Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1063-1070

THE EFFECT OF NICOTINAMIDE ON UNSCHEDULED DNA SYNTHESIS IN CULTURED HEPATOCYTES

F. R. Althaus, S. D. Lawrence, G. L. Sattler, and H. C. Pitot

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received June 28,1980

Summary: Unscheduled DNA synthesis of cultured hepatocytes in response to the direct acting carcinogen, methyl methanesulfonate, and to the procarcinogen, 2-acetylaminofluorene, was markedly increased when these cells were cultured in medium containing 25 mM nicotinamide. This effect of nicotinamide was apparently unrelated to the maintenance of intracellular nicotinamide coenzyme levels. The increase in unscheduled DNA synthesis mediated by 2-acetylaminofluorene in nicotinamide-treated hepatocytes could be partially accounted for by the maintenance of higher microsomal capacity for the metabolism of this procarcinogen. The ability of adult rat hepatocytes to respond to nicotinamide by increased unscheduled DNA synthesis was lost within a few hours after plating or upon short withdrawal of this vitamin from the culture medium.

INTRODUCTION

Unscheduled DNA synthesis (UDS) in response to UV light or chemical carcinogens is known to occur in adult rat hepatocytes in primary monolayer culture (1-7). Using biochemical and autoradiographic methods, Sirica et al. (6,8) found that stimulation of UDS by methyl methanesulfonate (MMS) or 2-acetylaminofluorene (AAF) in rat hepatocytes cultured on collagen gel/nylon meshes requires the presence of dexamethasone (10^{-5}M) and glucagon (10^{-6}M) or of a more complex mixture of hormones, fatty acids, and δ-amino-levulinic acid in a culture medium routinely supplemented with insulin. We have extended these findings of Sirica et al. (6,8) and demonstrated that a considerable stimulation of UDS by MMS and AAF occurs in hepatocytes cultured on collagen gel/nylon meshes in the absence, and an even higher response in the presence, of the medium supplements used by Sirica and

Abbreviations: UDS, unscheduled DNA synthesis; MMS, methyl methanesulfonate; AAF, 2-acetylaminofluorene; DMSO, dimethylsulfoxide; TCA, trichloroacetic acid.

0006-291X/80/151063-08$01.00/0

his associates. These results form the basis of the method used for measurement of UDS in this report.

Earlier studies from this laboratory had demonstrated the maintenance of cytochrome P450 at relatively low levels in primary hepatocyte cultures on collagen gels (9). However, within 24 h after explantation, the level of microsomal cytochrome P450 had dropped precipitously. Studies by Paine et al. (10,11) recently showed that the addition of nicotinamide to cultured hepatocytes resulted in the maintenance of cytochrome P450 levels at near control values for 24 h in culture. In light of these results and the requirement of cytochrome P450 for the microsomal metabolism of procarcinogens, we studied the effect of the addition of this vitamin to cultures of hepatocytes on UDS occurring in the presence of AAF and the direct-acting mutagen, MMS.

MATERIALS AND METHODS

Reagents: Leibovitz (L-15) medium, penicillin-streptomycin, fetal bovine serum, and Hi/Wo/Ba medium (10x concentrated) were purchased from KC Biological, Inc., Lenexa, KS. Isonicotinamide, insulin, crystalline bovine serum albumin, collagenase type I, Hepes, hydroxyurea, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co., St. Louis, MO. Nicotinamide was obtained from Calbiochem, Los Angeles, CA. Methyl methanesulfonate and urethane were obtained from Aldrich Chemical Co., Milwaukee, WI, and thioacetamide was purchased from the Fischer Scientific Co., Fairtown, N.J. 2-Acetylaminofluorene was a generous gift from Dr. E. C. Miller of the McArdle Laboratory. [Methyl-^{3}H]thymidine was from Amersham, Arlington Heights, IL.

