Alkylation of Intracellular and Extracellular DNA by Dimethylnitrosamine following Activation by Isolated Rat Hepatocytes

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ABSTRACT

Freshly prepared rat hepatocytes isolated by perfusion with collagenase were able to metabolize μM concentrations of dimethylnitrosamine to a methylating agent. The methylation of hepatocyte DNA in this system was complete within 2 hr, and after this time, the content of O6-methylguanine in the DNA declined, showing that the repair system for this product was active in the isolated hepatocytes. When extracellular calf thymus DNA was added to the incubated hepatocytes, this also became methylated. Methylation of this DNA was not due to cell lysis releasing activating enzymes into the medium, showing that the methylating species formed by the hepatocytes were active in the isolated hepatocytes. When extracellular calf thymus DNA was added to the incubated hepatocytes, this also became methylated. Methylation of this DNA was not due to cell lysis releasing activating enzymes into the medium, showing that the methylating species formed by the hepatocytes from dimethylnitrosamine is sufficiently stable to pass out of the cell in substantial amounts. These results support the possibility that alkylation of liver cells would not be confined to those cells metabolizing dimethylnitrosamine but could be extended to those cells which are in close proximity to the activating cells. These cells could include nonparenchymal cells which are known to be targets for the carcinogenic action of dimethylnitrosamine.

INTRODUCTION

Dimethylnitrosamine is a potent chemical carcinogen which requires metabolic activation for carcinogenic action (11, 28, 29, 34, 36). Such activation generates a methylating species, and the methylation of cellular components, particularly DNA, is thought to be responsible for the initiation of tumors. It is widely accepted that activation of dimethylnitrosamine involves an oxidative demethylation. Such oxidation leads to the unstable nitrosohydroxymethylmethylamine which eliminates formaldehyde generating a methyl carbion ion or equivalent methylating species (11, 13, 21, 28, 30, 38). Studies with methyl(acetoxyethyl)-nitrosamine, a relatively stable ester derivative which can be converted into the α-hydroxy intermediate by the action of esterases, support this concept (13, 20, 38, 46). However, multiple pathways leading to degradation (2, 9, 15, 16) have been suggested, and there is convincing evidence that there are several forms of dimethylnitrosamine demethylase, only one of which has a sufficiently low Km to have physiological importance (3-5, 36).

The metabolism of dimethylnitrosamine has been investigated both in vivo by following the fate of the labeled compound (12, 17, 44) and in vitro using tissue slices (10, 32, 44) or cell-free preparations (3, 21, 22, 27, 30, 36, 42). Tissue slice preparations have numerous disadvantages for biochemical studies, particularly since exposure to test substances may not be uniform throughout the tissue and since cell-free preparations may not accurately represent the reactions taking place within intact cells. Therefore, in the present work, we have studied the metabolic conversion of dimethylnitrosamine to a methylating agent by isolated rat hepatocytes. The experiments were carried out with a low concentration of dimethylnitrosamine to ensure that only physiologically relevant metabolism was measured. It was found that, under these conditions, the alkylating species derived from the carcinogen was sufficiently stable to permit significant methylation of extracellular DNA.

MATERIALS AND METHODS

Chemicals. Unlabeled dimethylnitrosamine was obtained from Aldrich Chemical Co., Milwaukee, Wis. Collagenase type II was from Worthington Biochemical Corp., Freehold, N. J., and Pronase (B grade) was from Calbiochem-Behring Corp., La Jolla, Calif. [3H]Dimethylnitrosamine (6.45 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. All other chemicals were from Sigma Chemical Co., St. Louis, Mo.

Preparation and Incubation of Hepatocytes. Hepatocytes were prepared by in situ collagenase perfusion of livers from female Sprague-Dawley rats (200 to 230 g) as described previously (45). This procedure yielded routinely 1.5 to 2.0 × 10⁶ cells from 2 rats. The hepatocytes were then incubated at a density of 2.5 to 3.0 × 10⁶ cells/ml in polycarbonate flasks (25-ml capacity) in a shaking water bath at 37° and were supplied with 95% O₂:5% CO₂. The incubation medium consisted of a modified Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 27.4 mM glucose, monosodium glutamate and pyruvate at 5 mM each, 3% bovine serum albumin, and plasma levels of amino acids. Calf thymus DNA was first dissolved in incubation medium and then added to the hepatocytes at appropriate times. Where indicated, cells were lysed by freeze-thawing twice in liquid N₂, and this resulted in >95% cell lysis as determined by trypan blue exclusion. At the end of the incubation, the hepatocytes were pelleted in a Sorvall GLC-2 centrifuge (1000 rpm for 1 min), washed once with medium, and frozen. Supernatants containing DNA were also frozen. Conditioned medium was prepared by incubating with hepatocytes for 2 hr and then removing the cells by centrifugation as above.

