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Antagonistic and synergistic interactions during the binding of binary mixtures of polycyclic aromatic hydrocarbons to the aryl hydrocarbon receptor

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ABSTRACT

In order to assess the potential of polycyclic aromatic hydrocarbons (PAHs) to interact with each other, benzo(a) pyrene (B(a)P) was incubated either alone or in combination with other isomeric 5-ring PAHs in precision-cut rat liver slices. At the end of the incubation, the slices were removed and the *O*-deethylation of ethoxyresorufin (EROD) was determined in microsomal preparations. The BP-mediated rise in EROD activity was suppressed in the presence of dibenzo(a,j)anthracene, dibenzo(a,c)anthracene and picene, whereas it was increased in the presence of pentacene. In the case of benzo(b)chrysene, benzo(c)chrysene and benzo(g)chrysene the effect was concentration-dependent with both antagonism and synergism being observed. The binding of B(a)P to the aryl hydrocarbon (Ah) receptor was similarly modulated by other PAHs. No correlation was evident between binding avidity of the PAH to the Ah receptor and either its potential for interaction or nature of interaction, e.g. synergism or antagonism. These interactions were also independent of the molecular shape (ring arrangement) of the 5-ring isomeric PAHs. Bearing in mind the role of the Ah receptor in chemical carcinogenesis, it may be concluded that interactions at the Ah receptor site may contribute to the well-established modulation of the carcinogenicity of one PAH in the presence of another.

1. Introduction

One of the most important groups of human chemical carcinogens are the polycyclic aromatic hydrocarbons (PAHs), being the products of the incomplete combustion of organic material. They are ubiquitous environmental contaminants present in the air and also in food, the latter being the major source of exposure for non-smokers (Phillips, 2005). It is a large group comprising compounds composed of fused aromatic rings that differ markedly in their carcinogenic activity. Small changes in structure may have profound effects on carcinogenicity; for example 5-methylchrysene is a potent carcinogen whereas chrysene is considered to be a weak carcinogen. Even isomers may be characterised by markedly different carcinogenic potency; benzo[*a*]pyrene B(a)P is a potent carcinogen whereas benzo[*e*]pyrene is at best a weak carcinogen (Hecht et al., 1974). PAHs are indirect-acting carcinogens that require metabolic activation to generate electrophilic intermediates that interact with DNA and mediate their carcinogenicity. The principal pathway of bioactivation proceeds through two cytochrome P450-mediated oxidations, catalysed primarily by the CYP1 family, and a hydration catalysed by epoxide hydrolase, resulting in the formation of dihydrodiol epoxides that function as the ultimate carcinogens (Shimada and Fujii-Kuriyama, 2004; Ioannides and Lewis, 2004).

Humans are exposed to mixtures rather than individual PAHs but carcinogenicity studies conducted in animal models almost always employ single pure compounds. This raises the possibility of interactions, both synergistic and antagonistic in nature, that can influence carcinogenic activity; it is conceivable that the carcinogenic activity of a PAH might be modified by the presence of another, non-carcinogenic member of the group. Indeed in animal studies, it was demonstrated that one PAH could exacerbate or suppress the carcinogenicity of another (Slaga et al., 1978; DiGiovanni et al., 1982). For example, in the mouse skin model the carcinogenicity of B(a)P was enhanced by its isomer benzo[*e*]pyrene but, in contrast, it was suppressed by the presence of dibenz[*a*,*c*]anthracene (DiGiovanni et al., 1982). Since such interactions have been also been observed in PAH-induced DNA damage, interactions can potentially occur at the initiation stage of chemical carcinogenesis involving both metabolic activation to electrophilic intermediates and their subsequent interaction with DNA (Rice et al., 1988; Hughes and Phillips, 1990; Smolarek et al., 1987; Lau and Baird, 1992; Marston et al., 2001; Cherng et al., 2001; Tarantini et al.,

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2011; Staal et al., 2007).

Current procedures employed in risk evaluation assume that no interactions are operative so that risk may be evaluated by summation of the carcinogenic activity of the individual PAHs making up a mixture, or by utilising B(a)P as the surrogate PAH (Relative Potency Factor, RPF) (Bostrom et al., 2002; Pufulete et al., 2004). However, such approaches are not concordant with observations seen in carcinogenicity studies in animal models; furthermore, it has been reported that the cytochrome P450-catalysed metabolism of one PAH may be modulated by the presence of others (Shimada and Guengerich, 2006; Shimada et al., 2007); thus it is reasonable to assume that the carcinogenic potential of a PAH mixture may not be reflected simply by the carcinogenic potential of its individual components.

In studies aimed at identifying biomarkers that could enable the potential prediction of interactions between PAHs, a series of structurally diverse PAHs were incubated with B(a)P, the prototypic and most extensively studied member of this class of carcinogens, in precision-cut rat liver slices, and the up-regulation of the CYP1 family was determined, as exemplified by the *O*-deethylation of ethoxyresorufin (Pushparajah et al., 2016). These studies revealed that the B(a)P-induced up-regulation of the CYP1 family could be modulated, both synergistically and antagonistically, by the presence of other PAHs. Since the up-regulation of the CYP1 family is regulated by the Aryl Hydrocarbon (Ah) receptor, a transcription factor localised in the cytosol (Köhle and Bock, 2009), it is conceivable that such interactions take place during the binding and/or activation of this receptor to its DNA ligand-binding form. The objectives of the present studies are two-fold: (a) to extend these studies by evaluating the ability of isomeric 5-ring PAHs to modify the B(a)P-mediated CYP1 up-regulation, so that the importance of molecular shape can be investigated, and (b) evaluate whether such interactions occur at the site of binding to the Ah reception.

