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Ah-Receptor-Independent Stimulation of Hepatoma 27 Culture Cell Proliferation by Polycyclic Aromatic Hydrocarbons

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Abstract—The proliferative effect of some compounds that are aryl hydrocarbon (Ah) receptor ligands was studied on hepatoma 27 cells with absent expression of Ah receptor. Compounds of the polycyclic aromatic hydrocarbon (PAH) class benzo/a/pyrene, 3-methylcholanthrene, 7,12-dimethylbenz/a/anthracene, and benzo/e/pyrene as well as β-naphthoflavone (β-NF) and chlorinated hydrocarbon Aroclor 1254 were studied. It was found that carcinogenic PAH and β-NF stimulate cell proliferation both under conditions of standard serum content and in a medium with low serum content. More efficient stimulation of proliferation was observed in the case of low serum content. Aroclor 1254 and benzo/e/pyrene did not stimulate cell proliferation. Stimulation of proliferation was accompanied by activation of the ERK1/2-dependent MAP-kinase cascade. Benzo/a/pyrene caused a decrease in the number of cells in G1 phase of the cell cycle and increase in number of cells in G2/M phases under conditions of cell growth in media with low serum content. Carcinogenic PAH and β-NF activated transcription factor AP-1, and in this case activation was more pronounced in cells grown in medium with low serum content. A possible mechanism of activation of proliferation by an Ah receptor-independent pathway is discussed.

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Key words: carcinogenesis, proliferation, AP-1, polycyclic aromatic hydrocarbons, Ah receptor

Chemical carcinogenesis is a multi-stage process with initiation and promotion as the main stages. Mutational changes in the cell genome, which predetermine tumor genotype of a formed clone, occur at the stage of initiation. The stage of promotion is the result of a non-genotoxic effect creating conditions for selective growth of initiated cells. The promotion phenomenon is usually studied on the model of double-stage carcinogenesis, when initiation and promotion stages are stimulated by different compounds. For "complete" carcinogens (substances stimulating tumor development without additional effects) the existence of the promotion stage is implied. It is not known whether a promotion stage follows the same scheme as in two-stage carcinogenesis (i.e. by a non-genotoxic mechanism), and, in what form, original or metabolized, a compound acts, i.e. whether functioning of the same regulatory systems change on the model of two-stage carcinogenesis. It is assumed that necessary properties of tumor promoters are their ability to stimulate proliferation, block apoptosis, and disturb functioning of intercellular gap junctions [1-3]. These effects for different type tumor promoters have been demonstrated both in cell culture and in experiments *in vivo*.

The main carcinogenic contaminants in the environment are polycyclic aromatic hydrocarbons (PAH) formed during the operation of internal combustion engines and from tobacco smoke; they are also found in foodstuffs after different types of cooking. Due to chemical inertness, they are accumulated and retained in the environment. For excretion from the organism, PAH undergo enzymatic oxidation by the monooxygenase enzyme system. This oxidation results in formation of intermediate highly active metabolites. It is known that similar metabolites, diol-epoxides in particular, are tumor-initiating agents. However, the mechanism of promotion is still unknown. In addition to formation of PAH metabolites, this class of carcinogens demonstrates an additional effect due to the interaction of the non-metabolized molecule of the substances with cytoplasmic aryl hydrocarbon (Ah) receptor. The latter activated by interaction with PAH is transported into the nucleus, where it functions as transcription factor activating genes encoding enzymes for metabolism of xenobiotics [4].

Abbreviations: Ah, aryl hydrocarbon; BP, benzo/a/pyrene; DMBA, 7,12-dimethylbenz/a/anthracene; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzodioxine; β-NF, β-naphthoflavone.

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The effect of Ah receptor long-term activation on the functions of an organism was studied with compounds characterized, among different known properties, only by interaction with Ah receptor. It was shown that the long-term introduction of Ah receptor ligands similar to chlorinated biphenyls and dioxins causes various disorders in the functioning of an organism, including promotion of carcinogenesis [2, 5, 6]. Recent investigations have shown that the activated Ah receptor, inducing signal transduction, interacts with different regulatory proteins such as retinoblastoma protein, NF-κB, and others, thus changing their functional activity [7-9].

