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Species Specific Hepatocarcinogen Inhibition of the Glucocorticoid Induction of Tyrosine Aminotransferase Gene in Mouse and Rat Liver

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Abstract—3′-Methyl-4-dimethylaminoazobenzene (3′-MeDAB) is a potent hepatocarcinogen in rats and a weak carcinogen in mice, whereas *o*-aminoazotoluene (OAT) is a potent hepatocarcinogen in mice but weak hepatocarcinogen in rats. They significantly suppress glucocorticoid induction of tyrosine aminotransferase (TAT) in the liver of sensitive animals and have minor effect on the induction of this enzyme in the liver of resistant animals (3′-MeDAB-treated mice and OAT-treated rats). The inhibitory effect of these carcinogens is realized at the level of gene transcription (decreased accumulation of TAT mRNA). This effect is mediated via reduction of DNA-binding activity of transcription factor HNF3 (without decrease of its content) without any involvement of the glucocorticoid receptor. It was shown that carcinogens influence DNA-binding activity of HNF3 via an unknown nuclear factor.

Key words: hepatocarcinogens, tyrosine aminotransferase, glucocorticoid induction, glucocorticoid receptor, transcription factor HNF3

Tissue and species-specific effects of many carcinogens are not well studied phenomena. Modern notions on mechanisms of carcinogenesis do not give a plausible explanation why a certain chemical may induce tumors only in certain organs of certain animal species. For example, the azo-dye N,N-dimethyl-4-aminoazobenzene (DAB) and its 3′-methyl derivative (3′-MeDAB) are highly carcinogenic for liver but not for other rat organs, but they are weakly carcinogenic for mouse liver, and are not carcinogens for cotton rat, guinea pig, chipmunk, hen, and others [1, 2]. In contrast to DAB and 3′-MeDAB, their isomer 2′,3-dimethyl-4-aminoazobenzene (old name *ortho*-aminoazotoluene, OAT) induces with high frequency hepatic and vascular tumors in some mouse strains, but it is almost ineffective in rats and other mouse strains [2-4]. Attempts to explain these discrepancies in the effectiveness of carcinogenesis by differences in metabolic activation of carcinogens are seriously questioned. We demonstrated that the activities of hepatic enzymes metabolizing aminoazo-dyes of OAT-sensitive and resistant mice are almost identical [5]. Moreover, enzymes of S9 fraction of rat liver activate OAT to mutagenic derivatives as effectively as corresponding enzymes of the mouse liver [6]. As shown in other laboratories, enzymes from human and cotton rat hepatocytes which are insensitive to the hepatocarcinogenic effect of 2-acetylaminofluorene, activate this compound to mutagenic metabolites even more actively than corresponding enzymes of hepatocytes from the sensitive Sprague-Dawley and Fischer 344 rats [7, 8].

In contrast to much attention paid to genotoxicity of carcinogens their epigenetic effects did not attract attention; however, it should be noted that many substances lacking mutagenic activity induce tumors via epigenetic mechanisms [9]. Many compounds which did not exhibit genotoxic activity in any known mutagenic tests were able to induce tumors in animals [10]. Thus, elucidation

Abbreviations: DENA) diethylnitrosamine; GRE) glucocorticoid response element; HNF3) hepatic nuclear factor 3; 4′-MeDAB) 4′-methyl-4-dimethylaminoazobenzene; 3′-MeDAB) 3′-methyl-4-dimethylaminoazobenzene; OAT) *o*-aminoazotoluene; TAT) L-tyrosine:2-oxoglutarate aminotransferase.

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of a mechanism responsible for these so-called "epigenetic" carcinogens may be important for understanding of mechanisms underlying effects of carcinogens possessing genotoxic activity. In this connection, studies of epigenetic effects induced by (genotoxic and non-genotoxic) carcinogens in tissues targets of sensitive animals are very promising.

One such effect of hepatocarcinogens which appears within several hours after their administration is impairment of glucocorticoid regulation of adaptive enzyme activity in hepatic cells. More than thirty years ago it was found that in liver the rat hepatocarcinogenic compounds aflatoxin B_1 (AFB_1), thioacetamide, DAB, and 3′-MeDAB specifically blocked increase in activity of some adaptive enzymes—tyrosine aminotransferase (TAT; L-tyrosine:2-oxoglutarate aminotransferase; EC 2.6.1.5), tryptophan dioxygenase (L-tryptophan:oxygen-2,3-oxidoreductase; EC 1.13.11.11), and others induced by glucocorticoid hormones. Non-carcinogenic 4-aminobenzene and 4′-MeDAB did not cause such effect [11-13]. AFB_1 and sterigmatocystin, another rat liver carcinogenic compound, suppressed glucocorticoid induction of TAT in rat hepatoma cells [14]. We found that OAT suppressed TAT induction in mouse liver [15]. In both cases the effect of carcinogen was specific and was not reproduced by its non-carcinogenic analogs [4, 15]. It was shown that OAT significantly influenced TAT induction only in those mouse strains which were sensitive to hepatocarcinogenic effect [4, 16]. These results suggest that suppression of glucocorticoid induction of TAT by some compound may be considered as a marker for hepatocarcinogenicity of this compound.

