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INDUCTION OF DNA REPAIR REPLICATION BY HYDROXYUREA IN HUMAN LYMPHOBLASTOID CELLS MEDIATED BY LIVER MICROSOMES AND NADPH

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SUMMARY

Hydroxyurea induces repair replication in human lymphoblastoid NC37 BaEV cells during incubation with liver microsomes and NADPH. Catalase reduces hydroxyurea-induced repair by more than 95%, suggesting that hydrogen peroxide derived from hydroxyurea in the presence of the metabolic activation system is involved in the DNA damage.

HU is widely used to suppress semiconservative DNA synthesis in procedures determining repair replication (1-3). This is based on the assumption that HU does not react with DNA in intact cells but exerts its effects on DNA synthesis and DNA structure by inhibiting ribonucleotide reductase and depleting endogenous nucleotide pools (4,5). In the course of measuring the induction of DNA repair replication by mutagens that require metabolism to become reactive (6), we noticed that in the presence of microsomes and NADPH HU interfered with the determination of mutagen -induced repair. Therefore, in the present study we re-evaluated the effects of HU in a test system for DNA repair replication that included drug metabolizing enzyme activity.

Abbreviations:

HU, hydroxyurea; G6P, glucose-6-phosphate; G6P-DH, glucose-6-phosphate dehydrogenase; FUdR, 5-fluorodeoxyuridine; BUdR, 5-bromodeoxyuridine; TdR, thymidine; PBS, phosphate buffered saline; TCA, trichloroacetic acid; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; SKF 525-A, 2-dimethylaminoethyl-2,2-diphenyl valerate.

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METHODS

Cell culture Human lymphoblastoid NC37 BaEV cells (NC37 cells infected with Baboon endogenous virus) were routinely grown as suspension cultures in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), and tylosin (50 µg /ml). 4-5 days prior to each experiment cells were transferred to medium without antibiotics. Immediately before the experiments cells were washed with medium lacking serum and glutathione and adjusted to a cell concentration of about 3×10^6/ml.

Metabolic activation system Liver microsomes from male 11 weeks old NMRI mice were prepared in 0.1 M phosphate buffer pH 7.4 after treating the animals with phenobarbital (0.1% in drinking water for 11 days), Clophen A50 (500 mg/kg body weight per os 5 days before sacrifice), or 3-methylcholanthrene (40 mg /kg body weight per os 2 days before sacrifice). The NADPH-regenerating system consisted of 1 mM NADP, 5 mM G6P, 2 mM $MgCl_2$ and 1.4 U/ml G6P-DH (final concentrations). NADP, G6P and $MgCl_2$ were dissolved in RPMI 1640 medium titrated to pH 10.4 to achieve a pH of 7.4.

Repair replication $5\text{-}10 \times 10^6$ cells were preincubated with 1 µM FUdR and 10 µM BUdR in RMPI 1640 medium lacking glutathione for 1 h. Subsequently, HU, microsomes, the NADPH-regenerating system and 10 µCi[^{3}H]TdR/ml (spec.act. ~ 40 Ci/mmol) were added. After 3 h the reaction was stopped by washing the cells in ice-cold PBS. Then cells were lysed in 0.5% SDS and digested with proteinase K according to (7). The digested lysates were added to 7.72 g of solid CsCl and brought to a refractive index of 1.4060 - 1.4065 by addition of 0.7 ml of 1 M and 1.7 ml of 0.1 M K_2HPO_4, pH 12.5. Following centrifugation of the solution in a Spinco 50 Ti rotor at 37,000 rpm for at least 40 h, about 45 fractions were collected from each gradient. 10 µg calf thymus DNA in 1.5 ml of 0.1 M K_2HPO_4 were added to fractions 1-10 and to the following even numbered fractions. The DNA was precipitated with 2.5 ml of 10% TCA and, after standing in ice water for at least 30 min, filtered onto Whatman GF/C glass fiber filters. The filters were washed extensively with 5% TCA and 6% pyrophosphate in 5% TCA, dried and counted with 5 ml toluene/PPO/POPOP scintillator in a Packard Tri-Carb spectrometer. Fraction 11 and the following odd numbered fractions were diluted with 0.7 ml of water and the absorbance was read at 260 nm. Repair replication was estimated by calculating the radioactivity incorporated into DNA of normal density and dividing it by the absorbance of the DNA peak. Results were expressed as cpm/absorbance unit (cpm/A, arbitrary units).

MATERIALS

FUdR, BUdR, calf thymus DNA and superoxide dismutase were purchased from Sigma; HU, glutathione, G6P, G6P-DH, catalase and proteinase K from Boehringer; RMPI 1640 and antibiotics from Seromed; fetal calf serum from Gibco; SKF 525-A from Smith, Kline and French; Clophen A50 from Bayer; 3-methylcholanthrene from Fluka; [^{3}H]TdR was from Amersham and CsCl from Serva.

