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EVIDENCE FOR THE INVOLVEMENT OF CYTOCHROME P-450-DEPENDENT MONOOXYGENASE(S) IN THE FORMATION OF GENOTOXIC METABOLITES FROM N-HYDROXYUREA

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Hydroxyurea induces DNA repair replication in the cytochrome P-450-containing C2Rev7 rat hepatoma cell line. Repair is severalfold increased by pretreatment of the cells with dexamethasone, which induces cytochrome P-450-dependent monooxygenase activities in these cells. In the dedifferentiated hepatoma line H5, which strongly expresses cytochrome P-448 but no cytochrome P-450-dependent enzyme activities, hydroxyurea is not genotoxic. The results support the notion that the formation of genotoxic metabolites from hydroxyurea is mediated by a cytochrome P-450-dependent enzyme.

We have recently shown that HU elicits DNA repair replication in human lymphoblastoid cells during incubation with a metabolic activation system consisting of liver microsomes from rodents and NADPH (1). Microsome-mediated genotoxicity of HU was enhanced by pretreatment of the animals with chemicals known to induce monooxygenase activities and was inhibited by SKF 525-A, suggesting the involvement of microsomal monooxygenase(s) in the metabolic activation of HU (1). The type of monooxygenase responsible for the production of genotoxic metabolites from HU remained unclear, however. Hepatic microsomes contain various monooxygenases which may be divided into two groups, the "cytochrome(s) P-448" and the "cytochrome(s)

Abbreviations:

BrdUrd, 5-bromodeoxyuridine; FdUrd, 5-fluorodeoxyuridine; DMN, dimethylnitrosamine; dCyd, deoxycytidine; FCS, fetal calf serum; HU, hydroxyurea; MMS, methyl methanesulfonate; PBS, phosphate buffered saline; SKF 525-A, 2-dimethylaminoethyl-2,2-diphenylvalerate; TCA, trichloroacetic acid.

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P-450". The former are inducible by polycyclic aromatic hydrocarbons and inhibited by e.g. 7,8-benzoflavone. The latter forms, which predominate in the liver, are not inducible by polycyclic aromatic hydrocarbons and not inhibited by 7,8-benzoflavone (2). Metabolism of HU by a monooxygenase of the former group appears unlikely, since these enzymes preferentially metabolize highly lipophilic, planar compounds. HU may be rather metabolized by a monooxygenase of the second group, which also accept hydrophilic, non-planar compounds as their substrates. Since hepatic microsomes contain both monooxygenase forms, an unequivocal assignment of the enzyme metabolizing HU to any of these forms was not possible.

In order to overcome this problem, we tested the ability of HU to induce DNA repair in the hepatoma cell lines C2Rev7 (3) and H5 (4). C2Rev7 cells are distinguished by the expression of cytochrome P-450-dependent enzyme activities (5), such as aldrin epoxidase and 7,8-benzoflavone-stimulated aryl hydrocarbon hydroxylase (6). Pretreatment of the cells with the synthetic glucocorticoid dexamethasone increases these cytochrome P-450-activities four- to eightfold (5). In contrast, cytochrome P-448-dependent monooxygenases are not detectable (5). H5 cells, on the other hand, selectively express P-448-dependent enzymes, like benz(a)anthracene-inducible aryl hydrocarbon hydroxylase, but no enzyme activities attributed to cytochrome P-450 (6). Thus, these hepatoma lines provide a unique test system in which the roles of the two monooxygenase forms in the metabolic activation of xenobiotics can be specifically determined. Since there is no method available to directly monitor the metabolism of HU in cell cultures, we investigated whether C2Rev7 or H5 cells form genotoxic metabolites from HU by measuring the induction of DNA repair synthesis in these cells.

METHODS

Cell culture The hepatoma clones C2Rev7 and H5 are derived from the Reuber H35 rat hepatoma clone H4IIEC3 (7) by Deschatrette et al. (3,4) and were kindly provided by Dr. M. C. Weiss (CNRS, Gif-sur-Yvette, France). The cells were grown in 100 mm Falcon-dishes at 37°C in an atmosphere of 95% air/5% CO_2. The growth medium consisted of equal volumes of modified Ham's F-12 and NCTC 135, 5% fetal calf serum, 100 units penicillin/ml, and 50 µg streptomycin/ml. For induction of monooxygenases, C2Rev7 cells were treated for 32 h with 2 µg dexamethasone/ml and H5 cells for 24 h with 5 µg benz(a)anthracene/ml prior to each repair experiment.

