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Genotoxicity of a carcinogenic metabolite of benzo[*a*]pyrene for cells of neuronal differentiation lineage

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Aim. Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) is the major metabolite of an environmental carcinogen, polycyclic aromatic hydrocarbon benzo[*a*]pyrene. The effects BPDE could have on neuronal cells progenitors are mostly uncharacterized. **Methods.** We have studied survival and morphology of cultured PC12 cells and mouse embryonic hippocampal neurons in the presence of BPDE. We have also used post-labeling to compare accumulation of BPDE adducts in cultured PC12 cells and fibroblasts. **Results.** The survival of cells and the level of adducts depended on the type of extracellular matrix to which the cells were attached. At tolerated BPDE doses, the adducts formed by this metabolite were efficiently repaired. In PC12 cells, BPDE toxicity and the level of adducts was generally lower than in fibroblasts. **Conclusions.** Overall, BPDE may be detrimental for developing neural tissue. However, the effects of BPDE on cells with the ability to differentiate into the neuronal lineage may depend on the cell microenvironment.

Keywords: DNA damage, DNA repair, genotoxicity, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, neuronal lineage.

Introduction. Benzo[*a*]pyrene (BP) is a well-known environmental mutagen and carcinogen. BP is metabolized by the consecutive action of cytochromes P450 (CYP1A1, CYP1B1) and epoxide hydrolase into several stereoisomers of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) [1], which then intercalate into DNA and react with purine moieties, predominantly with the exocyclic amino group of guanine [2]. Formation of BPDE adducts is cytotoxic and produces mutations and chromosome aberrations [3].

Although BP and BPDE action was extensively studied in human cells, only a limited number of cell lines have been characterized in this respect. In particular, little is known about the effects of these agents in cells of neuronal lineage, which may be important for assessing the environmental toxicity of BP. Here we analyze cyto-

toxic and genotoxic action of BPDE in two cell types capable of differentiation into neurons, PC12 rat pheochromocytoma cells and immature mouse embryonic hippocampal neurons.

Materials and methods. *Cell isolation and growth.* GM00637 cells (SV40-transformed human skin fibroblasts) and PC12 cells were obtained from the Coriell repository. All cell cultures were grown at 37 °C in 5 % CO₂. GM00637 cells were grown in DMEM supplemented with 10 % fetal calf serum, 10 U/ml penicillin and 100 mg/ml streptomycin. PC12 cells were grown in the same medium in suspension for 2–3 days, passed through a 40- μm filter, and seeded at 10⁵ cells/well into 24-well plates precoated with either poly-D-lysine, mouse laminin, their 10:1 mixture, or rat tail collagen (all from «Sigma-Aldrich», USA). The cells were then grown in DMEM High Glucose supplemented with 10 % horse serum, 5 % fetal calf serum, penicillin and streptomycin

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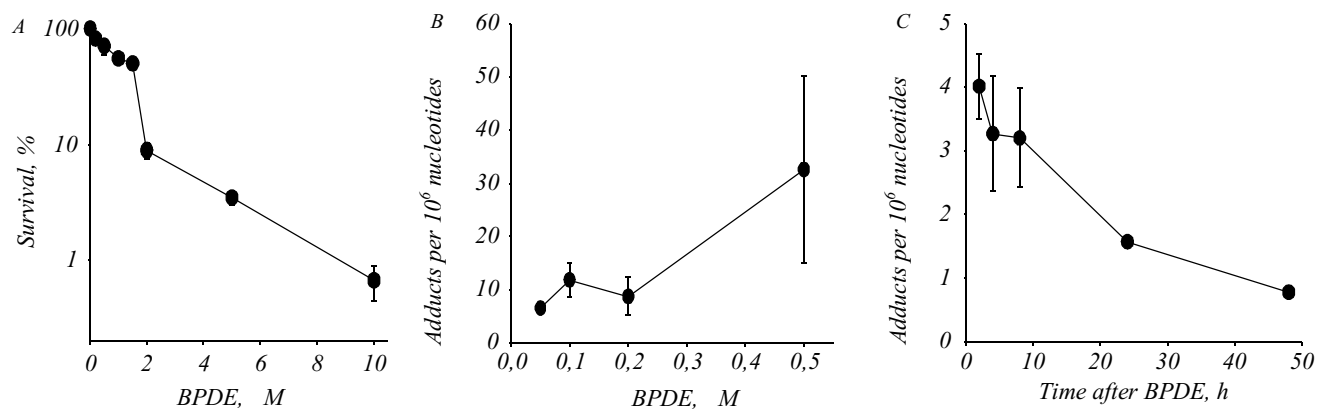


