ENZYMATIC ACTIVATION OF THE CARCINOGEN 4-HYDROXYAMINOQUINOLINE-1-OXIDE AND ITS INTERACTION WITH CELLULAR MACROMOLECULES

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SUMMARY: The carcinogen 4-hydroxyaminoquinoline-1-oxide (4HAQO) reacted with nucleic acid or protein following enzymatic activation. The enzyme activity was found in the cytosol fraction of rat ascites cells. Absolute requirement of ATP for the reaction suggests that a phosphotransferase catalyzes the formation of a phosphate ester of 4HAQO which then reacts spontaneously with the nucleophiles. The nucleic acid adducts formed in the cell free system were almost identical with those formed *in vivo*.

Binding of the residues of chemical carcinogens with nucleic acid or/and protein may be the primary event leading to carcinogenesis. The carcinogenic and mutagenic compounds, 4-hydroxyaminoquinoline-1-oxide (4HAQO) and its metabolic precursor, 4-nitroquinoline-1-oxide (4NQO) bind to nucleic acid and protein *in vivo*[1-6], however, these carcinogens are also able to interact with nucleic acid *in vitro*[7,8], to a lesser extent forming complexes which differ from the *in vivo* products[1,9]. These results suggest that *in vivo* interaction of 4HAQO with cellular macromolecules requires some metabolic activation of this carcinogen.

In this communication we report a cell free system which activates 4HAQO to react with nucleic acid or protein.

MATERIALS AND METHODS

Generally labeled 4HAQO-^{3}H (28 mCi/mmole) were generously provided by Dr. Y. Kawazoe, National Cancer Center Research Institute[10]. 4NQO-5,6,7,8,9,10-^{14}C (7.76 mCi/mmole) was purchased from Daiichi Pure Chemicals Co., Tokyo. CoA was the product of Boehringer Mannheim GmbH, Mannheim and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) was a gift from Takeda Chemical Industries, Osaka.

Ascites hepatoma cells (AH 130) of rat, 7 days after transplantation, were used as the source of the enzyme and nucleic acid[3].

The binding of the carcinogen to nucleic acid was measured by the amount of radioactivity of 4HAQO-^{3}H converted into an acid insoluble form. The routine assay mixture (0.1 ml) contained the following: 0.04 μmole of 4HAQO-^{3}H (28 mCi/mmole), 0.1 μmole of ATP, 1.0 μmole of phosphoenolpyruvate (tricyclohexylammonium salt, Boehringer), 4.0 μg of pyruvate kinase (glycerol solution, Boehringer), 0.5 μmole of $MgCl_2$, 1.0 mg of RNA from rat ascites cells, 0.1 μmole of dithiothreitol (DTT), 50 μg of sodium dextran sulfate 500 (Pharmacia), 50 μmoles of Tris-HCl buffer (pH 7.8) and enzyme. Incubation was carried out at 37°C in air for 60 min and was terminated by adding 1.2 ml of 0.1 M Tris-HCl buffer (pH 9.0) containing 0.5 % sodium dodecyl sulfate (SDS), 1 mM EDTA and 0.01 % Antiform AF (Dow Corning). The mixture was then added to an equal volume of phenol saturated with the same buffer and was mixed by bubbling of N_2 gas for 30 min. After centrifugation, nucleic acid was recovered from 1.0 ml aliquots of the aqueous layer by ethanol precipitation and was washed with cold 5 % trichloroacetic acid, ethanol containing 0.5 % CH_3COOK and ethanol successively by centrifugation. To assay the binding

Table I. Distribution of the Binding Activity in Subcellular Fractions of Rat Ascites Cells

Fraction	nmoles/ g cells	percentage distribution	nmoles/ mg protein
Homogenate	134	—	0.58
750×g sediment	0.9	0.7	0.01
Homogenate of 750×g sediment	0.9	0.7	0.01
10,000×g sediment	0.2	0.1	0.01
105,000×g sediment	7.6	5.4	0.05
105,000×g supernatant	130	93.1	1.98

Ascites cells were homogenized by a Emanuel-Chaikoff homogenizer[11] and subcellular fractions were obtained by differential centrifugation. For homogenization of 750×g sediment, a Branson Sonifier was used.

