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Inhibition of Gap Junction Intercellular Communications in Cell Culture by Polycyclic Aromatic Hydrocarbons (PAH) in the Absence of PAH Metabolism

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Abstract—We have studied the effect of polycyclic aromatic hydrocarbons (PAH) on gap junction intercellular communications (GJIC) in culture of hepatoma cells Hep G2 and G27. Carcinogenic PAH inhibited GJIC in both cultures in contrast to non-carcinogenic PAH. We showed that both constitutive and inducible expressions of mRNAs of Ah receptor and cytochrome P4501A1 (the main isoform involved in PAH metabolism) were absent in hepatoma G27 cells. We concluded that the initial, non-metabolized molecules of carcinogenic PAH are responsible for changes in GJIC through interaction with an unknown factor in the cellular membrane.

Key words: gap junction intercellular communications, polycyclic aromatic hydrocarbons, Ah receptor

Polycyclic aromatic hydrocarbons (PAH) are one of the major factors of environmental pollution. They are detected in combustion products of different types of fuels, in nutritional products, in tobacco smoke, and in other sources. High carcinogenic activity of PAH has been demonstrated with experimental animals. It is suggested that these compounds are responsible for high risk of development of lung and kidney cancers among smokers.

The absence of chemically active groups in PAH molecules suggests biological inertness of this class of compounds. However, highly reactive epoxides and phenols are generated from PAH during oxidation in the monooxygenase enzymatic system.

It is accepted that carcinogenic, mutagenic, and toxic effects of PAH are induced by chemically active PAH metabolites. Therefore, the studies of biological effects of PAH have been mainly focused on their cellular metabolism. In addition to the indicated above effects, it has been demonstrated that certain PAH can induce expression of enzymes involved in PAH metabolism (cytochrome P450, glutathione-S-transferase, quinone oxide reductase, and others). Interaction of the initial, non-metabolized PAH molecule with Ah receptor that is present in the cytoplasm is the first step of signal transduction during induction (see reviews [1, 2]). Thus, it was demonstrated that effect of PAH is mediated by the initial, chemically inert molecules of the compounds.

Carcinogenesis is a multi-step process, at least two stages of action of chemical compounds being distinguished in it: initiation and promotion (see review [3]). Certain compounds were found to be initiators, while some others possessed only promoting functions. Initiation is mediated by interaction of electrophilic metabolite with DNA molecule leading to change in DNA structure followed by mutation. The principal role of promoters consists of "switching-off" of the initiated cells from the regulatory control of the adjacent normal cells, thus providing the conditions for preferential growth of the initiated cells. The "promotion" stage is also referred to as epigenetic, since it is not associated with modification of the genetic apparatus and is induced

Abbreviations: BP) benzo[a]pyrene; B(e)P) benz[e]pyrene; MC) 3-methylcholanthrene; α-NF) α-naphthoflavone; PAH) polycyclic aromatic hydrocarbons; TCDD) 2,3,7,8-tetrachloro-*p*-dibenzodioxine; GJIC) gap junction intercellular communications.

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by non-genotoxic compounds [3, 4]. Change in gap junction intercellular communications (GJIC) is one of the major factors of promotion [5]. GJIC serve for coordination of functions of cells. Gap junctions (GJ) are pores in the cytoplasmic membrane formed by proteins connexins. Molecules with molecular weight up to 1.2 kD can pass through these pores [6]. Various forms of connexins exist; their expression depends on the cell type. The ability to abolish and to restore functioning of GJIC represents one of the systems of intercellular regulation within tissues [6]. Proliferative stimuli decrease the level of GJIC in cell culture independently of their nature (growth factors, chemical compounds) [7]. Functioning of GJIC is decreased in the majority of tumors in comparison to normal homologous tissues; in those tumors where the level of GJIC between tumor cells is high, GJIC are absent between tumor cells and the cells of adjacent normal tissue (see reviews [6, 8, 9]). Thus, the tumors with preserved functioning of GJIC between the cells inside the tumor remain insensible to regulatory influence of normal cells mediated by GJIC.