Our studies of the effect of OAT on transcription factors involved in glucocorticoid induction of TAT in different mouse strains revealed the existence of a certain interrelationship between OAT-induced decrease in TAT induction and decrease in DNA-binding activity of HNF3γ (hepatic nuclear factor 3γ) [16, 17]. Since HNF3γ belongs to one of the main families of transcription factors providing hepatocyte differentiation and phenotype maintenance of mature hepatocytes [18] it is reasonable to suggest than suppression of function of this factor may be important for the carcinogenic effect of OAT. Since members of the HNF3 family are expressed in several organs (preferentially in the liver) [19], the effect of OAT on HNF3γ may be related to tissue specificity of the carcinogen.

So, for subsequent study of impairments of glucocorticoid induction of TAT as the marker of subsequent development of hepatic tumor and the role of HNF3 as a component of the mechanism underlying specificity of hepatocarcinogenic effect, we investigated effects of 3′-MeDAB (a potent carcinogen for rats and weak carcinogen for mice) and OAT (a weak carcinogen for rats and potent carcinogen for mice) in the mouse and rat liver. We investigated effects of these carcinogens on TAT induction (which was evaluated by activity of this enzyme and its mRNA content), DNA-binding activity of transcription factors of HNF3 family in cell nuclear extracts, and also on the content of hormone- and DNA-binding activity of the glucocorticoid receptor.

MATERIALS AND METHODS

Male 3-6-month-old A/He mice sensitive to the hepatocarcinogenic effect of OAT [4] and male Wistar rats (150-200 g) from the animal house of the Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences, were used in experiments. Animals (3-6 per special plastic cage) had free access to water and food.

Induction and assay of TAT activity. Carcinogens were injected intraperitoneally as 0.1 M solutions in olive oil in the volume of 1 ml per 100 g of body weight (22.5 mg OAT, 25.0 mg 3′-MeDAB and 4′-MeDAB). DENA was dissolved in saline solution and injected in the dose 5 mg per 100 g of body weight. All the compounds were injected 20 h before administration of hydrocortisone or dexamethasone. Control animals received injection of an equivalent volume of the vehicle. Hydrocortisone (5 mg per 100 g body weight) and dexamethasone (0.5 mg per 100 g body weight), TAT inductors, were injected intraperitoneally. It was previously demonstrated that these doses of glucocorticoids induced almost identical increase in TAT activity [15]. Five hours after administration of these hormones the animals were sacrificed and TAT activity was assayed in liver cytosolic fraction as described [20]. One unit of enzymatic activity was defined as the amount of enzymes catalyzing formation of 1 μM *p*-hydroxyphenylpyruvate during 1 h. Protein concentration was determined by the method of Lowry [21].

Isolation of RNA and preparation of cDNA. Total RNA was isolated from liver of animals decapitated 3.5 h after the administration of inducers using phenolic extraction as described [22]; cDNA was obtained as described in [23].

Multiplex polymerase chain reaction (PCR). Multiplex PCR for detection of TAT mRNA in total RNA preparations was carried out in a total volume of 40 μl as described earlier [24]. The housekeeper gene encoding β-actin was used as the endogenous control for normalization of amplification products. Each PCR sample contained cDNA (corresponding to 10 ng total RNA) as a template, PCR buffer, 50 μM of each deoxyribonucleotide triphosphates, 20 pM of each primer for TAT amplification, and 2 U of Taq DNA-polymerase. Equal amount of primers (20 pM) for β-actin amplification were added after several cycles. For optimization of number of cycles required for visualization of the amplification products and termination of PCR at the exponential stage of amplification the reaction was stopped at various

cycles and the amplification products were analyzed by gel electrophoresis. Under our experimental conditions sufficient amplification included 26 and 32 cycles for β-actin and TAT, respectively. The amplification reaction was carried out under the following conditions: 94°C for 1 min; 55°C for 0.5 min; 72°C for 1 min. The reaction products (10 μl) were separated by electrophoresis in 2% agarose gel containing ethidium bromide (0.2 μg/ml). The gel was photographed, scanned, and evaluated using Scion Image software (National Institutes of Health, USA). Quantitative data were treated using Excel (Microsoft, USA) and Statistics (StatSoft, USA).

