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Benzo[a]pyrene-Dependent Activation of Transcription Factors NF-κB and AP-1 Related to Tumor Promotion in Hepatoma Cell Cultures

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Abstract—The activation by the carcinogenic polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (BP) of transcription factors NF-κB and AP-1 in hepatoma 27 and HepG2 cell cultures was studied. In contrast to the hepatoma HepG2 cells, cytochrome P450 isoforms and Ah-receptor are not expressed in the hepatoma 27 cells. The transcription factor NF-κB was activated only in the hepatoma 27 cells by BP treatment but not by its noncarcinogenic isomer benzo[e]pyrene (BeP). Conversely to NF-κB activation the transcription factor AP-1 was activated in the hepatoma HepG2 cells by cell treatment with BP but not in the hepatoma 27 cells. It is concluded that the NF-κB activation is caused by nonmetabolized BP molecule and not related to activation of the Ah-receptor. The transcription factor AP-1 seems to be activated as a result of the interaction of BP with the Ah-receptor. The realization of tumor promotion stage by carcinogenic PAHs treatment in dependence on the cytochrome P450 and Ah-receptor levels in the initiated cells is discussed.

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Key words: tumor promotion, cytochrome P450, Ah-receptor, NF-κB, AP-1

Tumor formation caused by carcinogens is a multistep process that includes initiation and promotion as principal stages. During the initiation stage, inherited changes in the cell genome occur which predetermine the tumor genotype of the generated clone. The promotion stage is now considered as a nongenotoxic effect determining a selective growth of the initiated cells. The promotion phenomenon is usually studied on a two-stage model of carcinogenesis, when the initiation and promotion stages are caused by different compounds. For "full" carcinogens (substances caused tumor without an additional exposure), the promotion stage is implicated. It is not known whether the promotion stage is realized by the same scheme as in the two-stage carcinogenesis (i.e., by the nongenotoxic mechanism), what form of the compound (original or metabolized) is acting, and whether there are changes in functioning of the same regulatory systems as in the two-stage model of carcinogenesis.

Polycyclic aromatic hydrocarbons (PAHs), due to their chemical inertness, can be accumulated and stored in environment and, thus, are carcinogenic pollutants of the environment. The elimination of PAHs from organisms is mediated by enzymatic oxidation by the monooxygenase enzyme system with the cytochrome P450 (CYP) as a functional link. The oxidation results in formation of highly active intermediate metabolites. These metabolites, in particular, diol-epoxides, are tumor-initiating agents. However, the mechanism of the promotion stage remains unclear.

In addition to effects of the PAH metabolites, there is another aspect of the action of this class carcinogens caused by interaction of a nonmetabolized compound with the cytoplasmic Ah-receptor. The Ah-receptor activated through the interaction with PAH is transported into the nucleus, where it acts as a transcription factor expressed the genes encoding the CYP isoforms and other enzymes of metabolism of xenobiotics [1]. Consequences

Abbreviations: BP) benzo[a]pyrene; BeP) benzo[e]pyrene; CYP) cytochromes of the P450 family; PAH) polycyclic aromatic hydrocarbon; TCDD) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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of the Ah-receptor activation are especially interesting because nongenotoxic compounds, such as dioxins or chlorinated biphenyls, possessing only promoter properties [2], are also ligands of the Ah-receptor. Initial forms of these compounds, similarly to such forms of PAHs, are chemically inert. Under physiological conditions, they are unable to interact with macromolecules, but, as distinguished from PAHs, are resistant to enzymatic oxidation.

Recent studies have shown that the activated Ah-1 receptor besides transmitting the inductive signal interacts with various regulatory proteins, such as the retinoblastoma protein, NF-κB, etc., and changes their functional activities [3-5].

It is accepted that the necessary properties of promoters are the following: the ability to stimulate cell proliferation, inhibition of apoptosis, and inhibition of gap junction intercellular communications (GJIC) [2, 6, 7]. These effects for different types of tumor promoters have been demonstrated in cell cultures and in *in vivo* experiments. We have earlier shown that under certain conditions carcinogenic PAHs can stimulate the cell proliferation in cell culture and inhibit intercellular gap junctions [8-10]. In the present work, we continued our studies on tumor-promoting properties of carcinogenic PAHs in cell culture. We studied the effect of the carcinogenic PAH benzo[a]pyrene (BP) on the activation of transcription factors NF-κB and AP-1. NF-κB is an important regulatory element that determines cell survival. Thus, induction of NF-κB has been shown to protect the cells during apoptosis induced by radiation, drugs, or tumor necrosis factor α (TNF-α) [11-13], as well as stimulate cell proliferation [14].

