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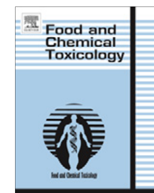

Assessment of the protective effects of selected dietary anticarcinogens against DNA damage and cytogenetic effects induced by benzo[a]pyrene in C57BL/6J mice

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ABSTRACT

The protective action in C57BL/6 J mice from orally administered ellagic acid (EA), benzyl isothiocyanate (BITC), an extract of epigallocatechins (Tegreen®) as well as chlorophyllin (CHL) against benzo[a]pyrene (B[a]P)-induced DNA damage and cytogenetic effects was investigated. In pilot experiment the comet assay indicated protective effects for all compounds, while such activity was confined to EA and CH with respect to B[a]P-DNA adducts and micronuclei. EA and CH were chosen for the main study where the levels of DNA adducts in liver after injection of 30 mg B[a]P/kg b.w. did not differ from those found for animals exposed to B[a]P and treated with the protective substances. In leukocytes no significant protective effect of CHL was detected while a 2-fold increase of adduct concentrations was observed after co-administration of EA. In the comet assay CHL or EA caused a 3-fold decrease of SSB, and a 2-fold decrease of FPG sites in comparison to animals treated with B[a]P. CHL or EA showed a significant protective effect against B[a]P-induced MN in polychromatic erythrocytes in bone marrow. In contrast, flow cytometry measurements in peripheral blood indicated the MN frequency after treatment with CHL or EA almost twice as high as that recorded for B[a]P alone.

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1. Introduction

Several compounds belonging to the group of polycyclic aromatic hydrocarbons (PAHs) are known animal and suspected human carcinogens (IARC, 1984, 1985, 2009). They have been implicated as major initiators of human lung cancer in smokers (Pfeifer et al., 2002; Pfeifer and Hainaut, 2003) and coke oven workers (Boffetta et al., 1997). Because of their widespread distribution in the environment and their presence in certain types of foods, like grilled meat, in concentrations that are sufficiently high (Larsson et al., 1983) to induce DNA damage in humans (Rothman et al., 1990; Van Maanen et al., 1994), PAHs may contribute to the induction of cancers also in the general population. One of the best studied PAHs is benzo[a]pyrene (B[a]P). Although human data specifically linking B[a]P to a carcinogenic effect are lacking, in view of overwhelming evidence from animal studies as well as its well established genotoxicity, this compound has been classified as a human carcinogen by IARC (2009).

Apart from carcinogens of anthropogenic or natural origin, the human diet contains a large number of substances with a chemopreventive potential. Although the most active principles have not been adequately identified, epidemiological data indicate that consumption of fruits and green vegetables can significantly reduce cancer risk (Kamineni et al., 1999; Michels et al., 2006; La Vecchia and Bosetti, 2006; Lila, 2007; Benetou et al., 2008; World Cancer Research Fund, 2009; Martinez-Gonzalez et al., 2009; Nagata, 2010). The aim of the present study was to investigate the efficacy of ellagic acid (EA), benzyl isothiocyanate (BITC), extract of epigallocatechins (Tegreen®) as well as the food additive chlorophyllin (CHL) in offering a protection against B[a]P-induced DNA damage and cytogenetic effects in C57BL/6 J mice. These agents have demonstrated anticarcinogenic action in rodents against selected carcinogens like aflatoxin, PAHs, nitrosamines, and heterocyclic amines (Castonguay, 1993; Hecht et al., 2002; Park and Surh,

Abbreviations: B[a]P, benzo[a]pyrene; BITC, benzyl isothiocyanate; BPDE, Benzo(a)pyrene diolepoxide; CHL, chlorophyllin; dN, deoxyribonucleotides; EA, ellagic acid; FACS, fluorescenceactivated cell sorting; FPG, formamidopyrimidine glycosylase; GT, glyceryl trioctanoin; i.p., intraperitoneal; Tegreen®, an extract of epigallocatechins; MN, micronucleus; MPCE, micronucleated polychromatic erythrocytes; NCE, normochromatic erythrocytes; PAHs, polycyclic aromatic hydrocarbons; PCE, polychromatic erythrocytes; p.o., per os; SCGE, single cell gel electrophoresis; SSB, single strand breaks.