RESULTS

When NC37 BaEV cells were incubated with 1 µM FUdR, 10 µM BUdR, 10 µCi[^{3}H]TdR/ml and 2.5 mM HU, only minute amounts of

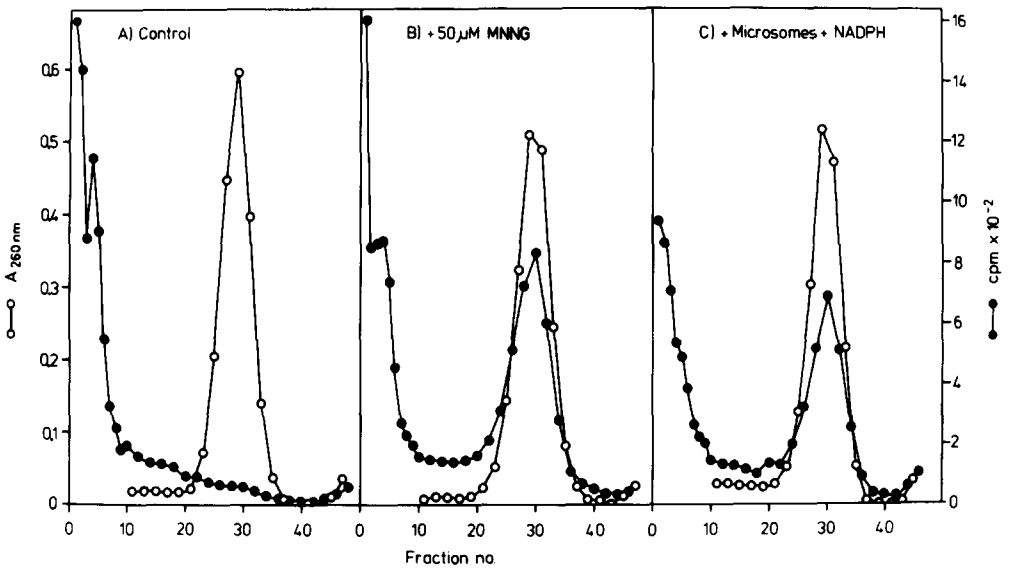
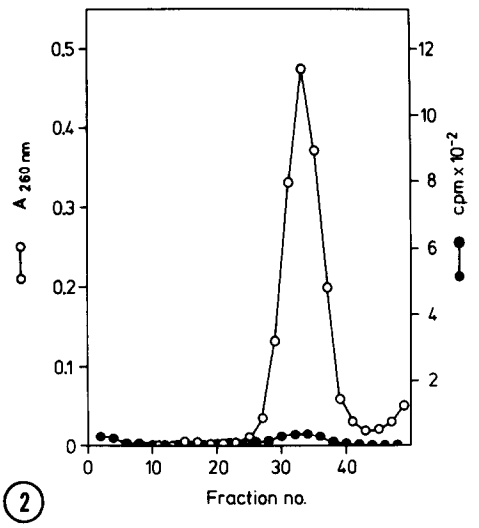


Fig. 1 Repair replication in NC37 BaEV cells. Cultures were incubated in the presence of 1 µM FUdR and 10 µM BUdR for 1 h. After addition of 2.5 mM HU (Fig. 1A-C), cells were exposed to medium (1A), 50 µM MNNG (1B), or phenobarbital-induced mouse liver microsomes (0.5 mg protein/ml) and a NADPH-regenerating system (1C) and labeled for 4.5 h with 10 µCi [^{3}H] TdR (40 Ci/mmol); cells were lysed, digested with proteinase K and sedimented in alkaline CsCl-gradients; the location of [^{3}H]-label at normal density (coincident with the absorbance peak) is indicative for repair replication.

radioactivity were taken up into DNA of normal density (Fig. 1A). Addition of liver microsomes from phenobarbital-treated NMRI mice and of a NADPH-regenerating system led to a marked incorporation of repair label (Fig. 1C). Obviously, the presence of the metabolic activation system gave rise to DNA damage with subsequent excision repair. Fig. 1B shows that the direct acting mutagen N-methyl-N'-nitro-N-nitrosoguanidine was similarly effective in inducing repair replication.