The binding activity was measured using 1.0 μmole of DTT and 1 mg of RNA. The reaction was terminated by adding 1.7 ml of SDS solution and the mixture was clarified by incubating at 37°C for 30 min prior to adding 1.7 ml of phenol solution. Other conditions for assay were as described in Materials and Methods. Fractions heated in boiling water for 10 min were used as controls. Protein was determined by the biuret reaction[12].

to protein, sodium dextran sulfate was omitted from the assay mixture, and acceptor RNA was replaced by 1 mg of bovine serum albumin. After the incubation protein was precipitated by adding cold 5 % trichloroacetic acid and was washed as above. The radioactivity of the washed pellet was measured by a liquid scintillation counter.

RESULTS

Characteristics of the Reaction

As shown in Table I, the binding activity was found in the 105,000×g supernatant fraction.

Requirements for the reaction catalyzed by the cytosol enzyme are summarized in Table II, and it is evident that ATP and Mg^{++} ions are essential requirements for the reaction. DTT

Table II. Requirements for the Binding Reaction

Components	Incorporation of 4HAQO-^{3}H (nmoles)
Binding to Nucleic Acid	
Complete	3.58
minus ATP and the ATP-regenerating system	0.04
minus Mg^{++}	0.16
minus DTT	2.72
minus dextran sulfate	2.98
minus RNA	0.12
RNA replaced by DNA	1.92
boiled enzyme	0.04
ATP and the ATP-regenerating system replaced by PAPS	0.05
plus SO_4^{--}	3.18
plus CoA and CH_3COO^-	2.16
plus CH_3COO^-	3.45
Binding to Protein	
Complete	1.51
minus ATP and the ATP-regenerating system	0.22
minus Mg^{++}	0.46
minus bovine serum albumin	0.93

Ascites cells, washed with 0.25 M sucrose containing 0.02 M Tris-HCl buffer (pH 7.5) and 1 mM $MgCl_2$, were homogenized with an equal volume of the sucrose solution by a VirTis 45 homogenizer and the cytosol fraction was obtained by centrifugation at 105,000×g for 1 hr. For determination of the reaction requirements the cytosol fraction was dialyzed against 0.25 M sucrose containing 0.02 M Tris-HCl (pH 7.5) overnight. The complete system and assay procedures were as described in Materials and Methods using 40 µl of the dialyzed cytosol enzyme (1.0 mg of protein) and 1.0 mg of RNA or bovine serum albumin. Where indicated, 0.1 µmole of PAPS, 0.5 µmole of Na_2SO_4, 0.1 µmole of CoA and 1.0 µmole of CH_3COOK were added.

prevented the oxidation of 4HAQO and contributed substantially to the full rate of the binding reaction. Dextran sulfate effectively protected acceptor RNA from degradation by nuclease. Factors responsible for sulfation or acetylation, such as PAPS, SO_4^{--}, CoA and CH_3COO^- did not stimulate the reaction. DNA and

bovine serum albumin were also able to accept the carcinogen.

Identification of the Adducts Formed in the Cell Free System

As reported previously[3], when the nucleic acid adducts isolated from 4NQO-exposed cells were hydrolyzed by acid or alkali, the degradation products showed characteristic patterns

