Caffeine Inhibition of Postreplication Repair of N-Acetoxy-2-acetylaminofluorene-damaged DNA in Chinese Hamster Cells

James E. Trosko, Phyllis Frank, Ernest H. Y. Chu, and Joyce E. Becker

Department of Human Development, Michigan State University, East Lansing, Michigan 48832 [J. T., P. F.]; Department of Human Genetics, University of Michigan, Ann Arbor, Michigan 48104 [E. H. Y. C.]; and McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706 [J. E. B.]

SUMMARY

The effect of caffeine on Chinese hamster cells in vitro, treated with various metabolites and derivatives of 2-acetylaminofluorene, was studied at the molecular level. With the use of an alkaline sucrose gradient centrifugation technique, parental and newly synthesized DNA in control and treated cells were studied in the presence and absence of caffeine. Caffeine synergistically affected only the sedimentation profiles of DNA synthesized in N-acetoxy-2-acetylaminofluorene-treated cells but not in the control cells or in cells treated with the various derivatives of 2-acetylaminofluorene. N-Acetoxy-2-acetylaminofluorene also affected the sedimentation profiles of parental DNA, but caffeine did not influence this effect. At the dose level used, caffeine had no apparent effect on the incorporation of thymidine into DNA in either the control or N-acetoxy-2-acetylaminofluorene-treated cells. These results supplement other reports that suggest that the N-acetoxy-2-acetylaminofluorene lesion in DNA of either human or Chinese hamster cells is repaired similarly to ultraviolet light-induced pyrimidine dimers.

INTRODUCTION

Presently, it appears that many, perhaps all, chemical carcinogens are potential mutagens (28). AAF2 is a potent liver carcinogen which is metabolically activated in rat liver. The sulfuric acid ester of N-OH-AAF appears to be the major ultimate reactive and carcinogenic derivative of AAF in the rat liver (30, 31). N-Acetoxy-AAF, which has been utilized as a synthetic analog of this ultimate carcinogen, reacts readily with RNA, DNA, and protein (1, 9, 12, 14, 15, 17, 24, 25, 29). The products of these reactions are also found in the livers of rats administered AAF or N-OH-AAF. N-Acetoxy-AAF and some of the other AAF derivatives have been shown to be mutagenic in Salmonella typhimurium (2) and in Chinese hamster cells (23). A considerable body of literature has been accumulated in recent years to document the close but complicated relationship between mutagenesis and DNA repair processes (see reviews in Refs. 4, 16, 27, and 43). Some molecular lesions induced in DNA by carcinogens can be repaired by an excision process which has been postulated to be an “error-free” repair mechanism. Replication of UV-damaged DNA produces temporary gaps in regions of daughter strands that are opposite the lesions (36). It has been proposed that the disappearance of these gaps and the formation of normal-molecular-weight DNA are due to repair by either a “recombination-type” mechanism in bacteria or a de novo “postreplication” mechanism in mammalian cells (5, 20). It has been postulated further that these latter processes are “error-prone” repair mechanisms (see reviews in Refs. 4 and 43). The absence of an effective excision repair mechanism in cells of human beings afflicted with the genetic disease, xeroderma pigmentosum (8, 39), is related to extremely high incidences of actinic skin cancers (35). N-Acetoxy-AAF-induced lesions in DNA are known to be repaired (38) and unscheduled DNA synthesis (40) is induced by N-acetoxy-AAF in normal but not in xeroderma pigmentosum cells.

Caffeine has been shown to act as an antimutagen in UV-irradiated Chinese hamster cells (41, 42), as well as an inhibitor of the postreplication repair of UV-induced DNA damage (7, 13, 21, 42). The studies reported here were designed to examine whether caffeine would inhibit the postreplication repair of DNA damage caused by various AAF metabolites and derivatives in Chinese hamster cells.

MATERIALS AND METHODS

Tissue Culture. An aneuploid clonal cell line (V79-4) originally derived from the lung tissue of a male Chinese hamster was used for these experiments. The V79-4 cells were grown in Eagle’s minimum essential medium, supplemented with 15% fetal calf serum. The origin, cytological, and growth characteristics of this cell line have been previ-
ously described (6). All experiments were performed with nonsynchronized, log-phase cells.