The transcription factor NF-κB is not a single protein but a family of hetero- or homodimeric complexes consisting of the Rel family proteins. In the majority of cells two types of complexes are mainly present: p65/p50 and p50/p50. In the inactive state, the dimer is located in the cytoplasm as a complex with IκB proteins. Upon activation, IκB proteins are phosphorylated with subsequent ubiquitination and proteosomal degradation, and this allows NF-κB to be transported into the nucleus and function as a transcription factor [15]. The transcription factor AP-1 is an integral regulator of proliferation independently of the initial stimulus of proliferation [16, 17]. It is a complex of so-called "early response" proteins to a stimulus for proliferation: (jun-jun or jun-fos).

We used cells expressing CYP and Ah-receptor (hepatoma HepG2) and cells lacking the expression of CYP and Ah-receptor (hepatoma 27). The effective stimulation of NF-κB was recorded only in the hepatoma 27 cells by the action of the carcinogen BP but not of its noncarcinogenic analog benzo[e]pyrene (BeP), whereas AP-1 was activated in the HepG2 cells but not in the hepatoma 27 cells. Based on these data, we suggest that the NF-κB activation should be mediated through interaction of a nonmetabolized molecule of PAH with an unknown acceptor. AP-1 should be activated either as a result of the Ah-receptor activation or as a result of production of PAH active metabolites and should be absent in the cells not expressing the Ah-receptor and CYP.

MATERIALS AND METHODS

Cell cultures. Human hepatoma HepG2 and rat hepatoma 27 cells were used. The HepG2 cells were obtained from the ATCC (American Type Culture Collection, USA). The cells were cultured in a DMEM + RPMI (1 : 1) medium supplemented with 10% fetal serum (HyClone, USA). The hepatoma 27 is a poorly differentiated transplanted tumor obtained by treatment of non-thoroughbred rats with chemical carcinogens [18]. For the cell culture, the hepatoma 27 cells were prepared from a subcutaneous tumor as follows: the tumor was removed under sterile conditions, and then a necrosis-free region was isolated, placed in a cooled trypsin–Versene (1 : 1) solution, incubated for 24 h at 4°C, washed with Hanks' solution, supplemented with the DMEM + RPMI (1 : 1) medium containing 10% fetal serum (Flow) + gentamicin (100 μg/ml), and planted into Carrel flasks. The medium was changed after 24 h to remove non-adhered cells.

BP (Fluka, Austria) and BeP (Ferak, Germany) were dissolved in acetone and added to the cell culture to the final concentration of 5 μg/ml. To the control cells, the same volume of acetone was added.

Binding of NF-κB, AP-1, and Sp-1 with a consensus oligonucleotide (Electrophoretic Mobility Shift Assay, EMSA). The protein extract was prepared from the nuclei as described in [19]. The binding reaction was performed using 10 μg nuclear protein in 20 μl of binding buffer (10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg poly(dI-dC), and a consensus oligonucleotide labeled by the 5′-end with ^{32}P ($(1\text{-}3)\cdot10^5$ cpm). The labeling was performed according to the protocol of the producer of consensus oligonucleotides (Promega Corp., USA). To prove the binding specificity, supershifts were used with antibodies to p50 and p65 (Santa Cruz Biotechnology, Inc., USA), as described in [20].

Protein–oligonucleotide complexes were analyzed in 6% native polyacrylamide gel containing 0.25-fold TBE buffer. The dried gel was exposed to a Kodak film (USA) at –80°C for 1-7 days.

Immunoblotting. The proteins were separated electrophoretically in 12.5% SDS-polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore Corporation, USA). Antibodies to IκB were obtained from Santa Cruz Biotechnology Inc. and to phospho-Ser32-IκB from Cell Signaling Technology Inc. (USA). The incubation with the antibodies to IκB continued for

1.5 h at room temperature, with those to phospho-Ser32-IκB continued for 12 h (in TBST buffer without milk) at 25°C. Peroxidase-conjugated rabbit antibodies (Amersham Pharmacia Biotech, Sweden) were used as secondary antibodies. The specific signal was developed using an ECL system (Amersham Pharmacia Biotech).