Preparation of liver nuclear extracts. The extracts were prepared as described earlier [25] with minor modifications [26]. After decapitation of animals livers were perfused with cold 10 mM Hepes buffer, pH 7.6, containing 25 mM KCl and 1 mM EDTA. All other procedures were carried out at 0°C. Livers were homogenized in 8-10 volumes of 10 mM Hepes buffer, pH 7.6, containing 25 mM KCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 2.1 M sucrose, and 10% glycerol. The homogenate (20-25 ml) layered onto 10 ml of the same medium in tubes for the SW-27 rotor of a Beckman L8-50M/E centrifuge (USA) was centrifuged at 24,000 rpm for 30 min. The resultant supernatant was removed, tube walls were dried with filter paper, and the sediment containing nuclei was suspended in 4 ml of 25 mM Hepes buffer, pH 7.6, containing 100 mM KCl, 0.1 mM EDTA, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. Nuclei were lysed by adding 400 μl of saturated (at 0°C) ammonium sulfate followed by gentle mixing. The lysate was transferred into tube for the SW-50 rotor and centrifuged at 34,000 rpm for 90 min for chromatin sedimentation. The resultant supernatant (3.5 ml) was treated with solid ammonium sulfate (882 mg) which was added in small portions during 30 min under mixing on ice. The mixture was centrifuged for 30 min at 34,000 rpm using the SW-50 rotor. The sediment dissolved in 500 μl of 25 mM Hepes buffer, pH 7.6, containing 150 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, and 10% glycerol was dialyzed twice (each time for 45 min) against 100 ml of the same buffer; aliquots (50 μl) frozen in liquid nitrogen were kept at −70°C and used once after thawing. In *ex vivo* study of the effects of OAT and 3′-MeDAB on the DNA-binding activity of HNF3 the media for nuclei isolation contained at various stages 1% DMSO or 1% saturated solution of OAT or 3′-MeDAB in DMSO. After addition at a certain stage of the isolation procedure, the carcinogen was present in all media used during subsequent stages.

Oligonucleotide gel retardation. Oligonucleotides corresponding to both strands of HNF3 binding site 5′-cagtCGAGTTGACTAAGTCAATAATCAGAATCAGT-CG-3′ [27] (small letters indicate overhang end) were synthesized by the H-phosphonate method using an automated ASM-102I synthesizer (Biosset, Novosibirsk, Russia); the resulting oligonucleotides were purified using electrophoresis in 12% denaturing polyacrylamide gel [28]. After annealing oligonucleotides were labeled using Klenow fragment of DNA-polymerase I and [^{32}P]dATP. The reaction mixture (10 μl) contained: 25 mM Hepes, pH 7.6, 150 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 2 ng of the labeled oligonucleotide, 4 μg of nuclear extract protein preincubated for 10 min at 0°C with calf thymus DNA (1 μg per 7 μg protein) that was sonicated. After the incubation at 20°C for 5 min the mixture was subjected to electrophoresis in 4% polyacrylamide gel in 0.5× Tris-borate buffer (TBE). Antibodies against HNF3β were kindly given by R. Costa (Rockefeller University, New York) [29] in the dilution for Western blotting. In the experiments with antibodies the nuclear extract was incubated with calf thymus DNA sonicated as described above, diluted with 25 mM Hepes buffer, pH 7.6, containing 150 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% gelatin, up to protein concentration 5 ng/μl. The diluted extract (4 μl) was incubated with antibodies (1 μl) at 0°C for 10 min, and then the labeled oligonucleotide (0.02 ng) was added in the dilution buffer. After incubation at 20°C for 5 min, the mixture was subjected to electrophoresis in 4% polyacrylamide gel. The dried gel was exposed to X-ray film for 2 weeks.

Preparation of liver cytosol fraction and determination of hormone- and DNA-binding activity of the glucocorticoid receptor. Animals were subjected to adrenalectomy and treatment with carcinogens. The time intervals before decapitation after the operation and carcinogen administration were 4 days and 24 h, respectively. Livers were perfused with cold 20 mM Tris-HCl buffer, pH 7.8, containing 50 mM NaCl, 1 mM EDTA, 1 mM monothioglycerol, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol, cut with scissors, and homogenized in the same buffer (6 ml of the medium per 1 g liver). The homogenate was centrifuged at 160,000*g* for 40 min. In the case of determination of glucocorticoid receptor DNA-binding activity, [$^3H_3$]triamcinolone acetonide was added to homogenate to final concentration 0.3 μM. For determination of number of glucocorticoid binding sites and apparent equilibrium dissociation constant (K_d), 50 μl cytosol (~70 μg protein) was incubated with 0.05-1.5 nmol [$^3H_3$]triamcinolone acetonide at 2°C for 4 h. Unbound hormone was removed by treatment with charcoal–dextran mixture [30]. The results were analyzed by the method of Scatchard [31] using the Excel program. For the determination of DNA-binding activity of the glucocorticoid receptor, 2 ng of ^{32}P-labeled DNA probe (cagtACTATAGGGACATGATGTTCCACACG-TCAC – GRE from the mouse gene encoding metallothionein I [32]) with specific radioactivity of 3300-3600 cpm/ng DNA was incubated with aliquots (4-10 μl) of cytosol fraction containing 10 mM $MgCl_2$ and poly(dI-dC) (250 ng/ml). Before probing the cytosol fraction was

activated at 25°C for 7 min. After the incubation at 20°C for 10 min, the samples were subjected to electrophoresis in 4% polyacrylamide gel containing 0.5× TBE and 0.3 μM triamcinolone acetonide. The electrophoretic procedure was run during 40-50 min at 10 V/cm. After the electrophoresis, the gel was transferred onto nylon membrane and radioautographed. The fragment corresponding to the band of receptor-bound probe retardation was cut out and its radioactivity was determined using a RackBeta 1209 counter (LKB-Wallac, Finland).