Simultaneously, there are reports showing that a number of non-genotoxic effects of Ah receptor ligands are independent of Ah receptor [10-13]. We used culture of hepatoma H27 cells resistant to the toxic effect of carcinogenic PAH [14] and devoid of Ah receptor and expression of PAH metabolizing enzymes [15] and showed that carcinogenic PAH inhibit intercellular gap junctions [16] and activate NF-κB transcription factor [17] independently of intracellular expression of Ah receptor. In continuation of studies on the effect of PAH on cell functions, we studied proliferative effects of some PAH on hepatoma 27 cells. The results show that carcinogenic PAH and β-NF stimulate proliferation of hepatoma 27 cells via an Ah receptor-independent pathway by activating the ERK1/2-dependent MAP kinase pathway. Transcription factor AP-1 is activated.

MATERIALS AND METHODS

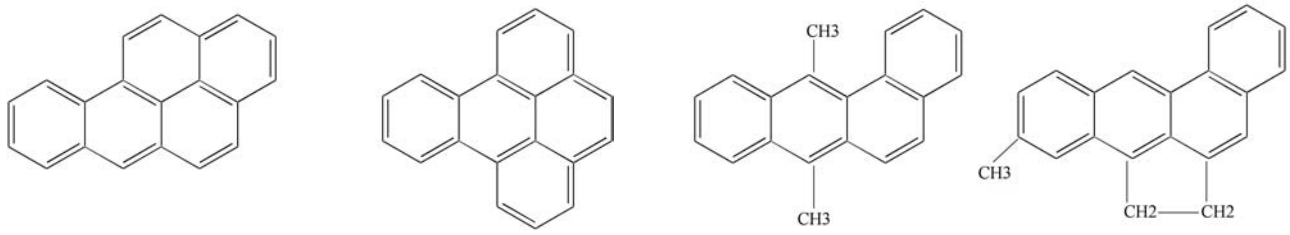
Rat hepatoma 27 cells were used in this work (hepatoma 27 is a lowly differentiated transplantable tumor obtained after chemical carcinogenesis of mongrel rats [18]). Cells were grown in DMEM and RPMI medium mixture (1 : 1) containing 10% embryonic calf serum (HyClone, USA). To transfer cells into the quiescent state, they were grown for 3 days in medium with 0.5% serum content.

The following PAH were used in this work: carcinogenic benzo/a/pyrene (BP), 3-methylcholanthrene (MC), and 7,12-dimethylbenz/a/anthracene (DMBA) and non-carcinogenic BP analog benzo/e/pyrene. All PAH were produced by Fluka (Austria). Formulas are given in Fig. 1. The mixture of chlorinated biphenyls, Aroclor 1254, was produced by Oxboro (USA). The compounds were dissolved in acetone, then the acetone solution was added to human albumin solution in the culture medium (25 mg/ml) to final concentration in albumin solution of 500 μg/ml; this solution was added directly to the cells under investigation to the final substance concentration of 5 μg/ml, and Aroclor 1254 to final concentration 1 μM.

Cell number was determined using fluorescent dye CyQUANT GR (Invitrogen, USA) specifically interacting with cellular DNA. The cells were analyzed according to Invitrogen's protocol. Briefly, 10,000 cells were plated into wells of 96-well plate containing 150 μl medium. The studied compounds were added to cells in experimental wells, while solvent was added to control cells. After the necessary incubation time, the medium was removed, and 100 μl CyQUANT GR reagent in buffer for cell lysis was added to each well. Samples were incubated for 30-45 min in the light at 37°C in a CO_2 incubator. The CyQUANT-GR fluorescence was measured on a Molecular F^{max} fluorescence microplate reader (excitation 485 nm, emission 530 nm). Results were processed in the Microsoft Excel program. Each point was the mean of four repeats.

Immunoblotting. Proteins were separated electrophoretically in 12.5% SDS-polyacrylamide gel and transferred onto Immobilon-P membrane (Millipore Corporation, USA). Antibodies to ERK1/2 and phospho-ERK1/2 were obtained from Abcam (USA). Incubation with antibodies was performed for 1.5 h at room temperature. Rabbit antibodies (AB) conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Sweden) were used as second antibodies. Specific signal was developed using the ECL system (Amersham Pharmacia Biotech).