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1996; Reddy and Randerath, 1990; Simonich et al., 2007; Sticha et al., 2000; Stoner and Morse, 1997). Proposed modes of action include, e.g., inhibition of carcinogens activation via inhibition of cytochrome P450 activity, or acceleration of metabolite degradation (e.g. BPDE) to inactive tetrols (Tachino et al., 1994), as well as diverse influence on factors involved in cell cycle regulation, proliferation or differentiation (Aggarwal and Shishodia, 2006).

A broad array of endpoints have been utilized in this study to assess genotoxic damage, including DNA adducts, single strand breaks and FPG-sensitive sites (comet assay) as well as the induction of micronuclei (MN) in blood and bone marrow polychromatic erythrocytes. In order to minimize the number of animals the study was designed in a step-wise fashion, whereby pilot experiments indicated the two most promising candidates for an in-depth investigation.

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene (#B1008-0; CAS: 50-32-8) and glyceryl trioctanoin (GT, #T9126; CAS: 538-23-8) were purchased from Sigma Aldrich (St Louis, MO, USA). AIN93 M rodent diet containing essential nutrients and supplemented with benzyl isothiocyanate (BITC, 1 g/kg, CAS: 622-78-6), chlorophyllin (CHL, 5 g/kg, CAS: 11006-34-1), ellagic acid (EA, 0.67 g/kg, CAS: 476-66-4) was provided by MP Biomedicals Inc. (Ohio, USA, #960397). AIN93 M diet with Tegreen® extract (containing ~75% tea catechins) added at concentration of 1.3 g/kg was purchased from Pharmanex (Provo, UT, USA). Standard rodent diet Murigran was supplied by Agropol (Motycz, Poland). Ribonuclease A and T1, prostatic acid phosphatase, snake venom phosphodiesterase I and nuclease P1 were obtained from Sigma Aldrich. Molecular biology grade phenol was purchased from VWR International (Darmstadt, Germany). The polyethyleneimine thin-layer chromatography plates were from Machery-Nagel (Düren, Germany) and proteinase K was from Roche (Rotkreutz, Switzerland). [^{32}P]-ATP was provided by GE Healthcare (Uppsala, Sweden) and the polynucleotide kinase by US Biochemicals (Cleveland, OH, USA). All other chemicals were of analytical grade and obtained from VWR International or Sigma Aldrich.

2.2. Animals

Female mice C57BL/6 J (6–8 weeks old, weighing between 19 and 21 g) were obtained from the Nofer Institute's breeding farm. The animals were housed under standard laboratory conditions: 21 ± 2 °C and 12:12-h light-dark cycle, food and water accessible *ad libitum*. The study was approved by the local Ethics Committee (resolution L/BD/224 of July 19, 2004). During all experiments body weight, water and fodder consumption were recorded thrice a week before B[a]P exposure and daily afterwards.

2.3. Pilot experiment 1 – characterizing routes of exposure, time- and dose-effect relationships

Pilot experiments were performed to determine suitable routes of administration and dosing schedules (gavage vs. intraperitoneal administration, B[a]P dose selection, sampling times). After one week of adaptation period, 80 animals were randomly divided into 10 groups. The mice were sacrificed 1, 4, 7 or 10 days after exposure to 30 mg B[a]P/kg b.w. dissolved in 100 µl of GT (gavage) or in 300 µl of GT (i.p. injection). Controls were sacrificed 4 days after GT administration. Immediately after sacrifice, liver, blood, stomach, lung and large and small intestine were collected, frozen and stored at −80 °C for later analyses of DNA adducts. For assessment of dose-effect relationship after lower, physiologically more relevant doses of B[a]P, 32 mice were randomly divided into 8 groups and administered 3, 10 or 30 mg B[a]P/kg b.w. in GT by gavage or i.p. Both the exposed and control animals were sacrificed 4 days after B[a]P administration, and tissues used for DNA adduct determination were isolated and stored as described above (Table 1). Dose-response relationship was also assessed as MN frequency in peripheral blood polychromatic erythrocytes (PCE by *fluorescence-activated cell sorting* (FACS) analysis). After collecting whole blood samples, erythrocytes were immediately isolated, then fixed with glutaraldehyde and stored at +4 °C until further analysis.