"Complete" carcinogens, i.e., compounds that induce tumors without the need for additional influences, are able to stimulate both the initiation and promotion stages. Some PAH are found to be complete carcinogens. The initiation process for PAH is relatively well studied; it is accepted that this stage of tumor development is realized through interaction of the DNA molecule with diol epoxides that are formed during PAH metabolism through cytochrome P450 in the monooxygenase enzymatic system. It is not yet established how and in what form PAH mediate the promotion stage. The effect of the studied compounds on GJIC is often considered to be a marker of promotion. We demonstrated earlier that carcinogenic PAH, in contrast to non-carcinogenic ones, alter GJIC in Hep G2 hepatoma cells [10]. The study of effects on GJIC and carcinogenicity of 8 PAH having different structures revealed that only carcinogenic PAH are able to alter GJIC. Benzo[a]pyrene (BP) was found to be the most effective among all tested compounds. It was demonstrated that the effect of carcinogenic PAH is time-dependent and develops gradually; GJIC were more effectively altered after 24-h than after 1-h exposure. It was suggested that GJIC were more effectively altered after 24-h incubation due to accumulation of active PAH metabolites.

Here we studied the effects of several PAH on GJIC under conditions of metabolism inhibition in hepatoma cells Hep G2 and G27. We showed, that cytochrome P4501A1 (the main enzyme of PAH metabolism) and Ah receptor (to date the only protein known whose interaction with PAH leads to biologically significant effects) are not expressed in G27 cell line. We demonstrated that GJIC inhibition depends on PAH structure. We concluded that a specific factor that interacts with carcinogenic PAH is present in cellular membranes; this interaction leads to alteration of GJIC. It is suggested that promotion stage is realized (at least partly) through interaction of non-metabolized PAH molecule with this factor.

MATERIALS AND METHODS

Cell culture. The cells of human hepatoma Hep G2 were kindly provided by Prof. Stavrovskaya (Russian Cancer Research Center, Russian Academy of Medical Sciences). The cells were cultivated in medium DMEM + RPMI (1 : 1) with addition of 10% fetal serum (Flow, UK). For detection of GJIC, the cells were cultivated as single colonies on glass slides. Hepatoma G27 is a lowly differentiated transplantable tumor obtained by treatment of non-thoroughbred rats with chemical carcinogens [11]. For cellular cultivation, the cells of hepatoma G27 were isolated from hypodermic tumor as follows: hepatoma G27 was ablated in sterile conditions, then necrosis-free fragment was isolated, put into chilled solution trypsin–versen (1 : 1), incubated 24 h at 4°C, and washed with Hanks' solution. Then medium DMEM + RPMI (1 : 1) with addition of 10% fetal serum (Flow) and 100 μg/ml of gentamicin were added and the cells were transferred to Korrel' flasks. The medium was changed 24 h later to eliminate non-adhering cells.

The studied PAH were first diluted in acetone, then acetone solution was added to albumin solution in the medium (25 mg/ml); final PAH concentration in albumin solution that was used for cell treatment was 500 μg/ml.

RNA isolation and RT-PCR. mRNA was isolated using TRIzol reagent (Gibco BRL Life Technologies). Concentration of total RNA was measured by optical density at 260 nm. cDNA was obtained in the reaction of reverse transcription with random hexanucleotide primer. cDNA was quantified using β-actin gene as a standard. Primers and conditions for amplification of specific genes are presented in Table 1.

Permeability of GJIC was measured by intracellular injections of fluorescent dye Lucifer yellow CH (Sigma, USA) in one cell of a cellular monolayer followed by monitoring of dye distribution to adjacent cells as described earlier [12]. Fluorescence was monitored using an Axiolab fluorescence microscope (Zeiss, Germany) with phase contrast optics and water immersion objective 40× and a CCTV video camera (Panasonic, Japan) connected to a computer. The colored cells were counted 2 min after dye injection.