***AP-1* gene transfection and determination of reporter gene activity.** Cells were transfected by plasmid containing luciferase reporter gene under control of responsive element AP-1. The plasmid was provided by A. V. Gasparyan. Transfection was carried out for 24 h at 37°C in the serum-free medium. In experiments on determina-



benzo/a/pyrene benzo/e/pyrene 7,12-dimethylbenz/a/anthracene 3-methylcholanthrene

Fig. 1. Formulas of PAH used in this work.

tion of AP-1 transcription activity (in the presence of serum), 12 h after transfection the medium was replaced with the serum-containing medium. PolyFect (Qiagen, USA) was used as a transfecting agent for hepatoma 27 cells. For control of transfection efficiency, cells were also transfected by plasmid containing β-galactosidase. Experiments were carried out for 48 h after transfection. Luciferase activity was measured according to a standard protocol (Promega, USA) on a Turner BioSystems 20/20 n luminometer (USA). Luciferase activity was calculated from the ratio of luciferase and β-galactosidase activities and expressed in relative units.

Cell distribution by cell cycle phases was studied using the flow cytofluorimetry technique. Hepatoma 27 cells removed from the plates using Versene solution (2 ml/plate) were incubated for 10 min, then the Versene was removed and trypsin was added (1 ml/plate) and cells were incubated for 1 min. After removal of trypsin, the cells were incubated in a CO_2-incubator for 10 min till complete detachment from the support. Then cells were transferred into centrifuge tubes and pelleted for 10 min at 1000 rpm. The pellet was fixed in 70% ethanol for 1 h. Then cells were washed free from ethanol using Hanks' solution and pelleted at 1000 rpm for 10 min. The pellets were treated with 400 μl buffer containing propidium iodide, detergent NP40, and RNase A. Cell distribution by cell cycle phases was analyzed in the FL2 apparatus. The results were processed using the WinMDI 2.9 program. Data are shown as means of at least three independent experiments.

RESULTS

Figure 2a shows data on cell proliferative activity caused by some PAH in hepatoma 27 cells during growth in standard serum-containing medium. It is seen that all carcinogenic PAH and β-NF nearly equally stimulate proliferation (at the second day of cultivation the cell number increased 1.2-1.5-fold). Non-carcinogenic PAH benzo/e/pyrene had no effect on proliferation, while chlorinated biphenyl (Aroclor 1254) inhibited proliferation. In the case of cell cultivation for three days in medium with low serum content, growth rate of control cells was somewhat slowed compared to cells grown in the presence of serum (Fig. 2b). Proliferation stimulation by carcinogenic PAH and β-NF is more efficient than in the medium with 10% serum. Thus, on the second day of cultivation, β-NF and DMBA increased cell number 4.8-fold, while BP and MC increased it 2.4- and 2.1-fold, respectively, compared to control. Benzo/e/pyrene, as in the case of incubation in the serum-containing medium, had no effect on proliferative activity, whereas Aroclor 1254 inhibited proliferation (Fig. 2b). Since stimulation of proliferation in the absence of serum significantly exceeds that in the serum-containing medium, we suppose that growth factors present in serum and the studied compounds activate the same signal pathway. It is known that growth factors activate the ERK1/2-dependent MAP-kinase pathway by phosphorylation of the final unit ERK1/2. We determined the change in the level of the ERK1/2 active phosphorylated form. The main pollutant of the environment – BP – was used as a marker compound. As shown in Fig. 3, BP increases phosphorylation of ERK1/2, which is indicative of activation of this signal pathway. Analysis of cell cycle changes in response to BP in hepatoma 27 cells kept for three days in serum-free medium showed relatively insignificant decrease in number of cells in G1 phase (from 70 to 64%) and significant increase in number of cells in G2/M phases (from 17 to 28%) (Fig. 4). Different signal systems can be activated in the nucleus upon mitotic signal transmission. We

