Activation of Constitutive Androstane Receptor under the Effect of Hepatocarcinogenic Aminoazo Dyes in Mouse and Rat Liver

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 9, pp. 316-319, September, 2007
Original article submitted December 6, 2006

Selective increase of DNA-binding activity of constitutive androstane receptor was detected in rat and mouse liver in response to aminoazo dyes exhibiting hepatocarcinogenic activity for these species (ortho-aminoazotoluene for mice and 3'-methyl-4-dimethylaminobenzene for rats). Competition of azo dyes with ^{3}H-5α-androst-16-ene-3α-ol (a well-known ligand of constitutive androstane receptor) for binding to liver cell cytosol proteins was studied. Ortho-aminoazotoluene and 3'-methyl-4-dimethylaminobenzene were better competitors for cytosol proteins from mouse and rat liver, respectively.

Key Words: *hepatocarcinogenesis; androstane constitutive receptor; species specificity; aminoazo dyes*

Aminoazo dyes are well known hepatocarcinogens for rodents, but, despite similar structure they are characterized by pronounced species and strain specificity of the tumor-inducing effect after administration to experimental animals. It is known, for example, that 3'-methyl-4-dimethylaminobenzene (3'-MDAB) is hepatocarcinogenic for rats, but causes almost no tumors in mice, while 2'-3-dimethyl-4-aminoazobenzene (ortho-aminoazotoluene, OAT) induces liver tumors in many mouse strains (SWR, A/He, DD, DBA/2, CBA) with high incidence and exhibits negligible carcinogenic effect in rats and mice of some other strains (AKR, CC57Br) [9,10]. The species and strain specificity of OAT and 3'-MDAB is not associated with their activation to mutagenic derivatives [1,11,14].

Among mechanisms responsible for species specificity, the effects of chemical carcinogens on the regulatory networks of target cells characterized by some species-specific features seem to be most important. Binding of these substances to certain receptor proteins can be step 1 in their action. Members of the orphan receptor family binding a wide spectrum of ligands and participating in numerous regulatory processes due to binding to the recognized effector gene DNA sites and due to reactions with other regulatory proteins [3] are candidates for the role of these proteins. Detection of these receptor proteins in mice and rats seems to be the key point in deciphering the mechanism of species-specific effect of hepatocarcinogenic azo dyes.

We studied the effects of species-specific hepatocarcinogens OAT and 3'-MDAB on DNA-binding activity of peroxisome proliferator receptor, constitutive androstane receptor (CAR), pregnane receptor, and hepatic X-receptor in mouse and rat liver, and the capacity of OAT and 3'-MDAB to bind to receptor exhibiting species specificity during activation with azo dyes.

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MATERIALS AND METHODS

The study was carried out on 3-6-month-old male SWR/J, A/He, and DD mice sensitive to hepatocarcinogenic effect of OAT and male Wistar rats (180 g) bred at vivarium of Institute of Cytology and Genetics. OAT, 3'-MDAB, and 4'-MDAB dissolved in olive oil were injected intraperitoneally in doses of 22.5 mg/100 g for OAT, 25 mg/100 g for 3'-MDAB and 4'-MDAB. Controls received the solvent.

Double-stranded oligonucleotides corresponding to transcription factor binding sites were used in the study (strand 2 is not presented): Ets: 5'-cagtTCGAACTTCCTGCTCGA-3' from rat *TAT* gene enhancer [5]; PPAR: 5'-gatcCAAAACTAGGTCAAAGGTCA-3' corresponding to consensus derived on the basis of 16 PPRES (type DR1) of various genes [8]; CAR: 5'-gatcGAAGTTCATGAGAGTTCA-3' from the rat *PIT-1* gene promotor [7]; PXR: 5'-gatcTATGAACTCAAAGGAGGTCAGT-3' from human *CYP3A4* gene enhancer [12]; LXR: 5'-gatcCAGTGACCGCCAGTAACCCCAGC-3' from mouse *SREBP-1c* gene promotor [13]. Small letters denote the added free ends, by which oligonucleotides after annealing were labeled with *E. coli* DNA polymerase I Klenow fragment in the presence of [α-^{32}P]-dATP. Oligonucleotides were synthesized on an ACM-102I automated biosynthesizer (Biosset) by the H-phosphonate method.

Liver cell nuclei extracts were prepared 2-3 h after injection of azo dyes as described elsewhere [2], except the stage of precipitation of nuclear extract proteins with ammonium sulfate (1.4 g ammonium sulfate was added to 3.5 ml supernatant obtained after chromatin precipitation, after which all procedures were carried out as described previously [2]). DNA-binding activity of nuclear proteins was evaluated by gel retardation assay [2]. The gel was dried and the binding picture was visualized on a Molecular Imager FX Pro Plus (BioRad).