Fig. 1. *A* – toxicity of BPDE for GM00637 cells (the cells were treated with 0–10 M BPDE for 14 h, and their survival was determined by the ATP assay); *B* – accumulation of BPDE adducts in DNA of GM00637 cells (the cells were treated with 0–0.5 M BPDE for 2 h, and the adducts were analyzed by postlabeling); *C* – removal of BPDE adducts from DNA of GM00637 cells (the cells were treated with 0.05 M BPDE for 2 h, and the adducts were analyzed by postlabeling after 2–48 h in BPDE-free medium). Mean \pm SD of 3 independent experiments is shown

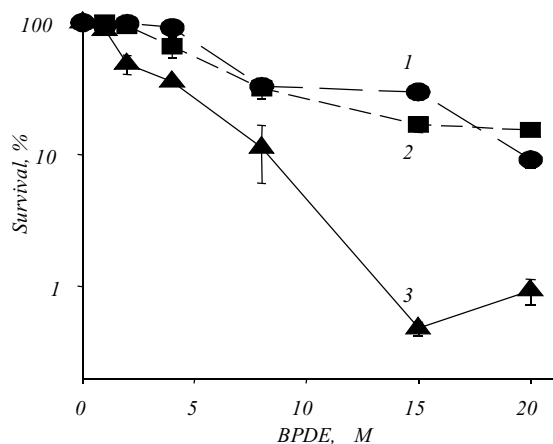


Fig. 2. Toxicity of BPDE for PC12 cells grown on the surfaces coated with poly-D-lysine (1), laminin (2) or their 10:1 mixture (3). The cells were treated with BPDE for 48 h. Mean \pm SD of 3 independent experiments is shown

as above. The relative amount of live cells was determined by their ATP content using the ATP bioluminescent somatic cell assay kit («Sigma», USA). To obtain mouse embryonic hippocampal neuron cultures, hippocampi from C57BL/6 E17.5 embryos were dissected, trypsinized, triturated in G3 medium (Neurobasal medium with B27 supplements, 25 M glutamate, 0.5 mM L-glutamine, and 10 g/ml gentamycin sulfate, penicillin and streptomycin as above) and passed through a 40- μ m filter as described [4]. The cells were seeded at 10^5 cells/well into 6-well plates precoated with a 10:1 mixture of poly-D-lysine and laminin in G3 medium. After 4 h, the medium was changed, and after 4 days, glutamate was removed.

BPDE treatment. BPDE (a mixture of *trans*(+) and *trans*(-) stereoisomers) and BPDE-adducted oligonucleotides were a kind gift from Dr. Nicolas Geacintov (New York University) GM00637 or PC12 cells were grown to confluence, washed with PBS and treated by 1–20 M BPDE (solution in DMSO) for the indicated time in the appropriate medium free of serum. If necessary, cells were then washed and transferred for the specified amount of time into serum-free, BPDE-free medium. For the control, the cells were grown without BPDE in DMEM, DMEM with serum, or DMEM with 2.5 % DMSO. Mouse embryonic hippocampal neurons were used at day 5 and treated with 10 M BPDE for 24 h.

Adduct quantitation by postlabeling. DNA was isolated from BPDE-treated cells using the DNeasy Blood and Tissue kit («Qiagen», USA). 5–20 μ g of DNA was hydrolyzed overnight at 37 °C with a mixture of snake venom phosphodiesterase and micrococcal nuclease, and then for 1 h at 37 °C with P1 nuclease as described [5]. The hydrophobic deoxynucleoside-BPDE adducts were extracted into butanol and phosphorylated with 10 U OptiKinase («Affymetrix», USA) and 7.5 MBq [³²P]-ATP. The reaction mixtures were separated by electrophoresis in 30 % non-denaturing polyacrylamide gels [5], which were visualized and quantified by phosphorimaging using Molecular Dynamics Storm 860 Imager. To quantify the adducts, a calibration curve was built using synthetic oligonucleotide standards containing a single BPDE adduct (5'-TCTTCTTCTGTXYCYCTCTTCTTCT-3'; X = G or *trans*(-)G-BPDE, Y = *trans*(+)A-BPDE or A) treated in the same way.