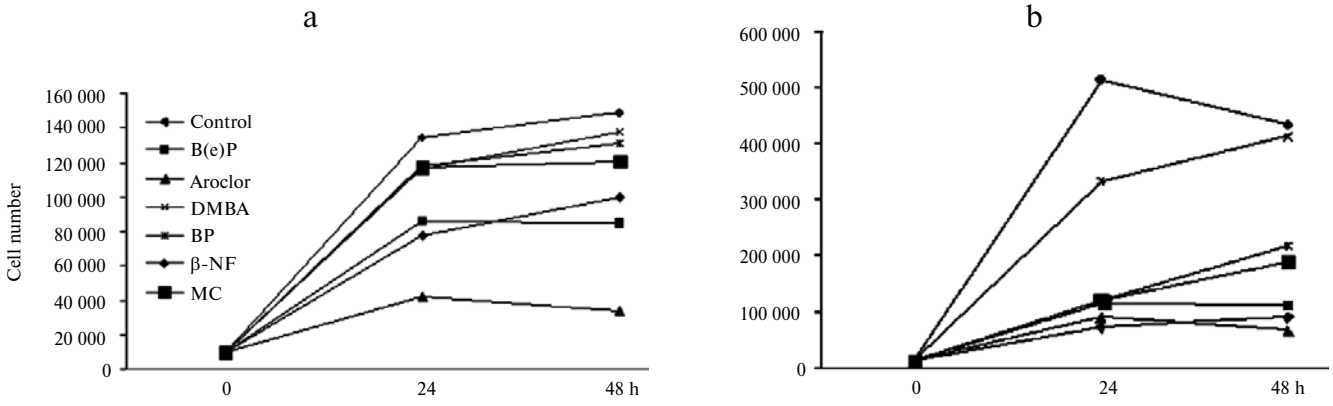


Fig. 2. Effect of the studied substances on proliferation of hepatoma 27 cells in medium with standard serum content (10%) (a) and in medium with low serum content (b). For cell transition to the quiescent state, they were grown for 3 days in the presence of 0.5% serum. The studied substances were dissolved in acetone and then added to the human albumin solution in the medium (25 mg/ml) to final concentration 500 μg/ml; this solution was added directly to the studied cells to final concentration 5 μg/ml, and Aroclor 1254 to final concentration 1 μM. Only albumin with acetone was added to control cells.

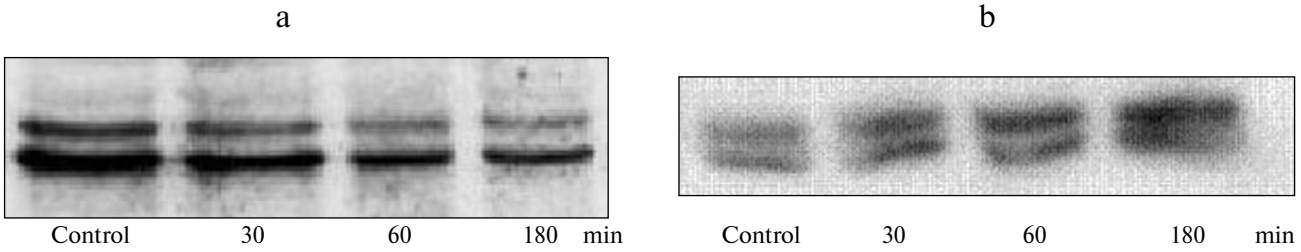


Fig. 3. Western blot of ERK1/2 (a) and ERK1/2 phosphorylated at threonine 202 and tyrosine 204 (b) in hepatoma 27 cells after 3 days of growth in medium with low serum content (control) and after addition of BP for different times of cultivation.