**Chemicals.** The carcinogens used (AAF, N-acetoxy-AAF, N-OH-AAF, 2-aminofluorene, 2-nitrosofluorene, N-hydroxy-2-aminofluorene) were generously provided by Dr. Elizabeth C. Miller and Dr. James A. Miller (McArdle Laboratory for Cancer Research, Madison, Wis.). DMSO was purchased from Fisher Scientific Company (Pittsburgh, Pa.). Caffeine was purchased from the Sigma Chemical Company (St. Louis, Mo.). Thymidine-methyl-3H was ordered from New England Nuclear (Boston, Mass.) and Schwarz/Mann (Orangeburg, N. Y.).

**Alkaline Sucrose Gradients.** To determine whether caffeine might interfere with the synthesis of new DNA (i.e., preventing small fragments from being linked together), the relative molecular-weight profiles of DNA molecules, synthesized in the presence or absence of caffeine, were determined by a modified alkaline sucrose gradient technique (26) described by Regan et al. (34). V79-4 cells were incubated at 37°C in thymidine-3H (5 μCi/ml; 15 to 20 Ci/mmol), with or without various concentrations of caffeine for 2 or 10 hr after treatment. Each experiment, which had duplicates of the treated cells, was repeated at least 2 times.

The 3.6-ml gradients of 5 to 20% sucrose contained 0.3 mM NaOH, 0.5 mM NaCl, and 0.01% EDTA. At the bottom was a cushion of 0.2 ml of 60% alkaline sucrose. At the top was 0.2 ml of 1 × NaOH into which approximately 3 to 12 × 10⁶ cells were gently layered. After remaining at 20°C for 1 hr, the samples were spun for 90 min at 30,000 rpm in a Beckman SW-56 rotor. The bottom of each centrifuge tube was punctured and 30 ± 1 fractions were collected on filter paper discs. The discs were washed with cold 5% TCA and then with ethyl alcohol. After drying, the discs were counted in a scintillation spectrometer.

To determine whether caffeine interfered with the repair of N-acetoxy-AAF-treated prelabeled DNA, cells were labeled with thymidine-3H for 24 hr. The medium was decanted and nonradioactive medium was added 2 hr prior to treatment. After the addition of N-acetoxy-AAF, nonradioactive medium, with or without 1 mM caffeine, was added to each Petri dish and the cells were incubated for another 6 hr. Cells were then collected and placed on a gradient as described above.

**DNA Synthesis Assays.** To determine whether caffeine treatment affected DNA synthesis (incorporation of thymidine-3H into TCA-insoluble material), the method described by Bollum (3) as modified by Regan and Chu (33) was used. To each 60-mm Petri dish 8 × 10⁵ cells were inoculated and incubated for 1 day before the assay. The old medium was decanted and 3 ml of new medium were added to each plate. Either 50 μl of DMSO or N-acetoxy-AAF in 50 μl of DMSO (35 μM, final concentration) were added to each plate. After 30 min, media were decanted and media containing thymidine-3H (3 μCi/ml; 6.7 Ci/mmol; New England Nuclear), with or without 1 mM caffeine, were added to control and N-acetoxy-AAF-treated cells. At various times after exposure of the cells to thymidine-3H or thymidine-3H plus caffeine, duplicate dishes for each treatment were sonically disrupted with a Sonifier tube was punctured and 30 ± 1 fractions were collected on 3-mm Whatman paper discs for scintillation counting.

**RESULTS**

**Sedimentation Profiles of Parental and Newly Synthesized DNA of Treated V79-4 Cells.** To determine what effects caffeine might have on the sedimentation profiles of newly synthesized DNA in treated cells, various metabolites and derivatives of AAF were given to the cells. After 30 min, thymidine-3H was added to the cells in the presence or absence of 1 mM caffeine for 10 hr. The results are shown in Chart 1. It is apparent that the DNA molecules that were synthesized in the presence of caffeine after the 30-min incubation with the model ultimate carcinogenic derivative of AAF (N-acetoxy-AAF) had a sedimentation profile which peaked as lower-molecular-weight DNA. AAF and various metabolites and derivatives (N-OH-AAF, N-hydroxy-2-aminofluorene, 2-nitrosofluorene, 2-aminofluorene), examined at equimolar concentrations, did not interact with caffeine, since they probably failed to damage DNA. However, it is possible that the damage caused was at a level that was undetectable with the alkaline sucrose technique. Although the N-OH-AAF has been shown to induce unscheduled DNA synthesis in human cells at high concentrations (19, 40), we were unsuccessful in attempts to measure any effect it might have had on the DNA with this gradient technique because, at this high concentration (0.1 mM), the amount of thymidine-3H incorporated into DNA was too low to measure.