Culture of adult rat hepatocytes: Hepatocytes having an initial viability of 80% as determined by trypan blue exclusion were isolated from the livers of adult male albino rats (Holtzman, 220-280 g, fed ad lib.) by the collagenase perfusion method of Berry and Friend (12) as modified by Bonney et al. (13). The hepatocytes were then plated at a cell density of 10-13 X 10^6 cells in 10 ml of L-15 medium (supplemented with 18 mM Hepes; 2 mg/ml albumin; 5% fetal bovine serum; penicillin, 100 µg/ml; streptomycin, 100 µg/ml; glucose, 1.5 mg/ml; and insulin, 0.5 µg/ml) onto 100-mm Falcon tissue culture dishes or onto 98-mm collagen gel/nylon meshes as described by Sirica et al. (14). It was noted that L-15 medium contains small amounts of nicotinamide (8.2 µM). A 100 X stock solution of nicotinamide or isonicotinamide in H_2O was added to the culture medium of freshly plated hepatocytes (0 h) where indicated. A medium change was routinely performed 4 h after plating to remove unattached cells. A second medium change followed 19 to 21 h after the initiation of cultures.

Treatment of cultures: Carcinogens (dissolved in 10 µl DMSO) and [methyl-^{3}H]thymidine (10 µCi/plate, 42-58 mCi/mmol) were added to the culture

medium 2 hours after the second medium change. In order to reduce [methyl-^{3}H]thymidine incorporation due to replicative DNA synthesis, hydroxyurea (10 mM) was added to the medium 2 hours prior to carcinogen treatment. Appropriate DMSO control cultures were run for each carcinogen tested.

Measurement of [methyl-^{3}H]thymidine incorporation into nuclear DNA: A series of experiments preceding this study was performed in order to improve procedures for the measurement of unscheduled DNA synthesis in cultured hepatocytes. [Methyl-^{3}H]thymidine radioactivity from non-nuclear DNA sources contaminate the DNA isolated from whole cells (5). We therefore tried to separate the nuclei from other cellular components. Up to 85% of the DNA present in the homogenate from cultured hepatocytes could be recovered by isolating nuclei prior to DNA extraction. The background radioactivity in DNA of control cultures could be reduced by a factor of 5 to 10 compared with procedures involving direct extraction of DNA from cells (data not shown).

At the end of each experiment, the cell monolayers were washed twice with 10 ml of ice cold phosphate-buffered saline (pH 7.4) containing thymidine (2 mM). Cells on plastic surfaces were then scraped off the plates with a rubber policeman. Cells on collagen gel/nylon meshes were recovered by short collagenase treatment (14). All the following procedures were carried out at 4°. Cells were pelleted and resuspended in 900 µl hypotonic buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0). After 10 min, cells were homogenized for 15 sec (Tissuemizer homogenizer, Tekmar, Cincinnati, OH, maximum speed). Triton X 100 was added to the homogenate to a final concentration of 1%. After 5 min of incubation, the homogenization step was repeated. The homogenate was centrifuged at 600 x g, and the pellet was resuspended in 0.25 M buffered sucrose (Tris-HCl 50 mM, KCl 25 mM, $MgCl_2$ 5 mM; pH 8.0). The method of Blobel and Potter (15) was used to isolate nuclei by high-speed centrifugation in sucrose. The pellets containing the nuclei were resuspended in 500 µl 10 mM Tris-HCl, pH 8.0, and stored frozen overnight. The purity of the nuclear fraction was checked by light microscopy after toluidine blue staining and by demonstration of DNA content. Nuclei were thawed and processed for DNA determination and scintillation counting. After addition of 500 µl Tris-HCl (10 mM, pH 8.0) and 500 µl 1 N KOH, the nuclei were incubated in 1 ml of 0.33 N KOH for 45 min (16) and neutralized with 300 µl 1 N HCl, and the DNA was precipitated in the presence of 10% trichloroacetic acid (TCA) and protein (0.5% bovine serum albumin). The pellets were collected by centrifugation. Hydrolysis of the DNA was achieved by heating in 5% TCA at 90° for 20 min. Any remaining precipitate was removed by centrifugation. Aliquots of the acid-soluble fraction were used for scintillation counting and measurement of DNA concentration by a slight modification of the diphenylamine procedure of Burton (17).

Measurement of microsomal cytochrome P450 concentration: Cells from two or three 100-mm plates were harvested in ice cold 0.25 M sucrose and homogenized in a glass-Teflon homogenizer (1000 rpm, 10 strokes). This was followed by treatment for 20 sec with a Tissuemizer homogenizer (Tekmar). This homogenate was centrifuged at 10,000 x g for 12 min. The resulting supernatant was spun at 105,000 x g for 90 min to sediment the microsomal fraction. Microsomes were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, and used for spectral analysis of cytochrome P450 as described by Omura and Sato (18). Protein concentration was determined by the method of Lowry et al. (19) with bovine serum albumin as the standard.