Intensity of the p65/p50 complex binding with the consensus oligonucleotide was assessed using the Scion Image program (version 4.0.2; Scion Corporation, USA).

RESULTS

Cell cultures of the hepatomas 27 and HepG2 were treated with BP (5 μg per ml of the medium). The nuclear protein fraction was isolated from the cells, and the binding reaction with the consensus oligonucleotide to NF-κB was performed (Fig. 1). As shown by reactions with the antibodies (supershift, data not presented), the treatment of the cells with BP induced the binding of two types of NF-κB complexes, p65/p50 and p50/p50. Figure 1 shows that 30 min after the addition of BP into the medium, the NF-κB binding was increased in the hepatoma 27 cells. The binding reached a maximum after 60 min of the treatment. Upon 90 min of the treatment with BP, the binding decreased. The treatment with BP of the hepatoma HepG2 cells for 30 min increased the binding very weakly. To test whether the observed activation of NF-κB was related with carcinogenicity of PAH, we compared the effect of BP with that of its noncarcinogenic analog BeP. In contrast to the carcinogen, BeP only slightly increased the binding after 1 h of the cell treatment (Fig. 2a). As a positive control of the NF-κB activation, we used TNF-α, which is a known activator of this transcription factor. The diagram (Fig. 2b) indicates the maximum BP-induced activation of NF-κB to be 50% of the TNF-α-induced activation. This suggests a rather effective NF-κB activation by BP. The traditional pathway of the NF-κB activation includes phosphorylation and the subsequent degradation of the inhibitory protein IκB [15]. Therefore, we also studied the level of IκBα phosphorylation in the hepatoma 27 cells treated with BP. The level of phosphorylated IκB was increased even after 30 min of the treatment with BP (Fig. 3). Degradation of total IκB was noted after 1 h of the treatment. Treatment of the cells with TNF-α resulted in stronger phosphorylation of IκBα.

Thus, the findings suggested that NF-κB was activated by nonmetabolized BP in the absence of the metabolic system in the hepatoma 27 cells, whereas in the

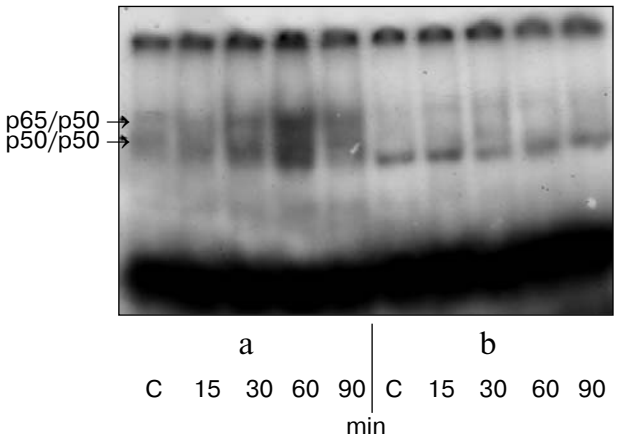


Fig. 1. Effect of benzo[a]pyrene (5 μg per ml of the medium) on NF-κB activation in the cells of hepatoma 27 (a) and HepG2 (b) upon treatment for 15, 30, 60, and 90 min. C, control cells treated with the solvent. The arrows indicate the binding of NF-κB.