2.4. Pilot experiment 2 – selection of candidates for in-depth study of chemoprevention

BITC, Tegreen®, CHL, and EA admixed into AIN93 M diet were given to groups of 4 mice for 1 week and during four days subsequent to B[a]P treatment. Calculated *ex post* dosages of the protective substances were as follows: BITC (125 mg/kg b.w./day), Tegreen® (165 mg/kg b.w./day), CHL (625 mg/kg b.w./day) and EA (84 mg/kg b.w./day). After 4 days of B[a]P administration at the dose of 30 mg/kg b.w. (by i.p. or gavage) the animals were sacrificed and biological material was immediately isolated and further processed for DNA adducts (liver and lung), MN test (bone marrow and peripheral blood), as well as comet assay (peripheral blood).

2.5. Main study – protective effects from CHL and EA

After 1 week of adaptation the animals were randomly divided into 5 groups (10 animals per group). After subsequent 1 week of pretreatment with appropriate diets (i.e. AIN93 M fodder without or with CHL (5 g/kg diet) or EA (0.67 g/kg diet)) the animals were exposed by i.p. administration to B[a]P dissolved in GT. After 4 days the animals were sacrificed and biological material was immediately isolated and further processed for DNA adducts (liver and leukocytes), MN test (bone marrow and peripheral blood), and comet assay (peripheral blood).

2.6. Analysis of DNA adducts by ^{32}P-postlabelling

Animal tissues were homogenized and DNA isolated as previously described (Lagerqvist et al., 2008). DNA adducts were analysed by the so-called dinucleotide monophosphate version of ^{32}P-postlabelling assay, as originally described by Randerath et al. (1989) and later modified by Lagerqvist et al. (2008). The adduct spots were detected and the associated radioactivity quantified using a Molecular Imager Pharos FX plus system with an imaging screen K, all from Bio-Rad, Hercules, CA, USA. DNA from 3 to 4 or 6 animals (depending on experiment) was used in 2–3 separate analyses and the data presented as mean ± the standard deviation between the animals. Background radioactivity in the expected location of the B[a]P adducts of vehicle treated animals was subtracted from the radioactive of the adduct spots detected in B[a]P-treated animals before calculation of adduct levels. Benzo(a)pyrene diolepoxide (BPDE)-treated DNA with an adduct level of 1.11/10^6 normal deoxyribonucleotides (dN) (Phillips and Castegnaro, 1999) was a kind gift from F.A. Beland, NCTR, FDA, Jefferson, Arkansas, USA and used as an external standard to correct for losses in the assay. Two samples of 4 µg standard DNA were analysed in parallel with each batch of samples and used for adduct level calculation:

$$\text{Adduct level of animal samples} = \frac{\text{intensity of adduct spots from animal samples} \times \text{known adduct level of standard}}{\text{intensity of adduct spots from external standard}}.$$

Table 1
Schedule and number of animals in pilot experiments on routes of exposure, time of sacrifice and dose-response for DNA adduct formation in mice.

Number of animals								
Time of sacrifice [days after B[a]P treatment]	B[a]P dose [30 mg/kg b.w.]							
	Control animals administered GT only							
	Gavage	i.p.	Gavage			i.p.		
1	–	–	8			8		
4	8	8	8			8		
7	–	–	8			8		
10	–	–	8			8		
	B[a]P dose [mg/kg b.w.]							
	Control animals administered GT only		3		10		30	
	Gavage	i.p.	Gavage	i.p.	Gavage	i.p.	Gavage	i.p.
4	4	4	4	4	4	4	4	4

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