Western-blot hybridization. Aliquots of nuclear extracts containing 10 μg of protein were diluted to 20 μl with the buffer for dialysis and mixed with 10 μl of 62.6 mM Tris-HCl buffer, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, and 0.01% Bromophenol Blue. After heating at 95°C for 5 min, samples were subjected to electrophoresis using a stepwise gel by the method of Laemmli [33]. The stacking gel contained 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 4% polyacrylamide; the separating gel contained 375 mM Tris-HCl, pH 8.9, 0.1% SDS, and 10% polyacrylamide gel. The upper and lower electrode buffers contained 25 mM Tris, 192 mM glycine, 0.1% SDS and 0.4× TBE, respectively. Semidry transfer onto nitrocellulose Hybond-ECL membrane (Amersham, England) was carried out during 1 h at 2 mA/cm^2 using a Semi-Phor apparatus (Hoefer Scientific Instruments, USA). The blot-buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. Western blot hybridization was carried out using an ECL-kit (Amersham) according to supplier's instructions. For prevention of non-specific binding, the membrane was incubated for 1 h in TBS-T buffer (20 mM Tris HCl, pH 7.6, 137 mM NaCl, 0.5% Tween-20) containing 5% human serum albumin. The membrane was washed 5 times with TBS-T, incubated for 1 h with antibodies against HNF3γ (Santa Cruz Biotechnology Inc., USA) (dilution 1 : 7000) or with primary antibodies against HNF3β [29] (dilution 1 : 1000), and then washed again. Then it was incubated for 1 h with secondary antibodies in TBS-T, washed again, and visualized using the ECL-kit.

Chemicals. The following chemicals were used in the study: [$^3H_3$]triamcinolone acetonide from Amersham (specific radioactivity 23 Ci/mmol); OAT, 3′-MeDAB, and 4′-MeDAB from Shostka Chemical factory (Ukraine) and Koch-Light (England); hydrocortisone acetate from Zdorov'e (Ukraine); DNase and RNasin from Pharmacia (USA); DENA, PMSF, DMSO, and dexamethasone from Serva (Germany); EDTA, EGTA, dithiothreitol, and Hepes from Sigma (USA); MoMLV RNA-dependent DNA-polymerase and Taq DNA-polymerase from Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences (Russia). Other chemicals of "chemically pure" and "special high purity" grades were from Russian suppliers.

RESULTS

Table 1 shows data on the effects of 3′-MeDAB, OAT, and their non-carcinogenic analog 4′-MeDAB on the glucocorticoid induction of TAT activity in mouse and rat liver. These compounds were administered 20 h before the analysis and the doses of carcinogens corresponded to those usually employed for tumor induction in experimental animals. Marked decrease in TAT induction (by two times and more) was observed only when the species-specific carcinogen was administered. "Foreign" carcinogen caused a weak effect on the enzyme induction in these animals and the non-carcinogenic analog was almost inactive in both rats and mice. The effect of tissue-specific carcinogens was realized at the transcription level; this is evidenced by analysis of hepatic TAT mRNA at the peak of induction [34] observed 3.5 h after the hormone administration (Fig. 1). In control mice and rats glucocorticoid administration caused 4-6-fold increase in TAT mRNA. Pretreatment of mice with OAT and rats with 3′-MeDAB caused two times less response to the hormone administration. In corresponding animals, "foreign" carcinogens caused insignificant effect on induction of TAT mRNA (Fig. 1) and the enzymatic activity (Table 1).

Increase in transcription of the TAT gene by glucocorticoids is determined by the interaction of a complex of transcription factors, where glucocorticoid receptor plays the leading role, with a certain regulatory region of this gene [35]. Glucocorticoid receptor is translocated

Table 1. Effect of hepatocarcinogens OAT and 3′-MeDAB and its non-carcinogenic analog 4′-MeDAB on the induction of TAT activity by hydrocortisone in A/He mice and Wistar rats

Experimental conditions	TAT activity, U/h per 100 mg protein	
	mice	rats
Vehicle	25 ± 2.0 (4)	28 ± 1.6 (5)
Vehicle + hydrocortisone	146 ± 7.3 (4)	144 ± 7.6 (8)
OAT + hydrocortisone	67 ± 7.7 (4)	114 ± 10.8 (5)
3′-MeDAB + hydrocortisone	117 ± 6.0 (3)	77 ± 5.1 (9)
4′-MeDAB + hydrocortisone	135 ± 12.2 (3)	146 ± 5.9 (5)

Note: Number of experiments is given in brackets. In each experiment two mice or one rat were used.