To determine what effect caffeine might have on the sedimentation profiles of parental DNA of V79-4 cells treated with N-acetoxy-AAF, cells were prelabeled with thymidine-3H for 24 hr, treated with N-acetoxy-AAF in nonradioactive medium for 30 min, and then incubated for an additional 6 hr with or without caffeine. The results are shown in Chart 2. It is apparent that there was a significant shift in the sedimentation profiles of the DNA of N-acetoxy-AAF-treated cells. However, the presence of caffeine had no detectable influence on the sedimentation profiles of parental DNA of control or treated cells as it had on the newly synthesized DNA.

The effects of various concentrations of caffeine on the sedimentation profiles of newly synthesized DNA in N-acetoxy-AAF-treated cells are shown in Chart 3. Concentrations of 0.1 mM caffeine or less were ineffective in producing a shift in the sedimentation profiles.

The results in Chart 4 demonstrate the effect of 2 hr posttreatment with 1 mM caffeine, followed by 8 hr in caffeine-free medium, on N-acetoxy-AAF-treated cells. It is apparent that the caffeine-induced profile shift in N-acetoxy-AAF-treated cells is not an irreversible effect if the caffeine is present only for 2 hr.

**The Effect of Caffeine on the Incorporation of Thymidine-3H in N-Acetoxy-AAF-Treated Cells.** To determine whether the sedimentation profile shift in N-acetoxy-AAF plus caffeine-treated cells was related to the synthesis of
DNA, the amount of TCA-precipitable material was measured under similar conditions (concentration of N-acetoxy-AAF was 5 times higher) as in the experiments for sedimentation profile studies. The results are shown in Chart 5. Caffeine had only a slight inhibitory effect on the incorporation of thymidine-$^3$H into the DNA of control cells after 6 hr. However, there appeared to be no synergistic effect of caffeine on the incorporation of thymidine-$^3$H in the N-acetoxy-AAF-treated cells.

**DISCUSSION**

These Chinese hamster cells in vitro were apparently unable to activate AAF and its derivatives into compounds that could damage DNA in a way that would have been detected using the alkaline sucrose technique. However, caffeine did synergistically interact with N-acetoxy-AAF by causing the cells to synthesize relatively low-molecular-weight DNA during the 1st S phase after treatment. These results are consistent with the observations that caffeine, at concentrations above 0.1 mM, acts specifically in the S phase of the cell cycle (10, 11, 32). Furthermore, the data in Chart 4 indicate that the caffeine-inhibited gap-filling process is not irreversible up to 2 hr. Lehmann (22) has
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It has been demonstrated that AAF-induced lesions appear to persist for a long time in rat liver DNA (18). A recent report indicates that N-acetoxy-AAF does, among other things, induce both single- and double-stranded breaks (37). Although we have no data indicating the amount of excision of these AAF-induced lesions, if any, that occurs in these Chinese hamster cells, the appearance of single-strand breaks in DNA in the presence of alkaline sucrose after 6 hr following treatment with N-acetoxy-AAF would be consistent with the observation that the N-acetoxy-AAF-induced DNA lesion does persist for a while.

The reduced molecular weight of nascent DNA in caffeine plus N-acetoxy-AAF-treated cells was not due to caffeine inhibition of thymidine-3H incorporation into DNA. Although N-acetoxy-AAF did inhibit the incorporation of thymidine-3H into DNA, caffeine did not modify this effect either way. Again, this result is identical to that found in caffeine-posttreated UV-irradiated cells (42).

It appears that, when lesions, such as those produced by UV or N-acetoxy-AAF are not excised to a great extent in Chinese hamster cells, gaps opposite these lesions are formed in the nascent DNA. Caffeine can prevent the de novo DNA synthesis necessary to repair these gaps (7, 13, 21, 22, 42). Assuming that this nonexcision, postreplica-
samples denote the standard error of the mean of 4 replicate plates for each point. Cells were exposed to 35 /IM N-acetoxy-AAF and the incorporation of thymidine-3H into TCA-insoluble material. Bars on the 6.5-hr chart represent the mean values.

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We wish to thank Mary Shaffer and Henryka Brania for their technical assistance and Dr. Elizabeth C. Miller and Dr. James A. Miller for their helpful discussions during this work. The senior author wishes to thank Dr. Van R. Potter, who provided both the opportunity and the physical space to accomplish this work.

ACKNOWLEDGMENTS

We wish to thank Mary Shaffer and Henryka Brania for their technical assistance and Dr. Elizabeth C. Miller and Dr. James A. Miller for their helpful discussions during this work. The senior author wishes to thank Dr. Van R. Potter, who provided both the opportunity and the physical space to accomplish this work.

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