DNA Isolation and Analysis. DNA was isolated from the hepatocytes exactly as described earlier (45) using a chloroform-isooamy alcohol procedure. The medium containing DNA was first adjusted to pH 7.4. NaCl by addition of 0.5 volume of water and then treated as for the hepatocytes. DNA was hydrolyzed in 0.1 M HCl for 30 min at 70°. After hydrolysis, each cell sample was divided into 2, and each medium sample was divided into 3 portions and then analyzed separately. The released purines were separated by high pressure liquid chromatography, using a Whatman Partisol 10/25 SCX column eluted with 0.02 M ammonium formate at 50° and a flow rate of 2 ml/min. Adenine and guanine were determined by measuring the absorbance at 260 nm in the appropriate fractions. Methylated bases were determined from their radioactivity using Formula 947 LSC cocktail (New England Nuclear) and a Beckman LS-3133T liquid scintillation counter. All experiments were carried out several times with duplicate analyses which agreed within ±15%. However, results could not be averaged between experi-
RESULTS

The primary hepatocyte preparations used in this study were found to be more than 98% hepatocytes by microscopic examination, and initial viability as determined by dye exclusion was more than 90%, decreasing only slightly over the time course of incubation (45). The purity of the preparation agrees well with previous reports of the lack of Kupffer cells (8) and the presence and synthesis of albumin by 98% of the cells (6) when hepatocytes were prepared in the same way as in our experiments. Also, hepatocytes incubated under similar conditions to those used in the present work synthesize and secrete albumin at a constant rate (6), attesting to the stability of the preparation over the relatively short time period used. Such primary hepatocytes prepared by collagenase perfusion retained the ability to metabolize dimethylnitrosamine to an alkylating agent, which in turn reacted with cellular DNA. Chart 1 is a representative chromatogram of hydrolyzed DNA from hepatocytes incubated with dimethylnitrosamine for 2 hr. Markers for the methylated bases were added so that their absorbance could be detected. The peaks preceding guanine consisted of pyrimidine oligonucleotides which were the sites of substantial alkylation. The fraction corresponding to 7-methylguanine also contained a large number of counts, in agreement with the extensive alkylation at that position in vivo (28, 34). Radioactivity was also found in fractions corresponding to O6-methylguanine, 7-methyladenine, 1-methyladenine, and 3-methyladenine.

Incubation of 2.5 x 10^6 cells/ml with 15 μM dimethylnitrosamine resulted in alkylation of cellular DNA. Table 1 shows the levels of 2 alkylated bases (7-methylguanine and O6-methylguanine) in the DNA. Results are shown as both the absolute amount per μmol of guanine and as the amount per μmol of guanine as DNA which allows for the small differences in the recovery of DNA from one point to another. The cellular alkylation was complete in 2 hr as indicated by the accumulation of 7-methylguanine, a relatively stable product in rat liver DNA (35, 37). This probably reflects the complete metabolism of the added dimethylnitrosamine by this time, since a further addition of labeled dimethylnitrosamine brought about an increase in alkylation (results not shown). O6-Methylguanine was also formed, but the level was not stable in the hepatocytes. O6-Methylguanine is enzymatically removed from DNA in rat liver in vivo (36, 37). The cellular O6:7-methylguanine ratio (Table 1) decreased with incubation time, indicating that the enzyme(s) responsible for removal of O6-methylguanine were active in the isolated hepatocytes.

When calf thymus DNA was added to the medium with hepatocytes and dimethylnitrosamine, alkylation of the exogenous DNA occurred (Table 1). Both 7-methylguanine and O6-methylguanine were formed in the extracellular DNA, such that total alkylation of the extracellular DNA exceeded that found in the hepatocytes. The methylation per μmol of guanine was lower in the exogenous DNA, probably because of the substantial dilution of the alkytating species into 10 ml of medium. The 7-methylguanine content in the exogenous DNA was approximately 10 times higher than the O6-methylguanine content and remained at that level for the 4-hr incubation (Table 1). Therefore, the enzyme(s) which act to remove O6-methylguanine from the cellular DNA were not present in the medium.

Experiments were carried out to determine whether the alkylation of exogenous DNA could be due to activation of dimethylnitrosamine by enzymes released into the medium by cell lysis. When cells were lysed by freeze-thawing and then incubated with dimethylnitrosamine and DNA, very little 7-methylguanine was formed (Table 2). Further, medium which was conditioned by incubation with hepatocytes for 2 hr did not by itself permit alkylation of DNA incubated with dimethylnitrosamine.