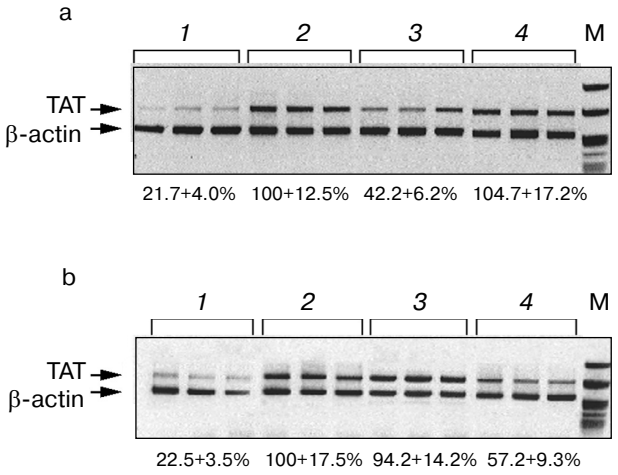

Fig. 1. Electrophoregram of products of multiplex PCR in 2% agarose gel. The level of TAT mRNA in the liver of A/He mice (a) and Wistar rats (b) was normalized versus β-actin; the averaged level of TAT mRNA after dexamethasone induction was defined as 100%. *1*) Control; *2*) dexamethasone induction; *3*) dexamethasone induction after pretreatment with OAT; *4*) dexamethasone induction after pretreatment with 3′-MeDAB. M, markers of molecular mass: plasmid pBIKS DNA hydrolyzed by restriction endonuclease *Bsp*RI.

from cytosol into nucleus and functions as the transcription factor only after hormone binding [36]. The effect of carcinogens on TAT induction might be realized via their possible influence on the glucocorticoid receptor. However, data of Table 2 show that carcinogen administration did not influence either K_d value for dissociation of triamcinolone acetonide–receptor complex or number of hormone binding sites and DNA-binding activity of glucocorticoid receptor in liver cytosol of mice and rats treated with the species-specific carcinogens.

It is known that besides glucocorticoid receptor the glucocorticoid induction of TAT gene also requires additional transcription factors including members of HNF3 family [37-39]. Using the method of DNA-probe retardation in gel we demonstrated previously that OAT administration to mice is accompanied by reduction of hepatic DNA-binding activity of factor HNF3γ [16, 17]. In this study we employed the same method for investigation of carcinogenic effects on DNA-binding activity in the liver of mice and rats.

Figure 2a shows a typical autograph, and quantitative data are given in Table 3. "Foreign" hepatocarcinogens (3′-MeDAB for mice and OAT for rats) did not influence DNA-binding activity of HNF3, whereas the species-specific carcinogens caused significant reduction in this parameter. Previously we demonstrated that a known inducer of hepatocarcinogenesis, DENA, possessing distinct chemical structure, also suppressed TAT induction in the liver [40]. The radioautograph shown at Fig. 2b demonstrates that DENA inhibits DNA-binding activity of HNF3 in rat liver nuclei, whereas non-carcinogenic 4′-MeDAB had no effect.

Use of highly specific antibodies against HNF3β revealed that this form is responsible for HNF3 DNA-binding activity in rat liver nuclei (Fig. 3). Earlier we found that the main DNA-binding activity of HNF3 in mouse liver nuclei can be attributed to the HNF3γ isoform [17]. In that study we also demonstrated that the decrease of HNF3 DNA-binding activity in mice pretreated with OAT was accompanied by some increase (but not decrease) of HNF3γ mRNA in the liver [17]. This suggests that the carcinogen influenced either translation of corresponding mRNA or modification of this protein. The latter seems to be more likely because HNF3 DNA binding activity is observed during the first hour after hepatocarcinogen administration [17].

In the present study using the method of Western-blot hybridization we demonstrated that administration of 3′-MeDAB and DENA to rats and OAT to mice did not influence content of HNF3β and HNF3γ (Fig. 4). This

Table 2. Effect of treatment of A/He mice with OAT and Wistar rats with 3′-MeDAB on parameters of glucocorticoid receptor in liver cytosol

Group of animals	Equilibrium dissociation constant of the complex receptor–[$^3H_3$]triamcinolone acetonide, nM	Number of binding sites for [$^3H_3$]triamcinolone acetonide, pmol per mg cytosol protein	DNA-binding activity of glucocorticoid receptor, cpm per 80 μg protein
Mice (control)	3.99 ± 1.08 (4)	1.92 ± 0.39 (4)	983 ± 58 (4)
Mice (OAT)	3.31 ± 0.85 (3)	1.88 ± 0.24 (3)	912 ± 98 (4)
Rats (control)	2.35 ± 0.39 (4)	1.33 ± 0.03 (4)	866 ± 29 (3)
Rats (3′-MeDAB)	2.54 ± 0.25 (4)	1.22 ± 0.39 (4)	813 ± 84 (3)

Note: Number of experiments is given in parentheses. In each experiment two mice or one rat were used.