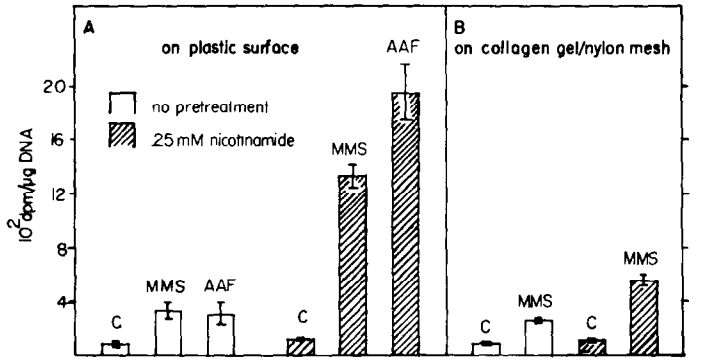

Figure 1. Unscheduled DNA synthesis in hepatocytes cultured in the presence or absence of 25 mM nicotinamide on plastic surfaces (1A) or on collagen gel (1B). Treatment of cultures was as described in the Methods section. Both MMS and AAF were present in the medium for 12 h at concentrations of 10^{-3} M. Appropriate controls (C) were run by treating cultures with 10 µl DMSO. The values in 1A represent the mean ± SD of 3 to 6 measurements obtained in separate cultures; the values in 1B are the means of duplicate determinations.

RESULTS AND DISCUSSION

Figure 1 shows the effect of MMS and AAF on UDS in adult rat hepatocytes maintained on plastic surfaces or on collagen gel/nylon meshes. Both MMS and AAF increased UDS considerably in these cells, and these effects were dependent on the dosage and the time of exposure (data not plotted). Maximum effects were observed between 5×10^{-4} and 10^{-3} M concentrations of both AAF and MMS. Higher doses of these carcinogens were visibly toxic to the cells and inhibited incorporation of [methyl-^{3}H]thymidine into nuclear DNA. Cells cultured on plastic surfaces exhibited greater levels of UDS than cells on collagen gel/nylon meshes (Fig. 1). This phenomenon might be due to unspecific binding of carcinogens to collagen, thus limiting the bioavailability of these carcinogens. The UDS response to MMS and AAF was greatly increased in both systems when nicotinamide (25 mM) was added to the culture medium (Fig. 1). A similar but somewhat smaller effect of nicotinamide was usually observed in experiments in which hepatocytes were treated on the first day of culture (see Table 1). In some experiments, nicotinamide treatment of control cultures also stimulated

TABLE 1. Effect of different times of nicotinamide treatment of cultured hepatocytes on the stimulation of UDS by MMS.

$5x10^{-4}$M MMS was routinely added to the medium 18 hours before harvest of cells except for the 13-h, 22-h, and 40-h exposures to 25 mM nicotinamide, where treatment with MMS was for the last 12 h of culture at a concentration of 10^{-3}M.

Nicotinamide treatment			
Exposure time (h)	Treatment interval (h)	Total culture period (h)	Stimulation of UDS by MMS[b] (-fold)
None	-	24	4.5
None	-	48	5.3
4	0[a]-4	48	5.8
13	27-40	40	4.0[d]
22	18-40	40	4.1[d]
24	0-24	48	8.4
24	0-24	24	6.8
31	0-4, 21-48	48	5.9
40	0-40	40	10.5[d]
48	0-48	48	11.1
95[c]	9-104	104	4.2[e]
104[c]	0-104	104	7.6[e]

a) 0 h indicates the time of plating freshly isolated hepatocytes.
b) Stimulation of UDS (values averaged from 2 to 6 experiments). Controls were identically pretreated and received 10 µl DMSO instead of MMS.
c) Medium changed every 24 h.
d) Stimulation of UDS by MMS in cultures not pretreated with nicotinamide was 4.2-fold.
e) Effect of MMS on UDS in the absence of nicotinamide was a 4.1-fold stimulation.

[methyl-^{3}H]thymidine incorporation (Figs. 1 and 2) to a slight but measurable extent. Thioacetamide and urethane (both tested at 10^{-5}M to 10^{-3}M concentrations) failed to elicit any detectable UDS response, and this was unaltered in hepatocytes pretreated with nicotinamide (data not plotted).