RESULTS

The effect of 3-methylcholanthrene (MC) on GJIC in Hep G2 cells is shown in Fig. 1. MC alters GJIC after 24-h exposure. Incubation of the cells with MC for 1 h

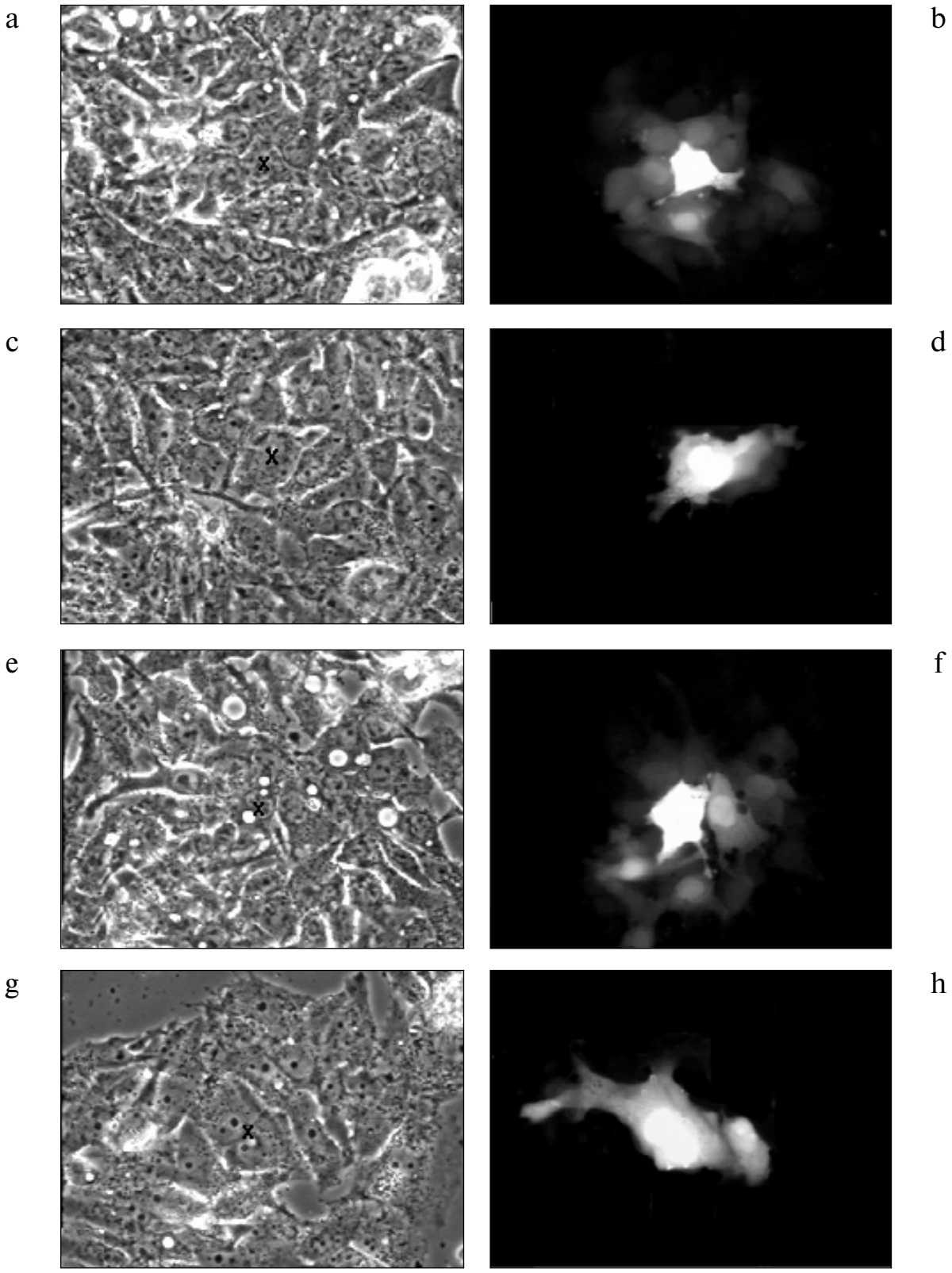

Fig. 1. Effect of 3-methylcholanthrene (MC) and α-naphtholflavone (α-NF) on the distribution of Lucifer yellow in Hep G2 cell culture. Concentrations: MC, 5 μg/ml; α-NF, 5 μM. Incubation time, 24 h. Phase-contrast (a, c, e, g) and corresponding fluorescent (b, d, f, h) images are shown; a, b) control; c, d) MC; e, f) α-NF; g, h) MC + α-NF. The cross designates the cell in which the dye was introduced.