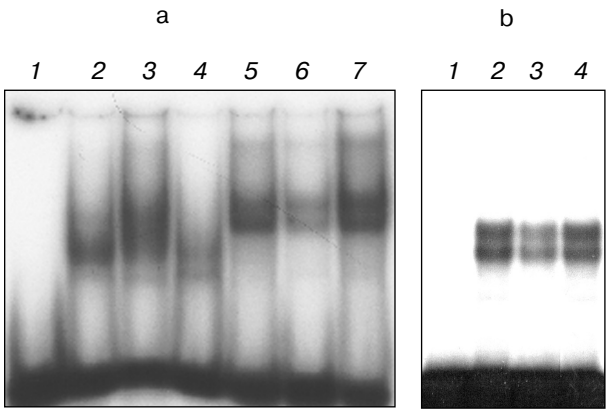

Fig. 2. Effect of administration of hepatocarcinogens and their non-carcinogenic analog on HNF3 DNA-binding activity in hepatic nuclear extracts of A/He mice and Wistar rats. a: *1*) Mobility of HNF3-binding labeled oligonucleotide (probe); *2*) probe retardation during incubation with hepatic nuclear extracts of control rats; *3*) probe retardation during incubation with hepatic nuclear extracts of OAT-treated rats; *4*) probe retardation during incubation with hepatic nuclear extracts of 3′-MeDAB-treated rats; *5*) probe retardation during incubation with hepatic nuclear extracts of control mice; *6*) probe retardation during incubation with hepatic nuclear extracts of OAT-treated mice; *7*) probe retardation during incubation with hepatic nuclear extracts of 3′-MeDAB-treated mice. b: *1*) Mobility of the labeled probe; *2*) probe retardation during incubation with hepatic nuclear extracts of control rats; *3*) probe retardation during incubation with hepatic nuclear extracts of DENA-treated rats; *4*) probe retardation during incubation with hepatic nuclear extracts of 4′-MeDAB-treated rats.

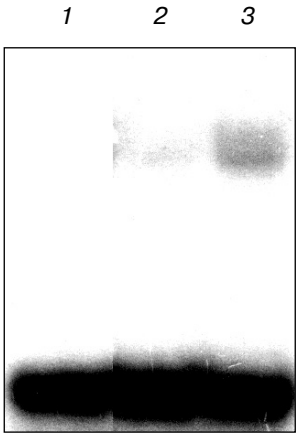

Fig. 3. Identification of HNF3 DNA-binding activity in rat hepatic nuclear extracts: *1*) mobility of HNF3-binding oligonucleotide (probe); *2*) probe retardation during incubation with hepatic nuclear extracts in the presence of antibodies against HNF3; *3*) probe retardation during incubation with hepatic nuclear extracts in the absence of antibodies.

suggests that the species-specific carcinogens cause modification of HNF3, which does not occur during administration of the "foreign" hepatocarcinogen.

For evaluation of a possible direct effect of the hepatocarcinogens on HNF3 proteins, we added OAT and 3′-MeDAB to the nuclear extract during various steps of its preparation. Figure 5 shows that direct addition of the hepatocarcinogens to nuclear extracts did not influence DNA-binding activity of HNF3. Consequently, HNF3 protein does not represent a direct target for OAT and 3′-MeDAB in nuclei of mouse and rat liver cells. However, addition of these carcinogens to liver homogenate caused the same species-specific effect on HNF3 DNA-binding activity as during *in vivo* administration. This effect was observed when the carcinogens were added to intact and lysed nuclei, but it is lost during their addition to nuclear lysates subjected to ammonium sulfate fractionation. This suggests involvement of some nuclear factor into the effect of the carcinogens on the DNA-binding activity of HNF3. It is possible that this factor is removed or irreversibly inactivated during ammonium sulfate fractionation. The results of this study also provide convincing evidence that the effect of OAT and 3′-MeDAB on HNF3 proteins does not require their metabolic activation because the compounds are still able to influence HNF3 proteins in fractions and preparations which are free of microsomal enzymes (localized in the cytoplasm).

DISCUSSION

In spite of similar structure, OAT and 3′-MeDAB have different carcinogenic effects on mice and rats. Induction of tumors in rats requires administration of 3′-MeDAB for 90 days at the daily dose of ~6 mg, whereas administration of OAT at the dose 10 mg for 300 days induces tumors only in one quarter of animals [2]. In

Table 3. Effect of OAT and 3′-MeDAB on DNA-binding activity of the transcription factor HNF3 in the liver cells of A/He mice and Wistar rats

Species	HNF3 DNA-binding activity, % of control	
	OAT	3′-MeDAB
Mice	42.3 ± 3.6% (7)	96.8 ± 6.1% (3)
Rats	103 ± 3.3% (3)	58.8 ± 1.3% (4)

Note: Number of experiments is given in parentheses. In each experiment from two to three mice or one or two rats were used. Data represent results quantitatively treated after radioautograph densitometry (a typical autograph is shown at Fig. 2).