Repair synthesis Cells were washed twice with prewarmed PBS and preincubated for 1 h in 4 ml William's medium E containing 10% FCS, 40 µM FdUrd, and 200 µM BrdUrd. After preincubation the medium was changed and the incubation continued for 18 h in the presence of FdUrd and BrdUrd (40 and 200 µM), the test compound, and 10 µCi [^{3}H]dCyd/ml (19-22 Ci/mmol). HU and DMN were dissolved in medium, MMS in dimethyl sulfoxide. For irradiation, cultures were rinsed, drained and irradiated with UV light (254 nm, 1 J x m^{-2} x sec^{-1}). Cell cultures pretreated with dexamethasone (C2Rev7) or benz(a)anthracene (H5) were continuously exposed to the inducer during preincubation and the subsequent labeling period. The cells were then washed with PBS and lysed in 3 ml lysis buffer (10 mM Tris, 10 mM NaCl, 10 mM EDTA, pH 7.9) containing 1% sodium dodecylsarkosine. Lysates were digested with proteinase K (50 µg/ml, 2 h at 50°C) and the DNA was precipitated by addition of 2.5 volumes ethanol containing 0.1 M potassium acetate. The precipitated DNA was washed with ethanol, dried and dissolved in 5.1 ml of $CsCl/Cs_2SO_4$ (4.8:1) in 0.1 N NaOH, refractive index 1.4000-1.4004, and mixed at high speed for 1 min. 5.0 ml samples were centrifuged in a Beckman VTi65 rotor at 48,000 rpm for at least 16 h (20°C). Following centrifugation, the lower half of each gradient, containing heavy DNA strands, was removed by means of a syringe. The remaining part, containing parental DNA strands, was brought to 5.0 ml with $CsCl/Cs_2SO_4$ solution and centrifuged as before. This rebanding step was repeated once to completely remove the large amounts of radioactivity incorporated into replicating DNA during the labeling period. The gradients were then fractionated from the top using a commercial density gradient fractionation system (Auto Densi-Flow IIC, Buchler, USA). About 24 equal-sized fractions were collected from each gradient. After diluting each fraction with 0.6 ml water, 0.25 ml were removed and mixed with 20 µg of calf thymus DNA in water. The DNA was precipitated with 2.5 ml 10% TCA and, after standing in ice water for about 30 min, filtered onto glass fibre filters by means of a Titertek multiple cell harvester (Flow Laboratories, Irvine, UK). The filters were extensively washed with 5% sodium pyrophosphate in 6% TCA, dried and counted with 2 ml toluene-based scintillator in a LKB 1216 Rackbeta liquid scintillation counter. The remaining part of each fraction was used to read the UV-absorbance at 260 nm. Repair replication was estimated by calculating the radioactivity incorporated into DNA of normal density. After converting the UV-absorbance units into µg DNA results were expressed as cpm/µg DNA.

MATERIALS

BrdUrd, FdUrd, dexamethasone, and calf thymus DNA were purchased from Sigma, München (FRG); HU and proteinase K from Boehringer, Mannheim (FRG); cell culture media from Flow, Meckenheim (FRG); FCS from Gibco, Karlsruhe (FRG); DMN and MMS from Merck-Schuchardt, Hohenbrunn (FRG); [^{3}H]dCyd from Amersham, Braunschweig (FRG); sodium dodecylsarkosine and CsCl (analytical grade) from Serva, Heidelberg (FRG); Cs_2SO_4 (suprapur) from Merck, Darmstadt (FRG).

RESULTS AND DISCUSSION

When C2Rev7 cells were exposed to HU for 18 h and labeled with [^{3}H]dCyd in the presence of FdUrd and BrdUrd, a concentration dependent increase in the specific radioactivity of parental DNA representing repair synthesis was observed (Fig. 1). This result demonstrates that the cells possess the capacity to metabolize HU to genotoxic products. DMN, which served as positive control since it is mainly metabolized by cytochrome P-450-dependent monooxygenase(s) (8), also caused repair (Fig. 1). Pretreatment of the cells with 2 µg dexamethasone/ml enhanced the induction of repair by both HU and DMN severalfold (Fig. 1). In contrast, neither HU (10-25 mM) nor DMN (20-50 mM) induced detectable DNA repair in H5 cells, even after pretreatment with benz(a)anthracene (data not shown).