To elucidate whether or not the microsome-mediated repair may be due to the presence of HU, cells were incubated with FUdR, BUdR, [^{3}H]TdR and the microsomal activation system, and HU was omitted. Assessing repair replication without HU required



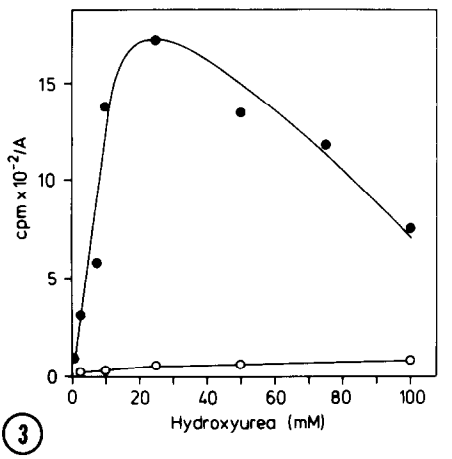


Fig. 2 Repair replication in NC37 BaEV cells determined in the presence of the metabolic activation system without HU. After preincubation with FUdR and BUdR cells were labeled for 4.5 h with 10 µCi [^{3}H] TdR (40 Ci/mmol) in the presence of phenobarbital-induced mouse liver microsomes (0.5 mg protein/ml) and a NADPH-regenerating system; cells were lysed, digested with proteinase K and sedimented in alkaline CsCl; fractions containing parental DNA were pooled and rebanded three times.

Fig. 3 Repair replication in NC37 BaEV cells induced by HU. After preincubation with FUdR and BUdR cultures were treated with HU in the presence (●-●) and absence (o-o) of phenobarbital-induced mouse liver microsomes (0.5 mg protein/ml) and labeled with 10 µCi [^{3}H] TdR/ml (40 Ci/mmol) for 3 h. After centrifugation in alkaline CsCl repair replication was estimated by measuring the amount of radioactivity incorporated into DNA of normal density and expressed as cpm/absorbance unit (cpm/A, arbitrary units).

three rebandings of the gradient fractions containing parental DNA in order to separate the large amounts of density labeled, semiconservatively synthesized DNA from parental (and possibly repair labeled) DNA. As shown in Fig. 2, very little radioactivity was incorporated in parental DNA under these conditions clearly indicating that HU had caused the repair observed when both the metabolic activation system and HU were present. The concentration dependence of repair induction by HU is shown in Fig. 3. An initial increase of repair up to 25 mM was followed by a subsequent decrease. When the activation system was omitted

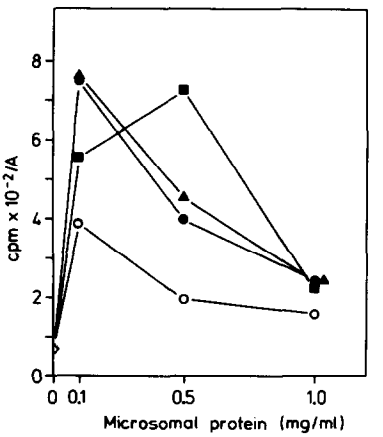


Fig. 4 The dependence of HU-induced repair replication on the amount of microsomal protein from differently treated male NMRI mice. After preincubation with FUdR and BUdR cultures were labeled with 10 µCi[^{3}H]TdR (40 Ci/mmol) for 3 h in the presence of 2.5 mM HU, a NADPH-regenerating system and microsomes from untreated (o-o), phenobarbital- (●-●), Clophen A50- (▲-▲), or 3-methylcholanthrene-treated (■-■) animals; induction of mice and determination of repair replication was as described in "methods".

a very low but consistent level of repair was detectable which increased threefold with increasing HU concentrations in the range of 2.5 - 100 mM, suggesting that a microsome-independent repair was due to the action of HU as well.

HU-induced repair occured also in the presence of hepatic microsomes from untreated mice and mice treated with Clophen A50 or 3-methylcholanthrene (Fig. 4), the microsomes from induced animals being more effective in mediating repair than those from uninduced ones. Repair induction was dependent on the presence of both microsomes and NADPH, and was reduced to 50% by SKF 525-A, an inhibitor of cytochrome P-450 (8). This adds further evidence to the notion that repair induction by HU is the consequence of microsomal monooxygenase-mediated metabolism (Table 1).

HU, like other compounds containing a free -NHOH group, causes the formation of hydrogen peroxide on exposure to oxygen (9). H_2O_2 has been shown to bring about many kinds of DNA lesions

Table 1

Repair replication in NC37 BaEV cells in the presence of 2.5 mM HU.

Additions	Repair replication (%)
None	4
microsomes[1]	2
NADPH[2]	9
microsomes[1] + NADPH[2]	100
microsomes[1] + NADPH[2] + SKF 525-A (0.1 mM)	47
microsomes[1] + NADPH[2] + glutathione (2 mM)	35
microsomes[1] + NADPH[2] + superoxide dismutase (10 µg/ml)	29

After preincubation with FUdR and BUdR for 1 h cells were incubated in the presence of 2.5 mM HU and the mentioned components; cells were labeled for 3 h with 10 µCi[^{3}H]TdR/ml (40 - 42 Ci/mmol), lysed, digested with proteinase K and sedimented in alkaline CsCl; repair replication was estimated as described in "methods"; each value represents the mean of at least two experiments.