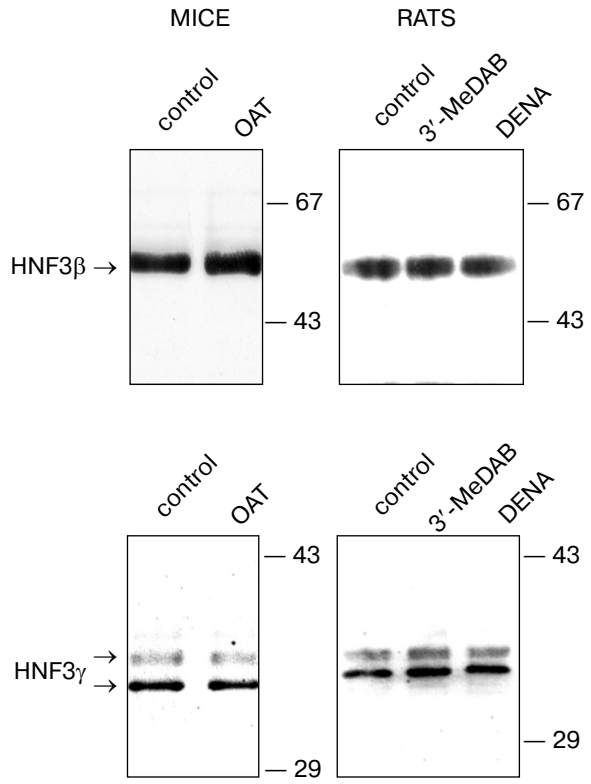

Fig. 4. Effect of hepatocarcinogens on the content of HNF3β and HNF3γ in hepatic nuclear extracts of A/He mice and Wistar rats.

mice OAT administration in the total dose 2000 mg/kg causes tumor induction in all animals, whereas 3′-MeDAB administration in the total dose 4786 mg/kg injected during the first 3 weeks of the postnatal development when mice are especially sensitive to induction of hepatic tumors caused tumors in some but not all animals [3]. Thus, even though the species-specificity of these carcinogens is not absolute, they clearly exhibit certain species preference.

The inhibitory effect of 3′-MeDAB on TAT induction by glucocorticoids was found during long-term feeding of rats with this carcinogen [11]. We demonstrated that this effect is not related to change in cell composition of the liver, which usually takes place during long-term feeding of experimental animals with the azo-dyes, and the effect of 3′-MeDAB appears during the first day of feeding (when the hepatocyte population remains virtually unchanged) [40]. We also found that OAT inhibits TAT induction in the mouse liver and there was a certain interrelationship between the magnitude of this inhibitory effect and susceptibility of animals to induction of hepatic tumors [4, 15]. So, we consider inhibition of glucocorticoid induction of TAT in the liver as an early manifestation of the effect of the hepatocarcinogens related to tumor induction [4, 15, 17].

Results of this study provide further experimental support for such a conclusion. They show that the carcinogens studied inhibit glucocorticoid induction of TAT only in those animals which are susceptible to tumor development provoked by these carcinogens (3′-MeDAB in rats and OAT in mice). The carcinogens did not influence glucocorticoid induction of TAT in resistant animals. Non-carcinogenic analog, 4′-MeDAB, did not influence glucocorticoid induction of TAT. Previously we demonstrated that DENA, which is carcinogenic for rat and mouse liver, inhibits glucocorticoid induction of TAT in both species [40], whereas 3′-MeDAB does not influence the induction of this enzyme in hamsters resistant to