Liver cell cytosol was obtained as described previously [2]. ^{3}H-androstenol with specific activity of 23 Ci/mM was prepared by the tritium exchange method. The cytosol (45 μl) was incubated with 1 μl ^{3}H-androstenol in DMSO (final concentration 20 μM) and 3-4 μl unlabeled competitors in DMSO for 4 h at 4°C. Free ^{3}H-androstenol was removed by precipitation with charcoal-dextrane suspension [2]. Radioactivity was measured in 5 ml dioxane scintillator on a RackBeta 1209 counter (LKB-Wallack).

RESULTS

Representatives of the orphan receptor family PPAR, CAR, PXR, and LXR were studied as proteins presumably binding azo dyes as ligands and modifying their activity in response to binding. Changes in DNA-binding activity of these receptors in mouse and rat liver in response to OAT, 3'-MDAB, and 4'-MDAB were studied. The DNA-binding activity of Ets transcription factor, not modified by OAT, 3'-MDAB, or 4'-MDAB [9], served as the negative control. Only one orphan receptor, CAR, changed its activity under the effects of these substances. This receptor was activated stronger in response to OAT, hepatocarcinogenic for mice (Fig. 1), in comparison with the control, while the increase in DNA-binding activity in response to 3'-MDAB, not carcinogenic for these animals, was much weaker. An opposite picture was observed in response to azo dye injections to rats (Fig. 1): CAR was stronger activated under the effect of rat hepatocarcinogen 3'-MDAB than under the effect of OAT, hepatocarcinogenic for mice. Activation of CAR under the effect of 4'-MDAB, a noncarcinogenic analog of these compounds, virtually did not differ from that under the effect of OAT, not carcinogenic for

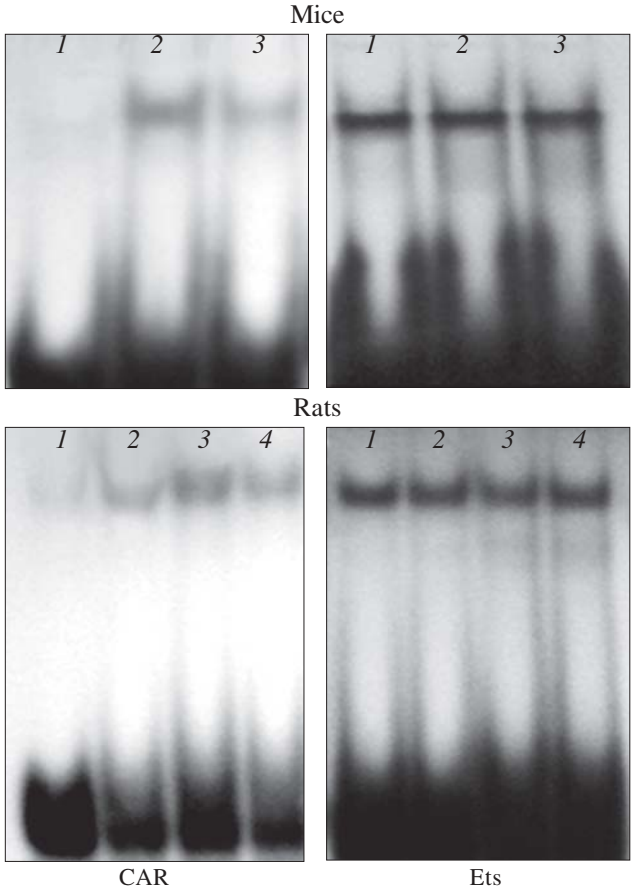

Fig. 1. Effects of injections of azo dyes to mice and rats on DNA-binding activity of CAR in extracts from liver cell nuclei. Probe retardation after incubation with extract from liver cell nuclei of control animals (*1*) and animals receiving 3'-MDAB (*2*), OAT (*3*), and 4'-MDAB (*4*).

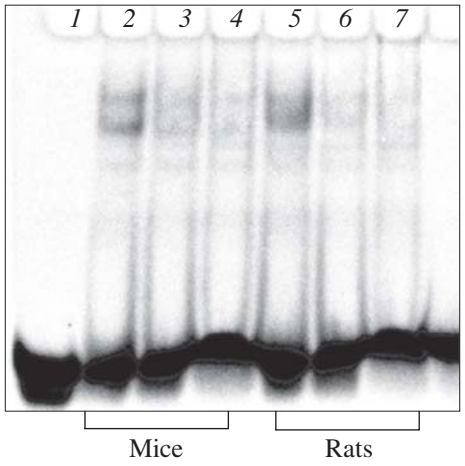

Fig. 2. Identification of DNA-binding activity of CAR in nuclear extracts from mouse (*2-4*) and rat (*5-7*) liver. *1*) mobility of labeled probe; *2*, *5*) probe retardation in incubation with nuclear extract without antibodies to CAR; *3*, *4*, *6*, *7*) the same in the presence of 1.3 μl antibodies to CAR (SantaCruz).

rats. Negligible activation in response to any aminoazo dye without selective activation was observed for other studied receptors, except PPAR, which was not activated at all. The same results were obtained on DD and SWR/J mice sensitive to OAT. Hence, we observed a clear-cut correlation between the species-specific tumorogenic effect of azo dyes and high capacity to CAR activation.