Table 1. Primer sequences used in this study

Gene	Nucleotide sequence of primers	Amplicon size	Reference
β-Actin	sense 5'-TGCAGAAGGAGATTACTGCC-3' antisense 5'-GCAGCTCAGTAACAGTCCG-3'	211	[27]
CYP1A1*	sense 5'-CCATGACCAGGAACTATGGG-3' antisense 5'-TCTGGTGAGCATCCAGGACA-3'	341	[28]
AHR**	sense 5'-TCCATGTAGCAGTGCCAGG-3' antisense 5'-ATATCAGGAAGAGGCTGGGC-3'	212	[28]

* CYP1A1, cytochrome P4501A1.
** AHR, Ah receptor.

has no influence on GJIC (Table 2). It can be suggested that the observed alteration of GJIC after 24-h treatment is mediated by MC metabolism by cytochrome P450 with generation of active metabolites. To verify this hypothesis, we studied the effect of α-naphthoflavone (α-NF), a well-known antagonist of Ah receptor and inhibitor of cytochrome P450 [13], on MC action. Table 2 and Fig. 1 show that α-NF has almost no influence on the quantity of colored cells and MC action. This observation indicates that GJIC alteration is induced by the initial PAH molecule. To study in more detail the role of metabolic activation of PAH in GJIC inhibition, we used a cell culture where the system of PAH metabolism is absent. We demonstrated earlier [14] that carcinogenic PAH are not toxic in hepatoma G27 cells, although this effect is typical in the majority of cells. It may be suggested that the absence of toxic effect is mediated by alteration of metabolic activation of PAH in hepatoma G27 cells, because the components of monooxygenase enzymatic system are not expressed in this cell line. To confirm this suggestion, we studied the expression of mRNAs of cytochrome P4501A1 and Ah receptor in these cells. The results of study of expression of β-actin (standard), Ah receptor, and CYP1A1 in hepatoma G27 and hepatoma 7777 cells (the second hepatoma was used as a positive control) are presented in Fig. 2. Both mRNAs of Ah receptor and CYP1A1 are expressed in hepatoma 7777 cells. Addition of the inducer stimulates synthesis of CYP1A1 mRNA. In hepatoma G27, mRNAs of Ah receptor and CYP1A1 are not expressed both in the native cells and in the cells treated with the inducer. Consequently, hepatoma G27 is a suitable model for the objectives of the experiment. We showed that hepatoma G27 cells possess functionally active GJIC; BP in dose 5 μg completely abolishes penetration of the dye into neighboring cells after 24-h exposure (Fig. 3). The effect is time-dependent, as in the Hep G2 model: GJIC are more altered after 24-h than after 1-h exposure (for the same concentration of the compound) (Fig. 4). The non-carcinogenic BP analog, benz[e]pyrene (B(e)P), does not influence GJIC at the same dose (5 μg) (Fig. 3). Table 2 demonstrates that the most effective ligand of Ah receptor, 2,3,7,8-tetrachloro-*p*-dibenzodioxine (TCDD), has a weak effect on GJIC functioning in hepatoma G27 cells in comparison to BP.