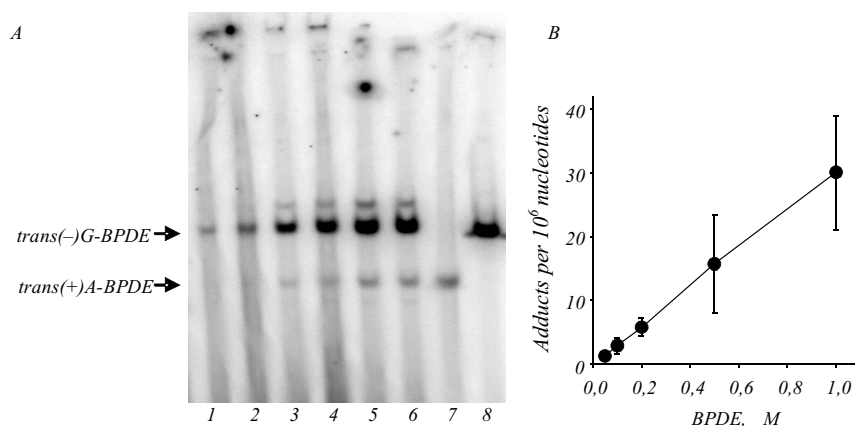


Fig. 3. Postlabeling assay for BPDE adducts: *A* – gel showing the accumulation of adducts in PC12 cells grown on collagen and treated with 0.05 (1), 0.1 (2), 0.2 (3), 0.5 (4), 1.0 (5), and 2.0 mM BPDE (6). Lane 7, *trans*(+)-A-BPDE standard; lane 8, *trans*(-)-G-BPDE standard; *B* – accumulation of BPDE adducts in DNA of PC12 cells. The cells were treated with 0–1 M BPDE for 2 h, and the adducts were analyzed by postlabeling. Mean ± SD of 3 independent

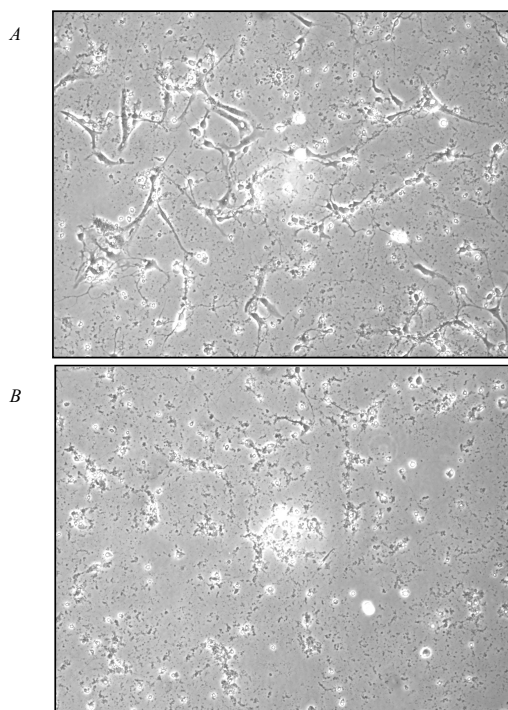


Fig. 4. Morphology of mouse embryonic hippocampal neurons after BPDE treatment: *A* – no BPDE; *B* – 10⁻⁶ M BPDE for 24 h. Phase contrast microphotographs with 150× magnification are shown

Results and discussion. BP has to be metabolized to manifest its genotoxic properties. As cells can differ significantly in their ability to activate BP, we have utilized its ultimate metabolite, BPDE, to study its effect on cells. As model cells of neuronal lineage, we have used PC12, rat pheochromocytoma cells that acquire sympathetic neuron-like morphology upon treatment with nerve growth factor [6], and immature neurons derived from mouse embryo hippocampus [4]. Immortalized human fibroblasts (GM00637) were used as control cells.

Both GM00637 and PC12 cells were killed by BPDE in a dose-dependent manner (Fig. 1, *A*, Fig. 2). How-

ever, PC12 cells were generally more resistant to BPDE than fibroblasts: while the survival of the latter was ~10% even at 2 M BPDE and < 1% at 10 M BPDE, the survival of PC12 was compromised to 10% at 10–20 M BPDE depending on the growth conditions.

Interactions of cells with extracellular matrix in living tissues can significantly influence the outcomes of cellular stress [7, 8]. PC12 cells normally require extracellular matrix coating to attach efficiently to plastic surfaces. We have found that the nature of this matrix affects survival of PC12 cells after BPDE treatment (Fig. 2). The cells showed little difference in survival when growing on poly-D-lysine or laminin but were more sensitive when grown attached to the poly-D-lysine: laminin 10:1 mixture (Fig. 2) or collagen (not shown).