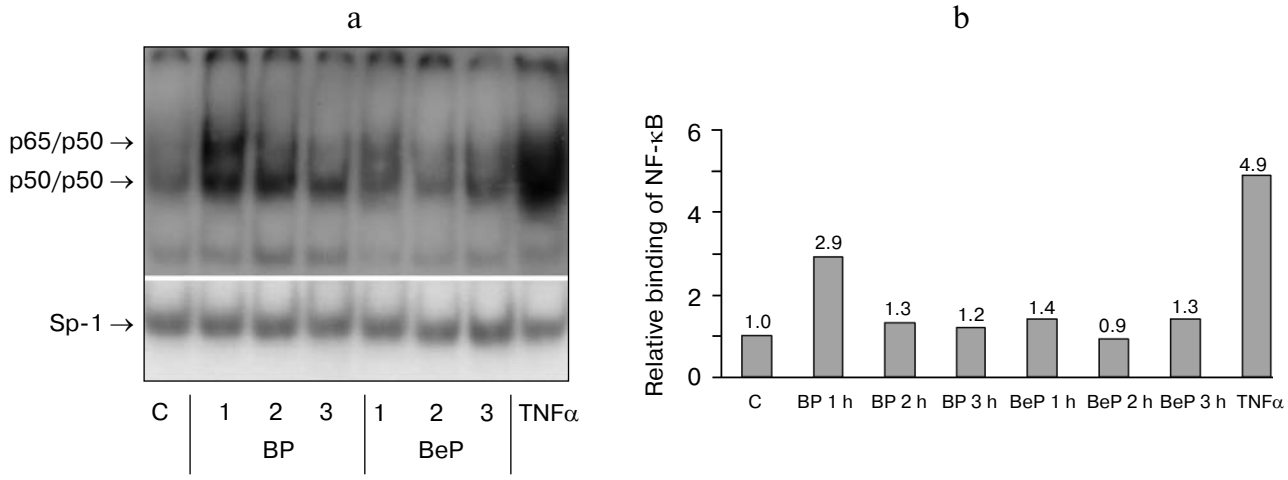


Fig. 2. a) NF-κB activation in hepatoma 27 cells treated with BP and BeP. C, control cells treated with the solvent; 1, 2, and 3, the treatment time (h). The arrows indicate the NF-κB binding. As a positive control of the NF-κB activation, we used cells treated with the known NF-κB activator TNF-α (10 ng/ml) for 1 h. To control the application of equal quantities of the nuclear protein extracts, the binding reaction with the transcription factor Sp-1 was used. b) Densitometry of intensity of the band corresponding to p65/p50 in Fig. 2a with respect to the control.

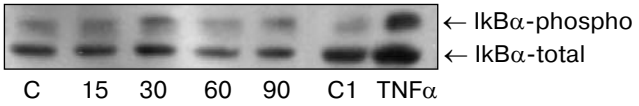


Fig. 3. Increase in the amount of phosphorylated IκBα upon incubation of hepatoma 27 cells with BP. The arrows indicate phosphorylated and total IκBα. As a positive control of the IκBα phosphorylation, the cells were treated with tumor necrosis factor α (TNF-α) at the concentration of 10 ng per ml of the medium for 1 h. C and C1, control cells treated with the solvent; 15, 30, 60, and 90, time of the exposure to BP (min).

hepatoma HepG2 cells with the expressed enzymes of metabolism NF-κB was activated much more weakly.

Afterwards, we studied the effect of BP on activation of the transcription factor AP-1 in the above-described cell cultures. In the hepatoma HepG2 cells, BP increased the AP-1 binding after 1 h and to a maximum after 2 h of the treatment (Fig. 4). The treatment with BP did not strengthen the binding in the hepatoma 27 cells.

DISCUSSION

BP is a "full" carcinogen, which induces both the initiation and promotion stages of carcinogenesis. The mechanism of the initiating action of BP type compounds is well studied. The initiation is related with mutations caused by active products of the oxidative metabolism of BP, first of all, diol-epoxides. The promotion stage of the carcinogenic action of "full" carcinogens is virtually uninvestigated.

In the model of two-stage carcinogenesis, the promotion stage is shown to be caused by nongenotoxic compounds, such as dioxins, barbiturates, and phorbol esters. Therefore, it was suggested that in the case of "full" carcinogens the promotion stage should be associated with an epigenetic action. The promotion includes stimulation of proliferation, inhibition of gap junction intercellular communications, and inhibition of apoptosis [2, 6, 7]. Activation of the transcription factors AP-1 and NF-κB is crucial for both stimulation of proliferation and inhibition of apoptosis [21].

We have shown that the carcinogenic PAH-BP activates the transcription factors NF-κB and AP-1. But the activation depends on the cell type. In the hepatoma 27 cells, we observed an effective activation of NF-κB along with the absence of AP-1 activation. The pattern was opposite in the hepatoma HepG2 cells: AP-1 was effectively activated along with a slight activation of NF-κB. These cultures are significantly different: the cytochrome P450 isoform and Ah-1 receptor are expressed in the hepatoma HepG2 cells, whereas their expression is absent in the hepatoma 27 cells. These proteins are now considered to be major factors determining the biological effect of PAHs. PAHs are oxidized by CYP and give rise to highly active metabolites interacting with various cellular components and changing their functions. A nonmetabolized PAH molecule interacts with the Ah-receptor and activates it. The activated Ah-receptor in a complex with the ARNT protein can act as a transcription factor and also interact with regulatory proteins changing their functions. In addition to PAHs, some chlorinated cyclic hydrocarbons are also ligands of the Ah-receptor, and TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is the most active. In contrast to PAHs, these compounds are not metabolized, and their biological effects seem to be associated only with activation of the Ah-receptor. Therefore, functions of the activated Ah-receptor are usually studied using TCDD. The TCDD-activated Ah-receptor increas-