DISCUSSION

The ability of PAH to alter GJIC in cellular culture has been described in several studies [15-18]. The results

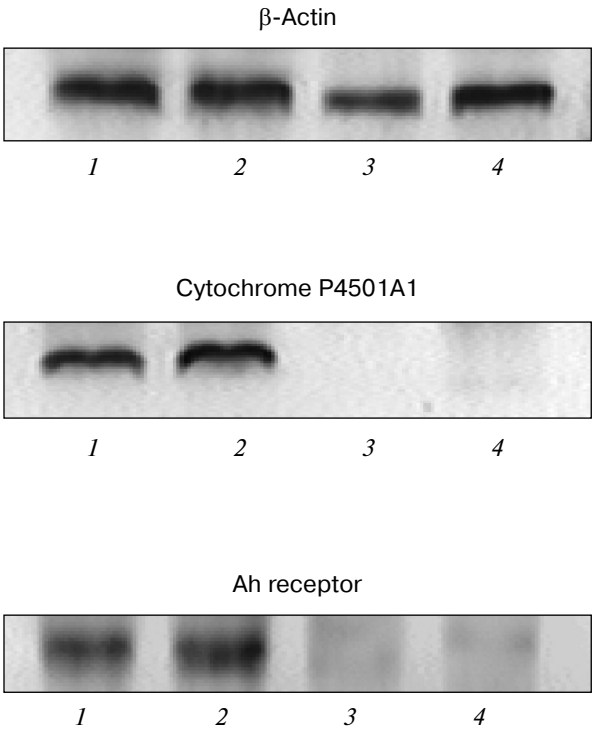

Fig. 2. Assessment of expression of β-actin, cytochrome P4501A1, and Ah receptor genes in hepatoma G27 and McA RH7777 cell cultures by RT-PCR. *1*) McA RH7777 (control); *2*) McA RH7777 + TCDD; *3*) G27 (control); *4*) G27 + TCDD. TCDD was used for induction at concentration 10^{-9} M. cDNA quantity taken for amplification was normalized by β-actin.