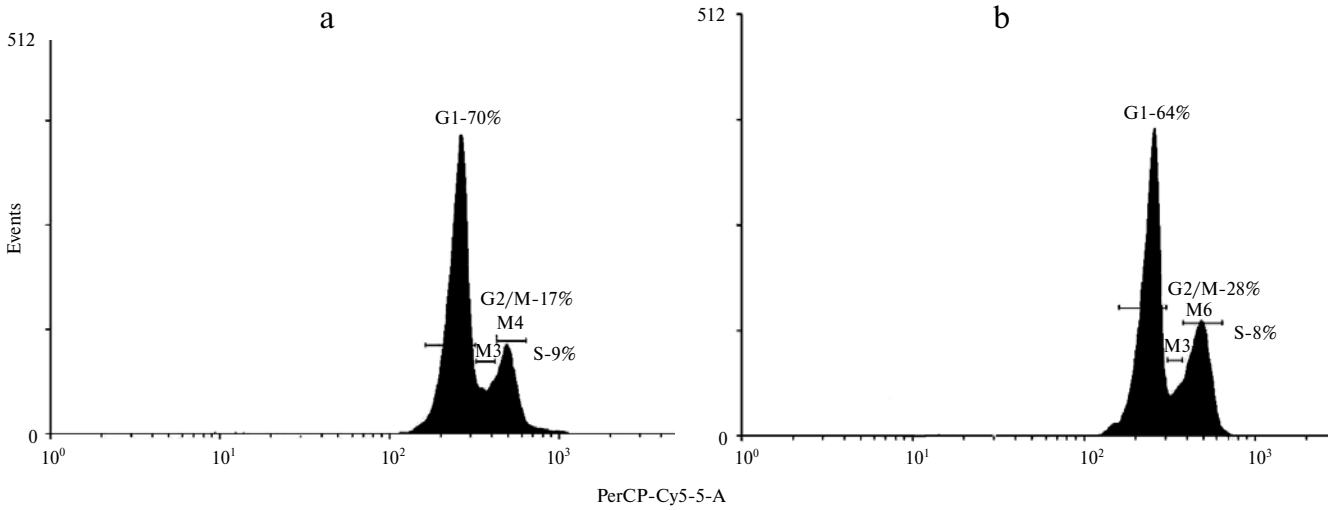


Fig. 4. Distribution of hepatoma 27 cells by different cell cycle phases in medium with low serum content: without (a) and with benzo/a/pyrene (b). The horizontal line shows DNA content in relative units. The vertical line shows cell number. Cells were cultivated for 3 days in the presence of 0.5% serum. Then BP or solvent (control) was added and cultivation continued for 48 h.

showed earlier [17] that carcinogenic PAH in hepatoma 27 cells activate transcription factor NF-κB involved both in antiapoptotic cell protection and activation of proliferation [9, 19]. Activation of so-called "quick response" genes *jun* and *fos* plays an important role in mitotic signal transmission. Protein products of these genes form transcription factor AP-1 involved in mitotic signal transmission in the nucleus. We studied activation of this transcription factor using cell transfection by a plasmid containing the reporter luciferase gene under control of responsive element AP-1 (see "Materials and Methods"). The results given in the table show that activation of AP-1 (in response to the studied substances) is more efficient in the medium with low serum content. In this case no correlation between AP-1 activation and stimulation of proliferation was found either with normal or high serum content. The most efficient activator of AP-1 is MC (table), whereas the most efficient stimulators of proliferation β-NF and DMBA exhibited lower AP-1 activating ability.

DISCUSSION

The results show that Ah receptor ligands with the exception of chlorinated biphenyl Aroclor 1254 stimulate cell proliferation by an Ah receptor-independent pathway. There are controversial data in the literature concerning proliferative effect of PAH and other Ah receptor ligands. For example, it was shown that in WB-F344 cells carcinogenic PAH [20] and naphthoflavones [21] stimulate proliferation. Stimulation of proliferation in these cells is accompanied by increased formation of cyclin D complex with cyclin-dependent kinases 4/6 and induction of cyclin A synthesis [22]. As shown in another work, MC stimulates proliferation of human lung tumor cells A549 [23, 24]. It is known that 2,3,7,8-tetrachlorodibenzodioxine (TCDD) stimulates proliferation in HepG2 cells by induction of *SOS1* gene expression, which is the Ras-activating protein [24a]. Upon *in vivo* introduction, TCDD stimulates growth of liver cells [25] and proliferation in rodent liver culture [26]. In culture of aorta mus-

Activation of transcription factor AP-1 under the influence of Ah receptor ligands in hepatoma27 cells