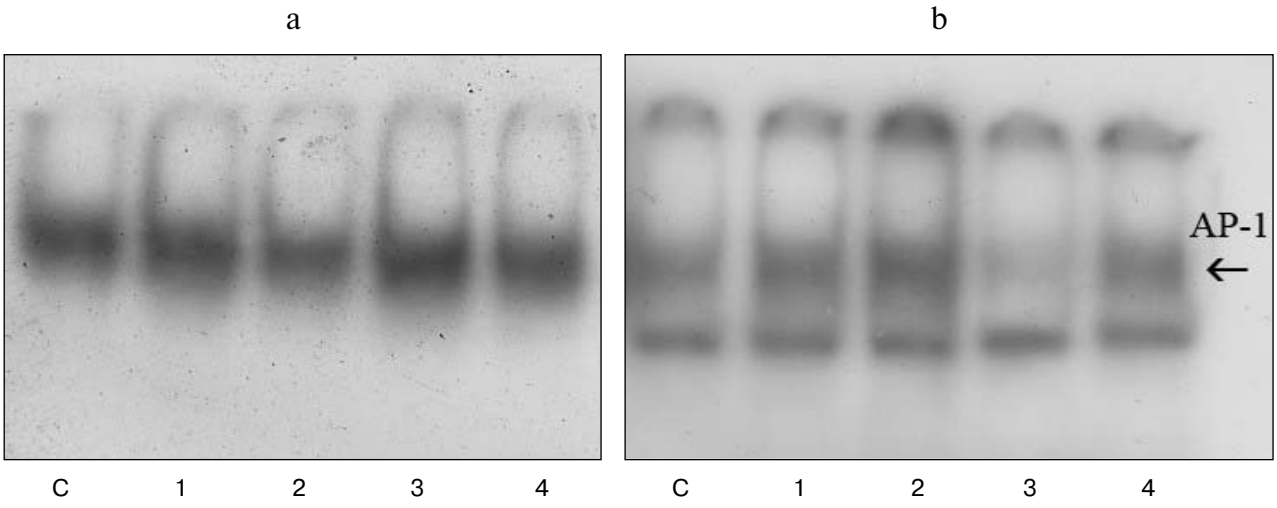


Fig. 4. Activation of AP-1 in hepatoma 27 (a) and HepG2 (b) cells. The cells were treated with BP (5 μg/ml) or the solvent (C, control) for different times. 1, 2, 3, and 4, treatment time (h). The arrow shows the AP-1 binding.

es the AP-1 binding with the DNA recognition site [22]. The TCDD-activated Ah-receptor has also been shown to interact with NF-κB and inhibit its functions [5].

However, the effect of carcinogenic PAHs on activation of the transcription factors NF-κB and AP-1 is studied insufficiently. In liver slices, BP activated AP-1 but not NF-κB [23]. BP stimulated the NF-κB expression in muscle cells of human and rat endothelium [24]. In the JB6P culture of mouse epidermal cells, diesel exhaust particles activated NF-κB but not AP-1. The authors suggest the effect to be caused by a mixture of PAHs, which is the major organic component produced on fuel combustion [25]. BP was shown to activate the gene *p53* transcription via NF-κB activation [26]. These works were not designed to determine the relationship between the effects and carcinogenicity of compounds and in what form, initial or metabolized, the PAHs were acting.

We have shown that the carcinogenic PAH-BP, unlike its noncarcinogenic isomer BeP, increases NF-κB binding with the DNA consensus site in the culture lacking both the system of PAH metabolism and Ah-receptor, which is the only acceptor of the unmetabolized molecule known by now.

Other researchers have also encountered biological effects of the Ah-receptor ligands in cells lacking this receptor. Thus, TCDD stimulated in cells the MAP kinase cascade independently of the Ah-receptor [27]. Apoptosis in T-cells lacking the Ah-receptor was caused by TCDD [28].