To compare the amounts of BPDE-induced DNA damage in PC12 cells and fibroblasts, we have used the post-labeling assay (Fig. 3, *A*) to determine the level of BPDE adducts at doses when most treated cells are alive (less than 0.5–1 M BPDE). The levels of *trans*(-)-G-BPDE in cells of both types increased in a dose-dependent manner (Fig. 1, *B*, Fig. 3, *B*), but PC12 cells always accumulated less adducts than fibroblasts did. After withdrawal of BPDE, the adducts were rapidly cleared from the cells' DNA (Fig. 1, *C*), consistent with the known efficient repair of this type of damage [9].

Finally, we have studied the effects of BPDE on mouse embryo hippocampal neurons. As the amount of cells that could be obtained was insufficient for reliable adduct or survival analysis, we have studied the morphology of the neurons after their attachment to the extracellular matrix-coated surface. After six days post-plating in the absence of BPDE, many cells formed well-defined bodies and extended neurites, some of

which even start to make connections (Fig. 4, A). In contrast, when during the last 24 h the cells were treated with BPDE, the observed cells were small, rounded or irregular in shape, lacking processes, and it is likely that most of them were dead or dying.

Conclusions. Our data indicate that BPDE is cytotoxic and genotoxic for cells of neuronal differentiation lineage, and thus BP may be detrimental for developing neural tissue. However, the effects of BPDE on these cells may be modulated by the cells' microenvironment, including the extracellular matrix with which they interact.

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Генотоксичність канцерогенного метаболіту бензо[а]пірену в клітинах нейрональної гілки диференціювання

Резюме

Мета. Бензо[а]пірен-7,8-дигідродіол-9,10-епоксид (BPDE) – основний метаболіт канцерогенного поліциклического ароматичного вуглеводню бензо[а]пірену. Вплив BPDE на попередники нервових клітин практично не охарактеризовано. **Методи.** Вивчено виживаність і морфологію клітин PC12 та ембріональних гіпокампальних нейронів миші в культурі за присутності BPDE. Методом електрофорезу радіоактивно мічених нуклеотидів проаналізовано накопичення адуктів BPDE у культивованих клітинах PC12 і фібробластах. **Результати.** Виживаність клітин і рівень адуктів залежать від типу позаклітинного матриксу, до якого прикріплюються клітини. За сублетальних доз BPDE спостерігається ефективна репарація адуктів, утворених цим метаболітом. Для клітин PC12 токсичність і рівень адуктів BPDE виявилися нижчими, ніж для фібробластів. **Висновки.** У цілому BPDE може порушувати нормальний розвиток нервової тканини, однак вплив BPDE на клітини, здатні до диференціювання по нейрональній гілці, скоріш за все, залежить від їхнього мікрооточення.

Ключові слова: пошкодження ДНК, репарація, генотоксичність, бензо[а]пірен-7,8-дигідродіол-9,10-епоксид, нейрональна гілка.

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Генотоксичность канцерогенного метаболита бензо[а]пирена в клетках нейрональной ветви дифференцировки

Резюме

Цель. Бензо[а]пирен-7,8-дигидродiol-9,10-эпоксид (BPDE) – основной метаболит канцерогенного полициклического аромати-

ческого углеводорода бензо[а]пирена. Влияние BPDE на предшественники нервных клеток практически не охарактеризовано. **Методы.** Изучены выживаемость и морфология клеток PC12 и эмбриональных гиппокампальных нейронов мыши в культуре в присутствии BPDE. Методом электрофореза радиоактивно меченных нуклеотидов проанализировано накопление аддуктов BPDE в культивируемых клетках PC12 и фибробластах. **Результаты.** Выживаемость клеток и уровень аддуктов зависят от типа внеклеточного матрикса, к которому прикрепляются клетки. При сублетальных дозах BPDE наблюдается эффективная репарация аддуктов, образуемых этим метаболитом. Для клеток PC12 токсичность и уровень аддуктов BPDE оказались ниже, чем для фибробластов. **Выводы.** В целом, BPDE может нарушать нормальное развитие нервной ткани, однако влияние BPDE на клетки, способные к дифференцировке по нейрональной ветви, может зависеть от их микроокружения.

Ключевые слова: повреждение ДНК, репарация, генотоксичность, бензо[а]пирен-7,8-дигидродiol-9,10-эпоксид, нейрональная ветвь.

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