Substance	Extent of AP-1 alteration comparing to control	
	medium with 10% serum content	medium with 0.5% serum content
Benzo/a/pyrene	2.02 ± 0.12	2.66 ± 0.12
3-Methylcholanthrene	3.75 ± 0.1	4.49 ± 0.1
7,12-Dimethylbenz/a/anthracene	2.04 ± 0.23	3.46 ± 0.35
Benzo/e/pyrene	1.14 ± 0.1	1.54 ± 0.1
Aroclor 1254	1.72 ± 0.15	2.18 ± 0.1
β-Naphthoflavone	2.54 ± 0.2	3.05 ± 0.1

cle cells, BP stimulates proliferation [27, 28]. On the other side, there are reports on inhibition of cell proliferation by Ah receptor ligands. Thus, in hepatoma 5L cells TCDD blocks the G1–S transition by inhibition of proliferation [29, 30]. MC inhibits proliferation in culture of human umbilical endothelial cells via stimulation of synthesis of inhibitory proteins p27 and p21 [31, 32] as well as of proliferation and differentiation of osteoblasts [33]. In hepatoma Hepa-1 TCDD decreased cells number in S phase and increased their number in G1 phase, which is indicative of inhibition of proliferation [34]. Since in some cases the Ah receptor ligands inhibit proliferation, Ah receptor is positioned as tumor suppressor [35].

It so happened in history that functions of Ah receptor as transcription factor attracted the most pronounced interest concerning biological effect of PAH and different Ah receptor ligands. Therefore, investigation of different Ah receptor ligand effects on cell functions was based on the supposition that they are due to Ah receptor activation. Because of this, in all studies associated with both stimulation and inhibition of proliferation, the observed effects are explained by Ah receptor activation. However, there is now sufficient information showing that some effects of Ah receptor ligands are realized by an Ah receptor-independent pathway. Thus, stimulation of dendritic cell proliferation by BP is independent of Ah receptor functioning [11]. In culture of human fibroblasts, TCDD inhibits expression of p16 suppressor protein independently of Ah receptor [10]. It is known that in mouse macrophages RAW 264.7, TCDD activates the ERK1/2-signal pathway [12] similarly to hepatoma cells (this work). In mouse cells with knocked out Ah receptor, TCDD stimulates activation of "immediate" response genes forming transcription complex AP-1 [13, 36]; the same effect of AP-1 activation by PAH was obtained in this work.

Differences are observed in effects of various Ah receptor ligands on cell functions. We have shown that chlorinated biphenyls using the example of Aroclor 1254 do not stimulate proliferation in hepatoma 27 cells, unlike studied PAH and β-NF. As in hepatoma 27 and Hep G2 cells, TCDD, unlike BP, did not break intercellular gap junctions [16, 37].

It was shown that different types of Ah receptor ligands have various effects on the cell functions. Thus, it was shown that in MCF-7 human mammary gland cells, PAH but not TCDD stimulate cell transition from G1 to S phase [38]. It was shown in the same cells but by different authors that MC stimulates activity of estrogen-dependent reporter luciferase gene, whereas chlorinated biphenyls, Ah receptor ligands, do not exhibit this activity [39]. It was shown that BP, rather than TCDD, disturbs intercellular interactions in human endometrium cells RL95-2 [40]. It was also shown that β-NF, unlike chlorinated biphenyls, retarded development of listeriosis via an Ah receptor-independent pathway [41].

Thus, it can be concluded that epigenetic effects of Ah receptor ligands are not limited only by Ah receptor activation. Evidently, there are different targets for effects of type PAH compounds, naphthoflavones, and other Ah receptor ligands, the interaction causing different changes in cell functioning.

The existence of not one but several targets of Ah receptor ligand effects, besides Ah receptor, is supported by the fact that in cells devoid of Ah receptor expression, β-NF and PAH stimulate proliferation and activation of a number of transcription factors, but simultaneously, in the same cells, β-NF does not inhibit intercellular gap junctions, while PAH cause this effect [42].