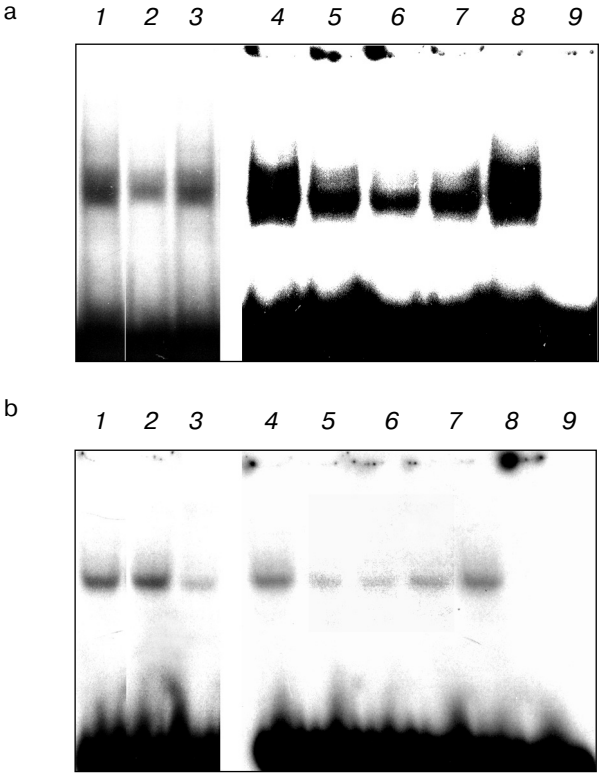

Fig. 5. Effect of 3′-MeDAB and OAT directly added to nuclear extracts from Wistar rat liver (a) and A/He mouse liver (b) at various stages of their isolation on HNF3 DNA-binding activity. *1-3*) Effect of the species-specific carcinogens added to homogenate: *1*) all solutions for extract isolation contain 1% DMSO; *2*) all solutions contain 1% of 3′-MeDAB solution in DMSO saturated at 0°C; *3*) all solutions contain 1% of OAT saturated in DMSO; *4-9*) effect of species-specific carcinogen added at various stages of extract isolation: *4*) all solutions for extract isolation contain 1% DMSO; *5*) carcinogen (3′-MeDAB for rats or OAT for mice) was added to homogenate and all subsequent stages; *6*) carcinogen was added to purified nuclei and all subsequent stages; *7*) carcinogen was added to nuclear lysate and all subsequent stages; *8*) carcinogen was added after ammonium sulfate fractionation of nuclear lysate; *9*) mobility of HNF3-binding labeled oligonucleotide (probe).

its hepatocarcinogenic effect [4]. Thus, the inhibitory effect of hepatocarcinogens on TAT induction by glucocorticoids is related to their tumorigenic action and, consequently, elucidation of the mechanism realizing this effect may help better understanding of the mechanisms of carcinogenesis.

Glucocorticoid receptor is one of the most evident targets of anti-glucocorticoid effect of any agent. However, our data suggest that neither 3′-MeDAB nor OAT influence the number of glucocorticoid receptors, their hormone-binding ability, and interaction with glucocorticoid response element (GRE) in both A/He mice and Wistar rats. Similar results were obtained previously during studies of the effect of OAT on glucocorticoid receptor in mice sensitive (SWR) or resistant (AKR) to its hepatocarcinogenic effect [41]. The other (indirect) evidence that the receptor is not the target for anti-glucocorticoid effect of hepatocarcinogens consists in the fact that carcinogens inhibit glucocorticoid induction of some enzymes (TAT, tryptophan dioxygenase, alanine aminotransferase) [11-15], but do not influence glucocorticoid induction of other enzymes (e.g., aspartate aminotransferase and phosphoenolpyruvate carboxykinase) in the same animals [42, 43].

Specificity of glucocorticoid control of various genes is achieved by the interaction of glucocorticoid receptors with other transcription factors which have binding sites near GREs, inside regulatory regions of these genes. In the case of TAT gene these factors include HNF3, C/EBP, and Ets [38, 44] and also a protein interacting with glucocorticoid modulating element (GME) [45]. In the previous studies [16, 17] we demonstrated that OAT inhibited DNA-binding activity of HNF3 only in mice sensitive to its hepatocarcinogenic effect. Studying effects of "self" and "foreign" hepatocarcinogens on DNA-binding activity of HNF3, we found that only a compound exhibiting marked carcinogenic activity for a given animal species was able to inhibit DNA-binding activity of HNF3 in the liver of animals of this species.

Thus, our data show that the inhibitory effect of hepatocarcinogens on the glucocorticoid induction of TAT can be attributed to reduction of activity of transcription factors of the HNF3 family. This is consistent with known data on the leading role of proteins of HNF3 in determination of magnitude of glucocorticoid induction of TAT [39]. Tumorigenic effect of hepatocarcinogens on the liver may also be attributed to their inhibitory action on HNF3, because members of this transcription factor family play the leading role in the formation and maintenance of phenotype of definitive hepatic cells and also in inhibition of hepatocyte proliferation [18, 46]. Involvement of members of the HNF3 family (expressed preferentially in the liver [19, 27] and to a lesser extent in some other organs) into the mechanism of hepatocarcinogen action may explain their organotropic effects. It is known that besides hepatic tumor OAT may also induce tumors in lungs, intestine, and vessels [1, 47], i.e., in organs expressing transcription factors of the HNF3 family [19]. Skeletal muscles and kidneys do not express HNF3 factor(s) [1] and are insensitive to the tumorigenic effect of OAT [19].

In the present study we demonstrated that inhibition of HNF3 DNA-binding activity by the hepatocarcinogens occurs via some changes in the state of hepatic nuclear proteins rather than reduction of their content. DNA-binding activity of various transcription factors is known to be modified by their covalent modification (phosphorylation/dephosphorylation, acetylation, etc.) or by noncovalent binding with low molecular weight ligands or during interaction with other regulatory proteins [48-51]. The mechanism by which hepatocarcinogens influence HNF3 DNA-binding activity remains unclear. Results of experiments on direct addition of OAT and 3′-MeDAB to liver homogenate, or to intact or lysed cell nuclei indicate that this transcription factor is not the direct target for the carcinogen effect. Perhaps, the effect of the hepatocarcinogens on HNF3 DNA-binding activity involves some nuclear protein. Identification of this protein in mice and rats may be very promising both for elucidation of the mechanism of the hepatocarcinogen effect on HNF3 DNA-binding activity and of the reasons underlying the species-specific effect of OAT and 3′-MeDAB.

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