The data in Fig. 2 seem to exclude the possibility that MMS requires metabolic activation to exert its stimulatory effect on UDS in hepatocytes *in vitro*. SKF525A, an inhibitor of microsomal mixed function oxidases,

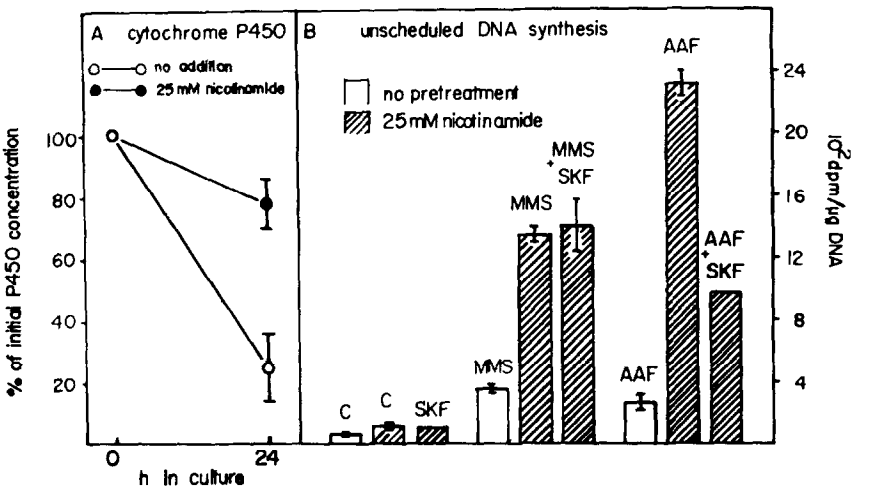

Figure 2. A. Effect of 25 mM nicotinamide on microsomal cytochrome P-450 concentrations of cultured hepatocytes. Cytochrome P450 concentrations in freshly isolated hepatocytes (0 h value) were 404 ± 22 pmol/mg microsomal protein (average of duplicate determinations obtained from 2 different cell batches). B. Effect of SKF525A (SKF, 40 µM) on UDS stimulated by MMS (10^{-3}) and AAF (10^{-3} M) in hepatocytes cultured in the presence or absence of 25 mM nicotinamide in the medium. Carcinogen treatment was for 12 h. SKF525A was added to the culture medium 2 h prior to carcinogen treatment. The data were obtained from duplicate determinations on the same preparation of cells.

clearly reduced stimulation of UDS by AAF, whereas UDS mediated by the direct-acting carcinogen MMS was not affected.

Paine et al. (11) have reported that maintenance of *in vivo* levels of cytochromes P450 in cultured hepatocytes is not related to the maintenance of intracellular nicotinamide coenzyme levels. In addition, isonicotinamide (10 mM) could also prevent the loss in P450 concentration in the initial culture period but did not reverse a 60% decrease in nicotinamide coenzyme levels occurring within 24 h after plating of the hepatocytes (11). The dose-response curve for the action of nicotinamide on MMS-mediated UDS (Fig. 3A) apparently excluded a causal relationship between maintenance of nicotinamide coenzyme levels and the effect of UDS. Moreover, isonicotinamide was found to be more active in enhancing UDS at lower doses (Fig. 3B).

The data in Table I show the requirement for the continuous presence of nicotinamide from the moment of plating to the harvest of cells. Beginning

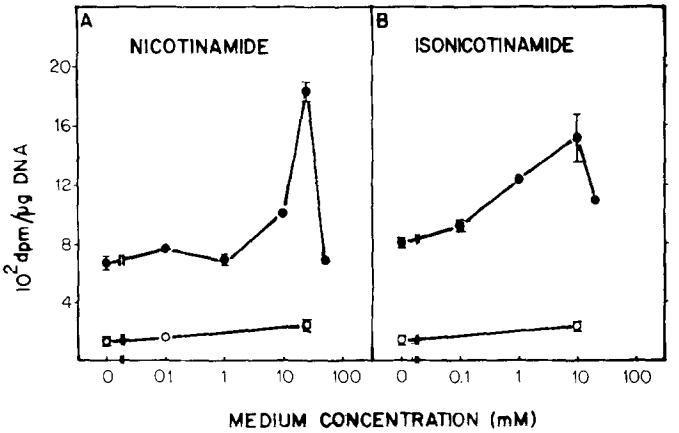

Figure 3. Effect of pretreatment of hepatocyte cultures with different concentrations of nicotinamide (3A) or isonicotinamide (3B) on UDS mediated by MMS ($5x10^{-4}$ M). Carcinogen treatment of cultures was for 18 h. The results in 3A and 3B were obtained from the same batch of cells. The averages of duplicate determinations are indicated by bars. 0——0, control; ●——●, MMS. $5x10^{-4}$ M.