There are still no data concerning the nature of these factors. Between the years 1990 and 2000, different research groups showed that the cytosol contains along with Ah receptor a certain protein with 4S sedimentation characteristic that binds in non-metabolized form carcinogenic PAH and β-NF, but not chlorinated ligands of Ah receptor [43-46]. The function of this protein is unknown. Perhaps binding to 4S protein is responsible for the PAH and β-NF proliferative effect, but it is necessary to determine how BP and other PAH inhibit intercellular gap junctions. The general scheme of non-genotoxic effect of Ah receptor ligands is shown in Fig. 5.

Concerning tumor-promoter effect of PAH, interaction with unknown acceptors can be no less important than interaction with Ah receptor. It was shown on the example of this work and other above-mentioned investigations that the Ah receptor-independent effects of BP include cell proliferation, activation of transcription factors responsible for antiapoptotic effect, such as NF-κB, and disturbance of intercellular gap junctions, which are necessary conditions for tumor promotion. The distinctions observed upon proliferative effect of Ah receptor lig-

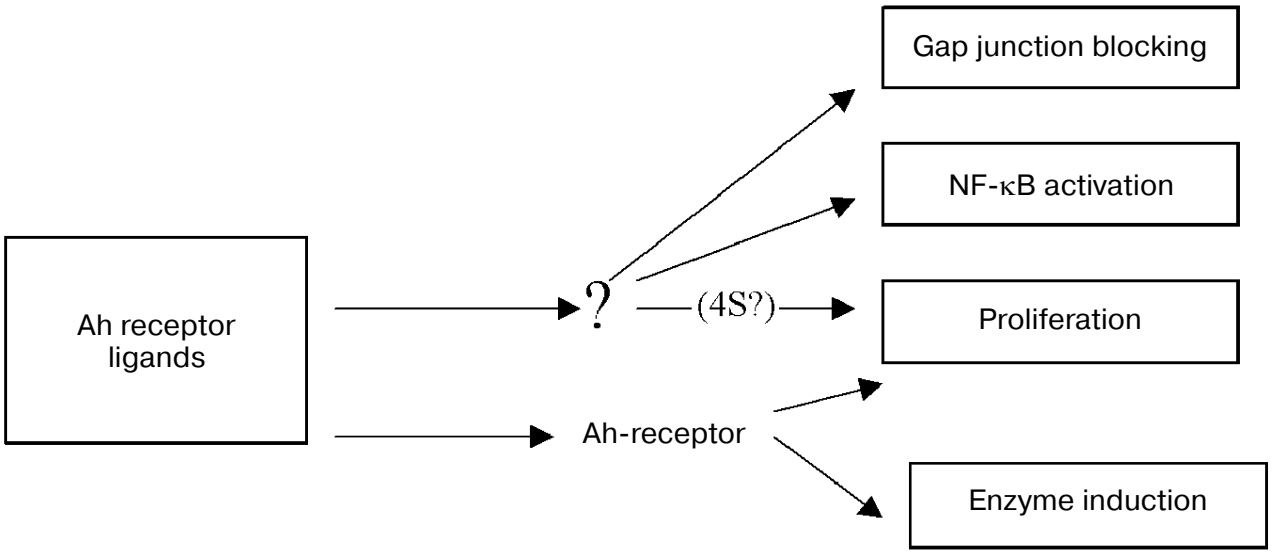


Fig. 5. Scheme of non-genotoxic effect of Ah receptor ligands on cell functions.

ands depending on cell type and tested compound are evidently caused by intracellular expression level of unknown acceptor(s) with which interaction results in proliferation. The tumor-promoting effect of Ah receptor ligands evidently also depends on the expression level in initiated cells of both Ah receptor and an unknown acceptor.

Expression of differentiation factors that evidently include Ah receptor usually decreases during carcinogenesis. Unfortunately, we did not find information concerning Ah receptor expression level at early stages of tumor transformation. In some tumors, the Ah receptor expression level is decreased compared to that in homologous normal tissue [47-49], or functionally defective protein is expressed [50]. If we suppose that decrease in Ah receptor expression occurs in early stages of carcinogenesis, then at the stage of promotion caused by PAH an important role belongs to an unknown factor, the interaction with which results in effects associated with promotion.

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