![Chart 1: Separation of methylated purines by high-pressure liquid chromatography. DNA was isolated from hepatocytes and hydrolyzed as described under "Materials and Methods." The bases were separated by high-pressure liquid chromatography, and each fraction was counted. A background of 20 cpm was subtracted from each fraction. Top, radioactivity; bottom, absorbance corresponding to the sample and appropriate marker bases.](image-url)
The methylated DNA in the medium could not simply be extracellular DNA. Therefore, cell lysis occurring during the 2 hr of incubation could not account for the extensive extracellular alkylation. The isolated hepatocytes to metabolize the added dimethylnitrosamine when these are cultured together with isolated hepatocytes and the carcinogen (19, 23, 39).

The ability of an alkylation species derived from dimethylnitrosamine to react at sites outside the cell in which it is generated can also be inferred by the production of microbial mutants in host-mediated assays (7, 14) and by the presence of alkylated hemoglobin in rats exposed to dimethylnitrosamine. Nevertheless, there is a substantial body of work indicating that there is a striking correlation between the abilities of organs to activate dimethylnitrosamine and the extent to which their DNA becomes alkylated by the carcinogen (18, 20, 28, 29, 32, 35, 43). It appears that the probability that reaction will take place prior to diffusion of the alkylation species through the blood stream to the organs is sufficiently high that organs made up of cells not possessing the metabolic activation system are unlikely to become alkylated. However, it appears quite probable that significant alkylation of cells within an organ could take place by reaction with a methylating species generated in another cell. This could be important in the alkylation of, for example, nonhepatocyte cells in the liver, since these cells may not be as competent as hepatocytes in activation of dimethylnitrosamine, which is known to induce cholangiocellular tumors and hemangiendothelial sarcomas as well as hepatocellular carcinomas in the rat (11, 18, 28). Therefore, further information concerning the stability of the alkylation species and its ability to pass through cell membranes would be of considerable interest. The isolated hepatocyte system provides a useful tool by which these factors can be studied.

It has been estimated that the half-life of nitrosodimethylmethyamine is less than 1 min under physiological conditions (13, 20, 38). A recent, more direct measurement indicated that, in phosphate buffer of pH 7, the half-life was estimated to be 10 sec and at pH 8, 1 sec (31). This value is likely to be even lower in the presence of cellular nucleophiles but still allows ample time for the diffusion or transport out of the cell observed in the present experiments. The actual methylating species is not known, but the involvement of diazomethane has been ruled out by deuterium labeling experiments (26). Park et al. (33) have provided evidence that the alkylation of DNA by a metabolite of N-nitrosodi-n-propylamine does not involve the

**DISCUSSION**

The present results confirm our previous finding that incubation of freshly isolated hepatocytes with dimethylnitrosamine results in alkylation of the hepatocyte DNA (45). The results in Table 1 are also in agreement with our observation that the extent of reaction with DNA is almost an order of magnitude less than that observed when liver DNA is alkylated by exposure to the same amount of dimethylnitrosamine in intact animals in vivo (35, 37, 45). This discrepancy was not due to inability of the isolated hepatocytes to metabolize the added dimethylnitrosamine in the time period of incubation (45). The present finding that there can be extensive reaction of the alkylation species derived from the nitrosamine with extracellular DNA provides a plausible explanation for this. When dimethylnitrosamine is metabolized in the liver, the close proximity of adjacent cells provides additional possibilities for the alkylation species to react with cellular DNA. The isolated hepatocytes are diluted into a substantial volume of incubation buffer in order to ensure sufficient oxygenation. Under these circumstances, a significant proportion of the alkylation species generated within the cell may diffuse out and react with either extracellular water or appropriate nucleophiles if they are available. Our results are also consistent with the induction of mutations in cells which do not metabolize dimethylnitrosamine when these are cultured together with isolated hepatocytes and the carcinogen (19, 23, 39).

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formation of a carbocation ion but, instead, proceeds via a bimolecular reaction. This is supported by the theoretical study of Andreozzi et al. (1) and may indicate the involvement of the alkyldioxydiol or alkyldioxyzoin ion in the reaction, but even a reaction of the initial α-hydroxy derivative is not ruled out (33). Analysis of the spectrum of products produced in DNA by reaction with N-methyl-N-nitrosourea and the activation form of dimethylnitrosamine reveals no differences, suggesting that the same methylating species is involved (24, 25, 35, 40, 41). The alkylation via N-methyl-N-nitrosourea is thought to take place via a predominantly SN2 mechanism (24, 25). Whether alkylation of DNA by dimethylnitrosamine also occurs by an SN1 reaction or by the bimolecular SN2 reaction suggested by Andreozzi et al. (1) and Park et al. (33) remains to be determined. The hepatocyte system described in this paper may prove valuable in further examination of this question.

REFERENCES

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