[1] phenobarbital-induced mouse liver microsomes (0.5 mg protein/ml)

[2] added as NADPH-regenerating system

(10-12) and to induce chromosomal aberrations and unscheduled DNA synthesis in human fibroblasts (13). Furthermore, H_2O_2 liberated from HU inactivated transforming DNA of B.subtilis (9). Therefore, we investigated whether H_2O_2 derived from HU in the presence of microsomes and NADPH might be involved in the HU-induced DNA damage. Our results indicate this to be the case (Fig. 5): The presence of 100 U catalase/ml during the incubation period nearly eliminated HU-induced repair replication. This inhibition was not due to cytotoxic effects of catalase, as the suppression of repair was accompanied by a slight increase of the residual (HU-resistant) DNA synthesis. In contrast, the small amount of repair replication observed in the absence of the activation

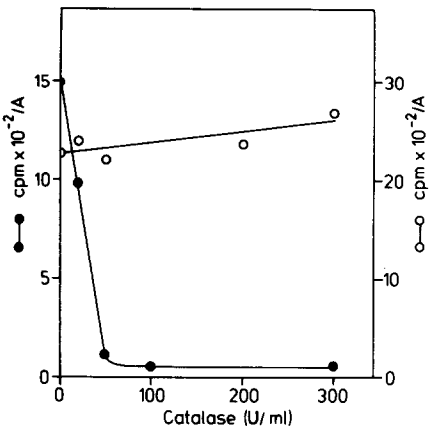


Fig. 5 Repair replication (●-●) and HU-resistant semiconservative DNA synthesis (o-o) in NC37 BaEV cells in the presence of 2.5 mM HU and catalase. After preincubation with FUdR and BUdR cultures were treated with 2.5 mM HU, catalase, phenobarbital-induced mouse liver microsomes (0.5 mg protein/ml) and a NADPH-regenerating system and labeled with 10 µCi [^{3}H]TdR (42 Ci/mmol) for 3 h; determination of repair replication was as described in "methods"; HU-resistant semiconservative DNA synthesis was determined by calculating the acid-insoluble radioactivity not incorporated into parental DNA.

system (Fig. 3) was insensitive to catalase and probably independent of H_2O_2 formation (results not shown).

It remains possible that some other reactive species formed as a consequence of H_2O_2 production was the ultimate DNA damaging agent. For example, $O_2^{\cdot-}$ radicals might have been involved as the addition of superoxide dismutase, which catalyzes the breakdown of $O_2^{\cdot-}$ radicals, inhibited HU-induced repair by 70%. Glutathione (2 mM) was similarly effective (Table 1).

DISCUSSION

HU, which is commonly used in procedures for measuring repair replication, is generally accepted to be free of repair inducing activity. This is in contrast to the findings presented in this paper. The discrepant results can be explained by the need for metabolism to convert HU to a reactive, DNA damaging product and the different metabolic capacities of the various cells and tis-

sues used. Most established cell lines possess very low levels of monooxygenase-activity, if any (14), and, thus, may not appreciably metabolize HU. In yeast (15), which contains cytochrome P-450 (16), HU was shown to be mutagenic. Furthermore, repair induction by HU may also vary in different cells, depending e.g. on their catalase content and on the levels of agents that interfere with the production or degradation of H_2O_2.

Recognition of HU-induced repair replication by measuring the incorporation of [^{3}H]TdR into DNA of non S phase cells ("unscheduled DNA synthesis") is difficult as these techniques can not discriminate unequivocally between repair and semiconservative DNA synthesis. Nevertheless, some recent findings might be taken as indications that repair induction by HU occurs also in other cells. Lampidis and Little (17) reported that HU enhanced ultraviolet light induced repair in human cells, and similar results were obtained by Smith and Hanawalt (7). In isolated rat liver cells, which retain their drug metabolizing activity, HU reduced the incorporation of [^{3}H]TdR to a surprisingly low extent (18,19). Martin et al. (20) observed a threefold increase in the uptake of [^{3}H]TdR into HeLa cell DNA in the presence of 10 mM HU when they added a postmitochondrial supernatant fraction from phenobarbital treated rats.

The present results caution against measuring DNA repair replication in the presence of HU and a drug metabolizing enzyme system for two reasons: a) the high "background" repair will considerably reduce the sensitivity of the method, and b) agents that influence the production or degradation of H_2O_2 (or H_2O_2 -derived radicals), such as metal ions, chelating compounds or radical scavengers, may alter the level of the "background" repair yielding equivocal results. Preferably, systems are to be

used which detect repair replication in the absence of HU. The BUdR-CsCl-gradient technique employed in this study allows the omission of HU but may require time consuming rebanding steps. An alternative is the use of density inhibited, confluent cultures of fibroblasts (21) which essentially show no semiconservative DNA synthesis.

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