Fig. 2 shows typical gradient profiles from which the results presented in Fig. 1 were derived. DNA synthesized semiconservatively during the incubation time was completely removed by the

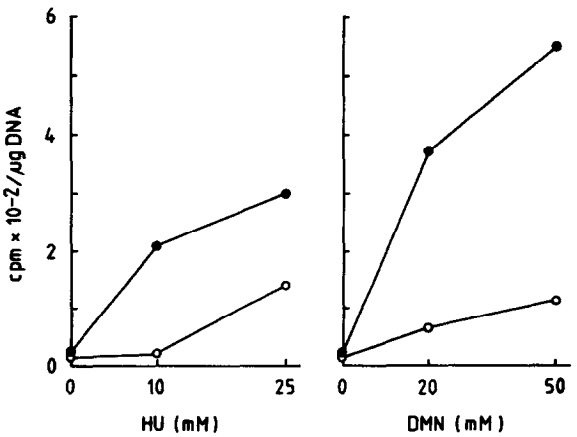

Fig. 1 Repair replication in C2Rev7 hepatoma cells treated with HU or DMN. Cells were grown in the absence (o–o) or presence (•–•) of 2 µg dexamethasone/ml for 32 h. After preincubation with FdUrd and BrdUrd the medium was changed and the cells were incubated for 18 h with HU or DMN, 10 µCi [^{3}H]dCyd/ml (22 Ci/mmol), FdUrd and BrdUrd in the absence (o–o) or presence (•–•) of 2 µg dexamethasone/ml. Following centrifugation of the isolated DNA in CsCl/Cs_2SO_4 gradients, repair replication was determined as described in "Methods". Data represent one typical experiment out of three.

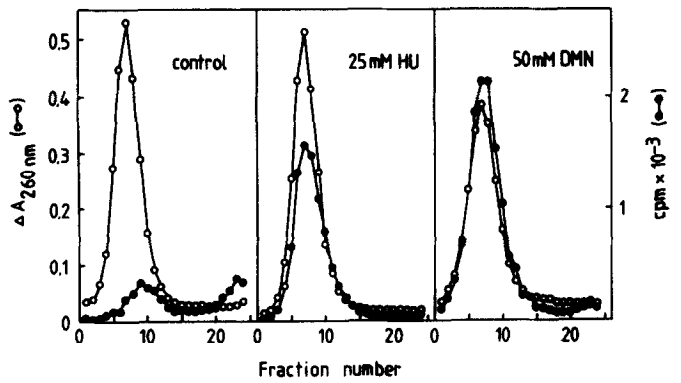

Fig. 2 $CsCl/Cs_2SO_4$ gradient profiles of DNA from C2Rev7 hepatoma cells treated with dexamethasone and HU or DMN as described in Fig. 1. Density increases from left to right. In each case the second reband is shown.

rebanding steps and did not appear as radioactivity peak at higher density in the profiles. In the control gradients of DNA from cells not treated with a genotoxic agent, the incorporated radioactivity was limited to a small peak with a density slightly higher than the density of parental DNA. The nature of this peak which is occasionally observed in CsCl gradients is not known (9). Repair induction by HU or DMN was indicated by the large amounts of radioactivity banding exactly with the UV-absorbance peak (parental DNA).

The apparent increase in HU- and DMN-induced DNA repair by pretreatment of the C2Rev7 cells with dexamethasone could be the consequence of a) enhanced metabolic activation of these compounds, b) an increase in the specific radioactivity of the dCyd-nucleotide pool(s), or c) an unspecific increase in the cellular capacity for DNA repair. However, the results shown in Table 1 indicate that b) and c) are very unlikely, since repair synthesis elicited by UV light or the directly methylating agent MMS increased not more than 25% after pretreatment of the cells with dexamethasone. The assumption that HU- and DMN-induced repair replication in the C2Rev7 cells is due to the metabolism of these compounds by a cytochrome P-450-dependent monooxygenase

Table 1 Effect of dexamethasone on repair replication induced by UV light or MMS in C2Rev7 cells

Treatment	Repair synthesis (cpm/μg DNA)	
	- dexamethasone	+ dexamethasone
UV (20 J/m^2)	2201	2744
MMS (1 mM)	5990	6809

Following pretreatment of the cells for 32 h with 2 μg dexamethasone/ml or solvent (0.2% methanol) and after UV irradiation or addition of MMS cells were incubated for 18 h with FdUrd, BrdUrd and 10 μCi [^{3}H]dCyd/ml (22 Ci/mmol) in the absence or presence of dexamethasone.

is further supported by the observation that HU, like DMN, also causes DNA repair in freshly isolated rat hepatocytes and another cytochrome P-450-containing hepatoma cell line (5), 2SFou (unpublished results). The lack of genotoxicity of HU in the dedifferentiated hepatoma clone H5 is in line with other reports demonstrating that in various cells of extrahepatic origin, such as HeLa, Syrian hamster embryo, 3T3 and A549, most of which are known to express only cytochrome P-448 (5,10,11), HU does not induce DNA repair (12,13, and unpublished results).

In conclusion, the present results suggest that HU is activated to genotoxic metabolites by cytochrome P-450-dependent monooxygenase(s). They further demonstrate the value of continuous cell lines expressing distinct monooxygenase species in elucidating the mechanisms by which genotoxic chemicals are metabolized in intact cells (5,14).

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