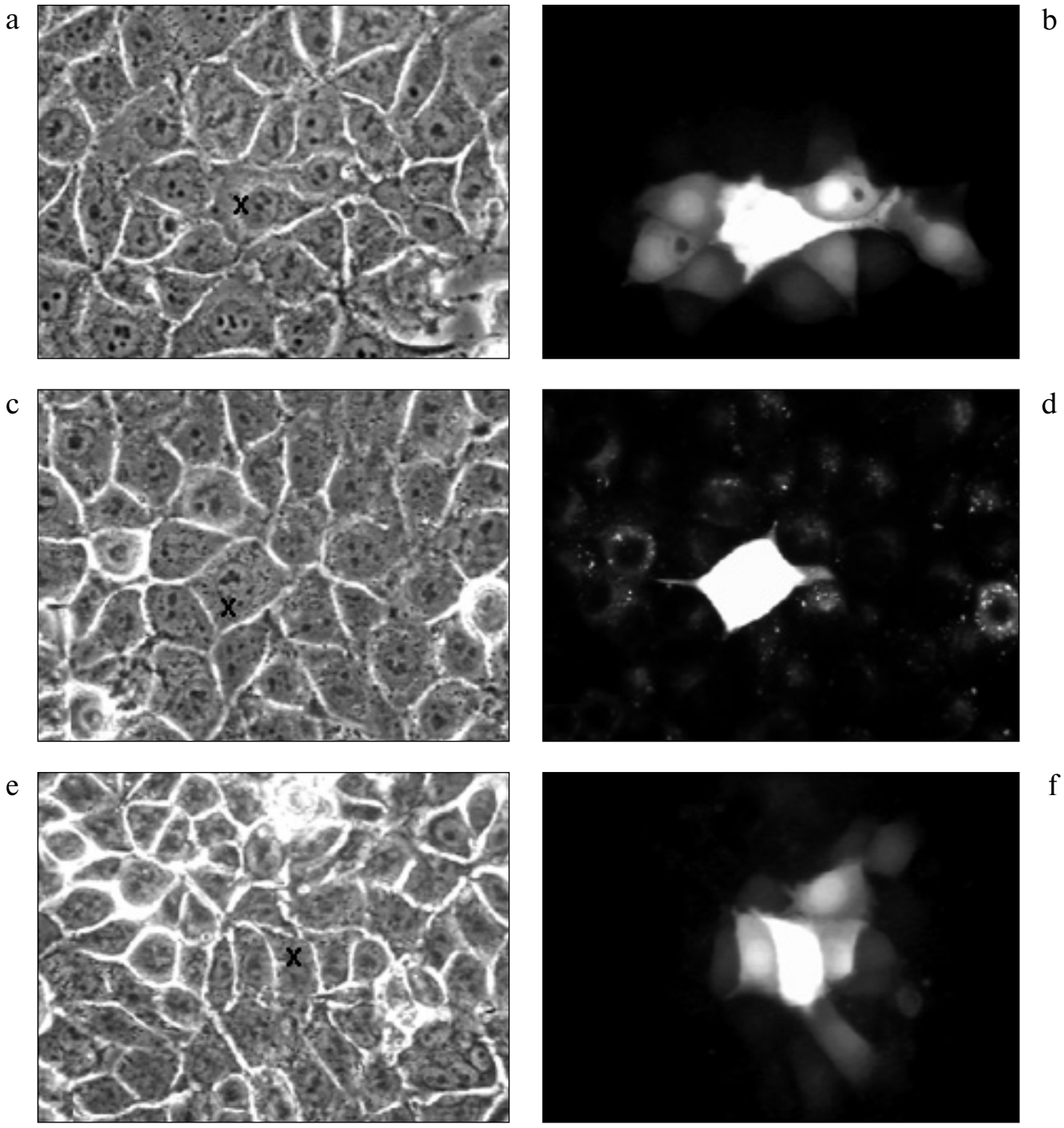

Fig. 3. Distribution of Lucifer yellow in G27 cell culture in the presence of benzo[a]pyrene (BP) and benz[e]pyrene (B(e)P). The concentration of each compound was 5 μg/ml, the incubation time was 24 h. Phase contrast (a, c, e) and corresponding fluorescent (b, d, f) images are presented. a, b) Control; c, d) BP; e, f) B(e)P. The cross designates the cell in which the dye was introduced.

of this and our previous study [10] also confirm the ability of PAH to influence alter GJIC. The rate of inhibition of GJIC by PAH depends on the structure of the compound indicating specificity of effects of the compounds. The following data indicate that the effect of PAH is not related to metabolic activation of the compound, but is mediated by the initial, non-metabolized PAH molecule: α-NF (a well known inhibitor of the monooxygenase enzymatic system) does not prevent the effect of PAH on GJIC during 24-h exposure of the cells, where

Table 2. Effect of different compounds on permeability of GJIC in hepatoma Hep G2 and G27 cells after exposure for 1 or 24 h (the effect is calculated in % of colored cells in comparison to the control)

Compound	% of control	Time of exposure, h	Cell type
MC, 5 μg/ml	98.3 ± 1.2	1	Hep G2
MC, 5 μg/ml	20.3 ± 5.9	24	Hep G2
α-NF, 5 μM	108.1 ± 12.6	24	Hep G2
MC (5 μg/ml) + α-NF (5 μM)	21.7 ± 5.3	24	Hep G2
TCDD, 10 nM	71.1 ± 3.5	1	G27
TCDD, 10 nM	98 ± 3.5	24	G27
BP, 5 μg/ml	11.5 ± 3.4	1	G27
BP, 5 μg/ml	0	24	G27
B(e)P, 5 μg/ml	89.7 ± 5.7	1	G27
B(e)P, 5 μg/ml	120 ± 17.6	24	G27

Note: Mean number of colored neighboring cells 2 min after dye injection in one cell of the monolayer ± S.E. (from 7-11 injections) was taken as 100% in the control. The mean number of colored cells in the control was 11 for Hep G2 and 7 for G27. BP, benzo[a]pyrene; B(e)P, benz[e]pyrene; MC, 3-methylcholanthrene; α-NF, α-naphthoflavone; TCDD, 2,3,7,8-tetrachloro-*p*-dibenzodioxine.

cytochrome P450 is present (Hep G2 cells); PAH alters GJIC in cell culture where mRNAs of cytochrome P450 and Ah receptor are not expressed (G27 cells). It appears that GJIC alteration develops gradually due to gradual penetration of the compound into the cellular membrane. The results of the study of PAH accumulation in cellular membrane favor this suggestion [19]. The authors demonstrated by confocal fluorescent microscopy that PAH accumulation occurs exclusively in the external cellular membrane, and these compounds are not detected in nuclear, mitochondrial, and lysosomal membranes. Saturation of the external membrane with PAH occurs 5 h after PAH injection.

