H, t, -CH₃), 3.25 (2H, s, CH₂-CO-), 7.20-7.33 (5H, m, -C₆H₅); EIMS m/z: 163 [M]+; Anal. Calcd for C₁₀H₁₃NO: C, 73.59; H, 8.03; N, 8.58. Found: C, 73.67; H, 8.00; N, 8.41.

Human colon tumor cell line (colo 205). Human colon tumor cell line (Human colon adenocarcinoma: colo 205, isolated from ascitic fluid of a 70-year-old Caucasian male with carcinoma of the colon was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were grown in 75-cm² tissue culture flasks at 37ÆC under a humidified 5% CO₂ atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin), respectively. These cells were grown loosely attached and in suspension.

Cell viability determination by using trypan blue exclusion. Cells were grown in 6-well plates at a density of 5x10⁵ cells/well for 24 h. Different concentrations of EPA were then added and the cells were grown at 37ÆC, 5% CO₂ and 95% air for 6, 12, 24 and 48 h. For determining cell viability, the trypan blue exclusion protocol was used. Briefly, about 10 µL of cell suspension in PBS were mixed with 40 µL of trypan blue, and the numbers of stained (dead cells) and unstained cells (live cells) were counted using a hemocytometer (18).

Preparation of colo 205 cytosols. About 1 x 10⁸ cells were placed in 2 mL of lysis buffer (20 mM Tris-HCl, pH 7.5 (at 4 °C), with 1 mM DTT, 1 mM EDTA, 50 µM PMSF, and 10 µM leupeptin) as previously described (18). The suspensions were centrifuged at 9,000 g for 1 min in a model 3200 Eppendorf/Brinkman centrifuge and the supernatant fraction was subsequently centrifuged at 10,000 g for 60 min. The supernatant was collected and kept on ice for NAT activity and protein determinations.

NAT activity determination. The determination of acetyl-CoA-dependent N-acetylation of AF was performed as previously described by Chang et al. (18).

Protein determination. Protein concentration in the human colon tumor cell cytosols was determined by the method of Bradford (19) with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

Effects of various concentrations of the EPA on the N-acetylation of AF in colo 205 cytosols. The reaction mixture consisted of 50 µL cytosol, 20 µL of recycling mixture containing AF at selected concentrations as substrate, and 10 µL of selected concentrations of EPA (18,20). The reaction was started by the addition of acetyl coenzyme A (Ac-CoA). The control reaction had 20 µL distilled water in place of Ac-CoA.

Time-course and concentration effects of EPA on the N-acetylation of AF in colo 205 intact cells. Colo 205 cells (5x10⁵ cells/ml) in selected media were incubated with 22.5 µM AF solution in individual wells of 24-well cell culture plate with or without EPA co-treatment for 6, 12, 24 and 48 h. At the end of incubation, the supernatant was immediately extracted with ethyl acetate/methanol (95:5), the solvent evaporated and the residue redissolved in methanol and assayed for AAF by using HPLC as described previously (18,20).

Effects of EPA on the cellular DNA in colo 205 cells. Cells were grown in 6-well plates at a density of 5x10⁵ cells/well for 24 h. Various concentrations of EPA were added and the cells were grown at 37°C, 5% CO₂ and 95% air for 6, 12, 24 and 48 h. For determining DNA content in cells, the PI stain and FACS analysis protocol were used (21).

Statistical treatment of data. Statistical analysis of the data was performed with an unpaired Student’s t-test.

Results

Effects of EPA on cell viability of human colon tumor (colo 205) cells. The results from trypan blue exclusion experiments indicated that <2% of colo 205 cells were stained when they were incubated in medium containing 10% FBS without EPA. In contrast, in the presence of EPA (0.1 - 2.0 mM), the number of viable cells were decreased with time and concentration of EPA (Figure 2).

Effects of EPA in the cytosol of colo 205 cells.

Figure 1. The structure of PA and N-ethylphenyl acetamide (EPA).
of N-acetylation of AF with or without EPA are given in Figure 3. The data indicated that N-acetylation of AF decreased with increasing concentrations of EPA.

Time-course and dose-dependent effects of EPA on N-acetylation of AF in colo 205 intact cells. When colo 205 cells were incubated with AF, AAF was produced in the culture media, whereas cells without AF or AF without cells did not lead to any detectable AAF in the media in any examined time (Figure 4). Increased time of incubation led to increased AAF production up to 48 h (Figure 4). In the presence of 0.1-2.0 mM of EPA, the N-acetylation of AF was decreased in a dose-dependent manner.
Effects of EPA on the DNA synthesis in colo 205 cells. Cells were incubated with EPA in 6-well plates and harvested by centrifugation. PI stain and FACS analysis for determination of DNA content were performed. EPA (0.1-1.5 mM) caused a decrease in DNA synthesis in the cell population (Figure 5).

Discussion

EPA affected the N-acetylation of AF in both examined systems (cytosols and intact cells). Cell viability, DNA synthesis and AAF production were decreased in a dose-dependent manner with increasing doses of EPA. Since NAT activity has been associated with the susceptibility to the carcinogenic effects of several aromatic and heterocyclic amines (4, 22-26), it was of interest to study the effects of EPA on the N-acetylation of AF in vitro. The results also show that EPA inhibits DNA synthesis in human colon cancer colo 205 cells based on the flow cytometric analysis. These results are associated with a decrease of viable cells. As shown in Figures 1 and 5, decreased DNA synthesis may also lead to cell death. The exact mechanism of EPA-induced apoptosis in this study is not clear.

Other investigators have demonstrated that humans display NAT acetylation polymorphism as shown following exposure to aromatic and heterocyclic amines which are related to urinary bladder (24,25). But N-acetylation also plays an important role for AF-induced carcinogenesis. Dogs treated with nonacetylated aromatic amines (AF) developed bladder cancer only (27,28), while dogs treated with N-acetylated aromatic amine (AAF) developed both bladder and liver tumors (29). It is well known that N-acetylation of AF is the first step for AF metabolism that is conducted by cytosolic NAT. The different results are attributed to the absence of NAT from dog’s blood. Therefore, the susceptibility of individuals to the effects of aromatic and heterocyclic amines may be related to the relative rate of N-acetylation and N-hydroxylation in the liver, the route of excretion of metabolites, and NAT activation and polymorphism in the target organs. Human tissues contain NAT1 and NAT2 protein both encoded by NAT1 and NAT2 genes located in chromosome 8, but separated by at least 25 kb (10, 30). AF is a common substrate for both NATs (31).

It has been reported that the attenuation of liver NAT activity is associated with the occurrence of breast and bladder cancer (22). Changes of NAT activity may also be associated with other diseases. Although the exact mechanism is not well understood, it is known that conversion of AF to mutagenic metabolites involves hydroxylation and acetylation in liver tissues, with additional acetylation of the circulating hydroxyl metabolites occurring in other tissues. Further studies will be required to elucidate the role of N-acetylation in the prevention of carcinogenesis, especially on the involvement of cytochrome P450 in the formation of mutagenic and carcinogenic products through the catalyzing conversion of AAF to 7-OH-AAF (32-35).

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References

34 Hayes MA, Roberts E, Safe SH, Farber E and Cameron RG: Influences of different polychlorinated biphenyls on cytoxicidal, mitoinhibitory, and nodule-selecting activities of N-2-fluorenylacetamide in rat liver. JNCI 76: 683-690, 1966.

Lin et al: EPA Inhibits 2-Aminofluorene N-Acetylation

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