2. Materials and methods

Cell culture lysis reagent, luciferase assay reagent (Promega, Wisconsin, USA), NADPH, ethoxyresorufin, resorufin (Sigma Co. Ltd., Poole, Dorset, UK), picene, pentacene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (LGC Promochem, Middlesex, UK), 12-well plates (Bibby Sterilin, Helena Biosciences, Sunderland, UK) and Earle's balanced salt solution (EBSS), foetal calf serum, gentamycin, and RPMI 1640 with L-glutamine culture medium (Invitrogen, Paisley, Scotland, UK) were all purchased. Benzo[*g*]chrysene B(g)C, benzo[*c*]chrysene B(c)C, benzo[*b*]chrysene B(b)C, dibenzo[*a,j*]anthracene D(a,j)A and dibenzo[*a,c*]anthracene D(a,c)A were generous gifts from Dr. A Seidel (Biochemical Institute of Environmental Carcinogenesis, Grosshansdorf, Germany). The purity of the PAHs employed in the present study was at least 99.0%. Young adult male Wistar albino rats (200 g) were purchased from B&K Universal Ltd. (Hull, East Yorkshire, UK); animals had free access to food and water and were housed at 22 ± 2 °C, 30–40% relative humidity in an alternating 12-h light:dark cycle with light onset at 07.00 h. Rats were killed by cervical dislocation, and livers were immediately excised and used to prepare precision-cut slices.

2.1. Precision-cut rat liver slices: preparation and incubation

Cylindrical liver cores (8 mm) were used to cut slices (250 μm) utilising a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as previously described (Hashemi et al., 1999). The multiwell system was used to culture slices using 12-well culture plates. Slices from five animals were pooled together. The culture medium (RPMI 1640) was essentially that described by Lake et al. (1993) and contained the following: foetal calf serum (FCS) (5%), L-methionine (0.5 mM), insulin (1 μM), hydrocortisone-21-hemisuccinate (0.1 mM) and gentamycin (50 μg ml^{-1}); one slice was placed in culture medium (1.5 ml) in each well. Incubation was performed for 24 h under sterile conditions, at a temperature of 37 °C and under an atmosphere of 95% O_2/5% CO_2, on a reciprocating plate shaker housed in a humidified incubator. To ensure that dead cells due to slicing are sloughed off, an initial 0.5 h incubation was carried out and the washed slices were transferred into fresh media. Three different slice pools, each comprising 4–10 slices, were used per time point. All PAHs were dissolved in DMSO and the concentration was the same in all incubations and never exceeded 2% (v/v). Slices were incubated with B(a)P alone (0.5 μM) and in the presence of a range of concentrations (0-50 μM) of the interacting PAH. Finally, control incubations were carried out in the presence of only DMSO.

2.2. Enzyme assays

On completion of the incubation, slices were carefully removed from the culture medium, homogenised, and microsomal and cytosolic fractions were prepared by differential centrifugation. The following assays were carried out on the microsomal fractions: the *O*-deethylation of ethoxyresorufin (Burke and Mayer, 1974) and epoxide hydrolase using benzo(a)pyrene 4,5-oxide as substrate (Dansette et al., 1979). Glutathione *S*-transferase was determined on the cytosolic fraction using 4-chloro-7-nitrobenzofuran as substrate (Ricci et al., 1994). Protein was determined on both fractions (Bradford, 1976).

2.3. AhR ligand-binding assay

Binding to the Ah receptor was determined using the chemical-activated luciferase expression (CALUX) assay. H1L1.1c2 cells (kindly donated by Drs. M. Denison, University of California, Davis, USA, and A. Roda, University of Bologna, Italy), were cultured (7×10^4 cells/ml) in 24-well plates for 24 h until 50–70% confluent; cells were cultured in α-MEM (minimum essential medium) supplemented with 10% FBS and penicillin-streptomycin-neomycin antibiotic solution. Cells were then incubated with B(a)P alone or in the presence of another PAH for 24 h at 37 °C and 5% CO_2 in a humid environment, and subsequently washed with PBS; a 100 μl aliquot of the cell culture was then incubated with the lysis reagent (100 μl) for 15 min and the ensuing lysates were centrifuged (13,000*g* for 2 min). Luciferase activity in the supernatant was determined using the Promega stabilised luciferase assay reagent as indicated by the manufacturer. Luminescence was read in a Packard Lumicount microplate luminometer with PlateReader software (Packard Instrument Company) and normalised for cell number. TCDD (10^{-9} M) was employed as a positive control attaining 100% binding. Cell numbers and viability were counted in aliquots (10 μl) of the cell suspension which were mixed with equal volume of trypan blue (4%) in a Modified Fuch's Rosenthal counting chamber, under phase contrast light microscopy. Blue cells were scored as dead/non-viable whereas non-coloured cells were scored as live/viable.

2.4. Statistical evaluation

Results are presented as mean ± SD. Statistical evaluation of the interactions in the binary mixtures was achieved by comparing the mean values of groups using the "least significant difference" LSD test, utilising the Minitap package, version 14.0 (MINITAB Inc., State. College, PA), as we have previously described (Pushparajah et al., 2016).

3. Results

The structures of the interacting 5-ring PAHs are depicted in Fig. 1.

In preliminary studies using LDH leakage as marker, it was established that the PAHs did not compromise the viability of rat liver slices; the only exception was B(b)C as a result of which lower concentrations were used. When rat liver slices were incubated for 24 h with 0.5 μM B(a)P in the presence of a range of concentrations of B(c)C and

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