nicotinamide treatment a few hours after the plating of cells or withdrawing it for a short period of time from the culture medium abolished the stimulatory action on UDS compared with cultures treated continuously for nearly the same period or even shorter times with nicotinamide. This indicates that the capacity of cultured hepatocytes to respond to nicotinamide by increased UDS is lost within a few hours after plating and when nicotinamide exposure is interrupted in late culture periods.

The studies reported in this communication demonstrate that nicotinamide added to primary cultures of adult rat hepatocytes profoundly enhances UDS induced by a representative procarcinogen, 2-acetylaminofluorene, and a direct-acting mutagen, methyl methanesulfonate. Although a significant portion of the UDS stimulated by 2-AAF in the presence of nicotinamide could be inhibited by the inhibition of microsomal metabolism by SKF525A, a substantial component of UDS stimulated by this procarcinogen as well as by the direct-acting mutagen, MMS, is not affected by this inhibitor. Thus, the effects of nicotinamide on UDS stimulated by these agents cannot be accounted for entirely by the metabolism of the compound to a chemically reactive form.

A recent report by Durkacz et al. (20) indicates a relationship of poly(ADP-ribose) metabolism to the process of DNA excision repair in L1210 cells. Poly(ADP-ribose) is the polymerization product of a nuclear enzyme reaction involving the ADP-ribose unit of NAD^+. The effect of nicotinamide on NAD metabolism and the possible involvement of poly(ADP-ribose) in DNA repair mechanisms of cultured hepatocytes is currently being investigated in our laboratory.

ACKNOWLEDGEMENTS

This work was supported by contract N01-CP-85609 and grants CA-07175 and CA-22484 from the National Cancer Institute and in part by a Public Health Service International Research Fellowship (5 F05 TW02756-2) awarded to F.R.A.

REFERENCES

1. Williams, G.M. (1976) Cancer Letters 1, 231-236.
2. Williams, G.M. (1977) Cancer Res. 37, 1845-1851.
3. Casciano, D.A., Farr, J.A., Oldham, J.W. and Cave, M.D. (1978) Cancer Letters 5, 173-178.
4. Michalopoulos, G., Sattler, G.L., O'Connor, L. and Pitot, H.C. (1978) Cancer Res. 38, 1866-1871.
5. Yager, J.D. and Miller, J.A. (1978) Cancer Res. 38, 4385-4394.
6. Sirica, A.E., Hwang, C.G., Sattler, G.L. and Pitot, H.C. (1979) Proc. Am. Assoc. Cancer Res. 20, 142.
7. Williams, G.M. and Laspia, M.F. (1979) Cancer Letters 6, 199-206.
8. Sirica, A.E., Hwang, C.G., Sattler, G.L. and Pitot, H.C. (1980) Cancer Res., in press.
9. Michalopoulos, G., Sattler, G.L. and Pitot, H.C. (1976) Life Sci. 18, 1139-1144.
10. Paine, A.J., Williams, L.J. and Legg, R.F. (1979) Life Sci. 24, 2185-2192.
11. Paine, A.J., Hockin, L.J. and Legg, R.F. (1979) Biochem. J. 184, 461-463.
12. Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506-520.
13. Bonney, R.J., Becker, J.E., Walker, P.R. and Potter, V.R. (1974) In Vitro 9, 399-413.
14. Sirica, A.E., Richards, W., Tsukada, Y., Sattler, C.A. and Pitot, H.C. (1979) Proc. Natl. Acad. Sci. USA 76, 283-287.
15. Blobel, G. and Potter, V.R. (1966) Science 154, 1662-1665.
16. Martin, C.N., McDermid, A.C. and Garner, R.C. (1978) Cancer Res. 38, 2621-2627.
17. Burton, K.A. (1956) Biochem. J. 62, 315-323.
18. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370-2385.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
20. Durkacz, B.W., Omidiji, O., Gray, D.A. and Shall, S. (1980) Nature 283, 593-596.