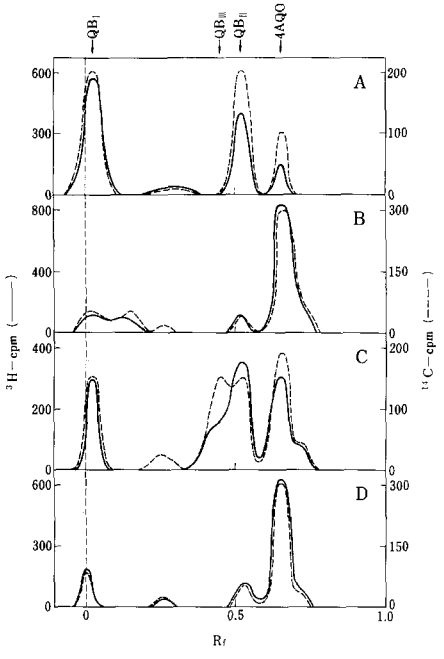

Fig. 1. Analyses of the carcinogen-nucleic acid adducts. The nucleic acid adducts were prepared using the cytosol enzyme according to the binding assay procedures described in Materials and Methods and were hydrolyzed either by acid or alkali together with the adducts isolated from ^{14}C-4NQO-exposed cells[3]. The hydrolysates were chromatographed with 1 mg of unlabeled RNA adduct-acid hydrolysate using the solvent system ethanol-water (4 : 1, v/v) on Whatman No.1 filter paper. Radioactivity was measured as reported previously[3]. 0.15 mg of enzymatically formed RNA adduct and 0.40 mg of *in vivo* formed RNA adduct were treated with (A) 50 µl of 1 N HCl or (B) 30 µl of 1.0 N KOH at 100°C for 1 hr in N_2 atomosphere. 0.24 mg of enzymatically formed DNA adduct and 0.40 mg of *in vivo* formed DNA adduct were treated with (C) HCl or (D) KOH as above. Full line: hydrolysates of ^{3}H-labeled adducts prepared in the cell free system; broken line: hydrolysates of ^{14}C-labeled adduct formed *in vivo*.

on paper chromatography. Upon acid degradation the RNA adduct yields three products, QB_I, QB_{II} and 4-aminoquinoline-1-oxide (4AQO), while the DNA adduct yields the same three products and an additional one, QB_{III} (Fig. 1A and C, broken line). It has been reported that the products designated as QB are apparently quinoline derivatives linked with purine bases[3]. By the treatment with alkali, a considerable amount of 4HAQO residues bound to nucleic acids are split off as 4AQO (Fig. 1B and D, broken line).

To compare the identity of the adducts formed by the cytosol enzyme *in vitro* with those formed *in vivo*, their hydrolysates were examined by paper chromatography. The RNA adduct formed in the cell free system containing 4HAQO-^{3}H showed essentially the same pattern to that of the RNA adducts isolated from 4NQO-^{14}C-exposed cells (Fig. 1A and B). The DNA adduct formed in the cell free system either lacked or contained only small amounts of the component QB_{III}, which was found in the acid hydrolysates of the DNA adduct formed *in vivo* (Fig. 1C); otherwise both DNA adducts showed the same degradation pattern (Fig. 1C and D).

DISCUSSION

The binding mechanism of 4HAQO with nucleic acid or protein catalyzed by the cytosol enzyme is not clear. However, absolute requirement of ATP for the reaction suggests that a phosphotransferase catalyzes the formation of a phosphate ester of 4HAQO which then reacts spontaneously with nucleic acid or protein. Recent studies have demonstrated that the carcinogen N-hydroxy-2-acetylaminofluorene (N-hydroxy-AAF) reacts with nucleic acid following enzymatic phosphorylation or sulfation in the presence of ATP or PAPS (or SO_4^{--} and ATP)[13-15]. N-hydroxy-

AAF is also acetylated by acetyl-CoA non-enzymatically[16]. Since PAPS, SO_4^{--}, CoA or CH_3COO^- do not stimulate the binding reaction of 4HAQO, esterification by sulfate or acetate may not participate in activation of 4HAQO in the system reported here.

Analysis of the adducts revealed that the cell free system was able to produce nucleic acid adducts which were almost identical with those formed in vivo. Although one can not exclude the possibility of the existence of a nuclear enzyme which is responsible for modification of DNA adducts, the cytosol enzyme may participate in in vivo binding of 4HAQO with nucleic acids, and subsequently play an important role in the initiation of 4HAQO carcinogenesis.

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