The effects on GJIC of isomers with similar structures and the same solubility in lipids (determined by the coefficient of distribution in the system octanol/water), carcinogenic BP and non-carcinogenic B(e)P, are different. These data provide evidence that this effect cannot be explained by unspecific alteration of membrane structure by highly lipophilic PAH. Hence, it is suggested that a factor specific to certain PAH, like BP, exists in the cellular membrane. It is worth noting that the properties of this factor differ from that of Ah receptor, since the most effective Ah receptor ligand, TCDD, was not active towards GJIC in concentrations at which it stimulates induction.

We demonstrated earlier that PAHs (BP and benzo[a]anthracene) that alter GJIC in hepatoma Gep H2 cells [10] stimulate proliferation of G27 hepatoma cells [14], while non-carcinogenic phenanthrene has no influence on GJIC and cellular proliferation. Possibly, GJIC alteration and stimulation of proliferation by PAH are mediated by the same molecule. It was demonstrated that the increase in connexin expression leads to inhibition of proliferation [20, 21]. It appears that connexins are not inert pore components that limit pore size, but they play a functional role. The observation that incorporation of functionally active connexin gene "normalizes" tumor cells in culture in the absence of adjacent normal cells favor this suggestion [22, 23]. This effect is accompanied by an increase in expression of an inhibitor of the cell cycle, p27 protein [24]. Consequently, "switching-off" of connexins may lead to alteration of proliferative activity of the cells that we have observed earlier [14].

Thus, the results of this work indicate the presence of a new factor (in addition to Ah receptor) that interacts with the initial PAH molecules. This interaction leads to GJIC alteration and stimulation of proliferation.

Several authors concluded earlier [19, 25, 26] that alteration of GJIC is the most sensitive indicator of the toxic effect of PAH. In all these studies, cultures that metabolized PAH were used. Our data were obtained in hepatoma G27 cells, where PAH are not metabolized and are not toxic, but the ability of PAH to alter GJIC is preserved, and provide evidence for the conclusion that GJIC alteration should not be considered as an indicator of PAH toxicity. Apparently, toxic effect is realized through formation of active metabolites, while GJIC alteration is mediated by the initial, non-metabolized PAH molecule.

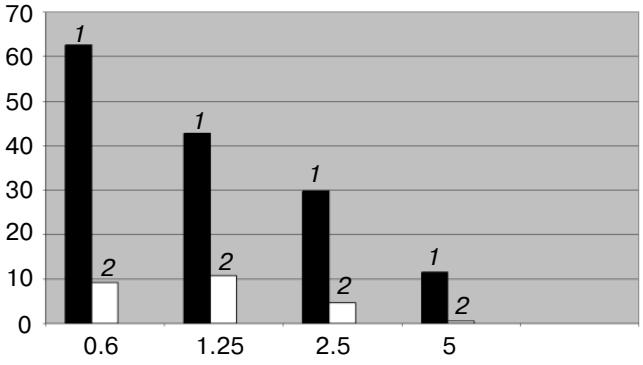

Fig. 4. Concentration dependence of the influence of BP on the level of intercellular communication in hepatoma G27 cells. Horizontally, BP concentration in μg/ml; vertically, the number of colored cells after incubation with BP in percent of the control cells. *1*) 1-h incubation with BP; *2*) 24-h incubation with BP.

The abilities to stimulate cellular proliferation and inhibit GJIC and apoptosis are thought to be essential properties of promoters [3]. The ability of the initial non-metabolized PAH molecule to stimulate cell proliferation and to inhibit GJIC provides evidence to consider that PAH mediate the promotion stage in this initial form during carcinogenesis induced by this group of compounds.

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