Experiments with addition of specific antibodies to CAR showed that this protein was responsible for DNA-binding activity. Since antibodies added to the reaction mixture were directed to the DNA-binding domain of CAR, the band corresponding to the complex of CAR with labeled probe was weaker (Fig. 2).

Our data on selective increase in DNA-binding activity of CAR under the effect of species-specific hepatocarcinogens prompt investigation of the capacity of these carcinogens to specifically bind this intracellular receptor. We studied competition of OAT and 3'-MDAB with androstenol (5α-androst-16-ene-3α-ol), a known ligand for CAR [6], for binding mouse and rat liver cytosol proteins. Progesterone and clofibrate (highly specific PPAR ligand) were selected for negative control; as was expected, they did not compete for androstenol binding (Fig. 3). In contrast to clofibrate, OAT and 3'-MDAB competed for binding (Fig. 3), and hence, were capable of binding CAR. In rat liver, 3'-MDAB (carcinogen for rats) competed with ^{3}H-androstenol much stronger than OAT (carcinogen for mice), while an opposite picture was observed in experiments with mouse liver cytosol. Presumably, hepatocarcinogenic aminoazo dyes directly bind CAR, but with different efficiency, depending on the origin of protein. The cause of this selective binding and activation is not clear. We hypothesize that a role in this phenomenon is played by non-identity of the CAR ligand-binding domain in mice and rats differing by 10% amino acids including 3 amino acid substitutions in a domain consisting of 33 amino acids and forming a ligand-binding pouch [4].

Hence, species-specific carcinogenic effect of hepatocarcinogenic aminoazo dyes is presumably associated with CAR activation in the liver of mice and rats.

The study was supported by the Russian Foundation for Basic Research (grant No. 06-04-48575).

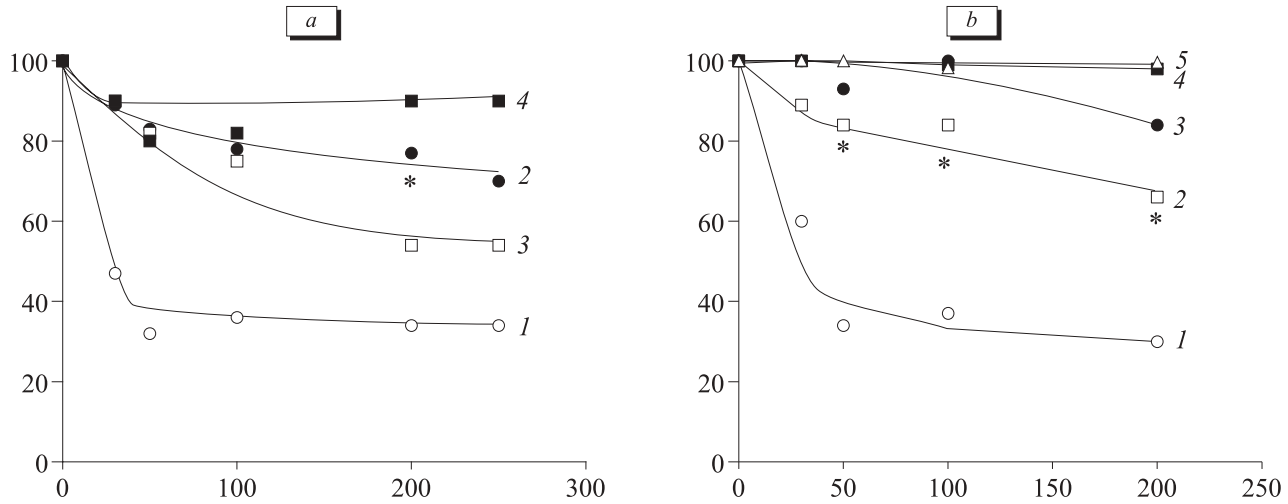

Fig. 3. Binding of ^{2}H-androstenol in liver cell cytosol of mice (*a*) and rats (*b*) in the presence of 5-200-fold excess of unlabeled androstenol (*1*), 3'-MDAB (*2*), OAT (*3*), clofibrate (*4*), and progesterone (*5*). Abscissas: excess of unlabeled competitor. Results of 3-5 independent experiments are presented in % of bound ^{3}H-androstenol without competitors. *p<0.05 compared to OAT.

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