In the presence of TCDD, the "early response genes", such as *fos* and *jun*, were activated through a number of mechanisms, and at least one of them did not depend on the Ah-receptor [29]. Activation of MAP kinases by TCDD treatment was also recorded in the culture of cells lacking the Ah-receptor [5].

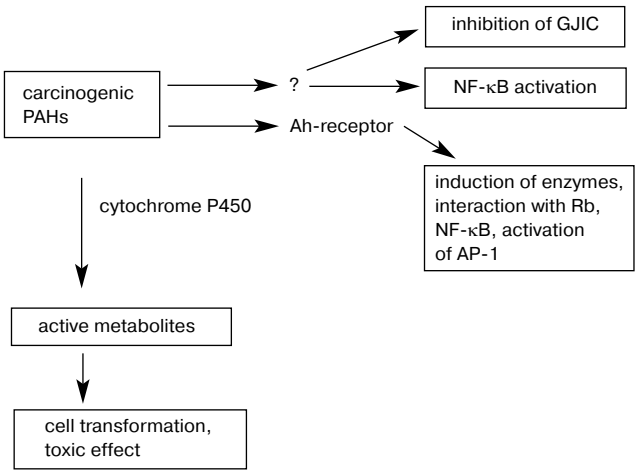
In mouse hepatoma Hepa 1c1c7 cells the antiapoptotic effect was not inhibited by α-naphthoflavone, which is concurrently an inhibitor of PAH metabolism and antagonist of the Ah-receptor [30]. BP similarly activated the MAP-kinase cascade in the cells of both normal mice and the mice with knocked-out Ah-receptor [31]. In human macrophages, BP stimulated TNF-α expression, which was not reduced by antagonists of the Ah-receptor, and the authors concluded that this effect should be unrelated with the Ah-receptor activation [32]. The carcinogenic activity of 7,12-dimethylbenz[a]anthracene was the same in the mice with the Ah-receptor and the mice with this gene knocked-out [33].

Studies on PAH binding with cell proteins have revealed that, in addition to the Ah-receptor, a 4S protein exists which efficiently interacts with PAHs [34], but functions of this protein are still unclear.

All the data presented suggest that in the cells, in addition to the known pathways realizing the biological effect of PAHs, there is, at least, one more pathway involving interaction between the initial nonmetabolized molecule of the compound with an unknown constituent of the cell. The NF-κB activation is not the only result of this interaction. We have earlier shown that gap junction intercellular communications are inhibited by BP treatment and some other carcinogenic PAHs [9, 10] in both the hepatoma HepG2 and hepatoma 27 cells. Because the gap junction intercellular communications are inhibited similarly in the cells of the two hepatomas, it is concluded that this effect of carcinogens is not related with the NF-κB activation (Scheme).

The difference in the BP-induced activation of the transcription factors AP-1 and NF-κB in the hepatomas HepG2 and 27 cells may be explained as follows. In the hepatoma HepG2 cells, both the Ah-receptor and an unknown acceptor of BP activating NF-κB are present. Because the activated Ah-receptor can interact with NF-κB and inhibit its functioning [5], the Ah-receptor is more active in this competition in such cells as HepG2 which possess this receptor. As a result, BP has a very little effect on NF-κB activation. In the hepatoma 27 cells, there is no Ah-receptor, and nothing prevents the NF-κB activation. The AP-1 activation seems to be mediated through activation of the Ah-receptor, and this is in agreement with the above-presented data on the TCDD-induced activation of AP-1 [22]. Therefore, AP-1 is activated in the hepatoma HepG2 cells and not activated in the hepatoma 27 cells lacking the Ah-receptor.

Exposure to chemical carcinogens during the early stages of transformation results in generation of cell clones with different expression levels of the Ah-receptor and CYP. It seems that in the clones with a low expression of CYP and Ah-receptor carcinogens stimulate the proliferation and inhibit apoptosis as a result of the NF-κB activation. This provides for conditions for the preferential growth of these transformed cells. This may explain the usually decreased expression of CYP and Ah-receptor



Scheme of supposed action of carcinogenic PAHs (Ah-receptor ligands) on cells

in tumors induced by chemical carcinogens [35]. In the transformed cells with the retained expression of CYP and Ah-receptor, carcinogens stimulate the proliferation through activation of AP-1.

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