Inhibition of Mitochondrial Protein Synthesis during Early Stages of Aflatoxin B1-induced Hepatocarcinogenesis


Laboratories of Biochemistry, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT

Experiments were designed to determine the in vivo effects of a single 6-mg/kg dose aflatoxin B1 on rat liver mitochondrial transcription and translation processes. With the use of intact hepatocytes and also a highly active mitoplast system for incorporation, it was observed that both mitochondrial transcription and translation activities are inhibited progressively even after 24 hr of carcinogen administration. Electrophoretic patterns of mitochondrial translation products show some qualitative changes during early periods of carcinogen administration. At later stages (>12 hr), however, there is a general inhibition of many of the products, although by this time there is a qualitative and quantitative recovery in the synthesis of mitochondrial proteins imported from the cytoplasm. These results, along with the data showing considerably high levels of aflatoxin B1 binding to mitochondrial DNA suggest that mitochondrial genetic system is one of the direct targets during experimental carcinogenesis.

INTRODUCTION

The role of mitochondria in the production and maintenance of cancer phenotype has been the subject of argument ever since the pioneering work of Warburg (27), which showed fundamental changes in the metabolic patterns of tumor tissues. Over the years, a number of studies have demonstrated altered mitochondrial content (8, 13), structure, and function (11, 12, 28) in a variety of tumor cells. It has been shown that mitochondria from tumor cells have altered ultrastructural organization, membrane composition, abnormal ion transport, and altered biochemical properties (for a discussion, see Ref. 22). Leukemic leukocytes and several solid tumors have been shown to contain unusual types of oligomeric and catenated forms of mitochondrial DNA (9, 18, 29). Another line of investigation suggests that mitochondria may be one of the cellular targets of attack by carcinogenic agents (22). Substantial amounts of carcinogens administered to experimental animals (2, 31) or incubated with tissue culture cells (4) not only are localized in the mitochondrial fraction but also are covalently bound to mitochondrial nuclear acids. Wunderlich et al. (31) found that hepatic carcinogen such as N-nitrosodimethylamine preferentially methylates mitochondrial DNA under both in vivo and in vitro conditions. Furthermore, experiments of Baker and Weinstein (4) showed that the dihydrodiol-epoxide derivative of benz(a)pyrene covalently modifies mitochondrial DNA at efficiencies 40 to 90 times greater than those for the nuclear DNA. Recent experiments in our laboratory showed the presence of a unique monoxygenase system, different from the microsomal activity in rat liver mitochondria, which can activate hepatic carcinogen AFB, into electrophilic reactive forms (19). The activated components bind to mitochondrial DNA, RNA, and proteins (19, 20). All of these results taken together suggest that the mitochondrial genetic system may be the direct and possibly important target of carcinogens. In an attempt to verify this possibility, we have now studied the extent of AFB attack on the mitochondrial genome and the resultant effects on mitochondrial transcription and translation processes under in vivo conditions.

MATERIALS AND METHODS

Materials. Amino acids, nucleotide triphosphates, chloramphenicol, cycloheximide, o-mannitol, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. RNase-free sucrose and electrophoresis grade SDS were from Polysciences Inc., Warrington, Pa. Electrophoresis grade acrylamide, bisacrylamide and other reagents used for electrophoresis were from Bio-Rad, Richmond, Calif. AFB was from Calbiochem/Behring Corp., La Jolla, Calif. Tissue culture media and reagents were from Grand Island Biological Co., Grand Island, N.Y. [35S]Methionine (1200 Ci/mmol) was from American Radioc chemicals Co. [3H]GTP (15 Ci/mmol) and [3H]CTP (20 Ci/mmol) were from New England Nuclear, Boston, Mass. All other chemicals used were of analytical grade.

Animals and Carcinogen Administration. In all the experiments, 5- to 7-week-old male Sprague-Dawley rats (150 to 200 g) were used. AFB, dissolved in dimethyl sulfoxide was injected i.p. at a dose of 6 mg/kg. The control animals received equal volumes of dimethyl sulfoxide. The treated and untreated animals were anesthetized with ether and sacrificed by cervical fracture. The livers were either removed and used for isolating mitochondria or perfused in situ for preparing hepatocytes.

Preparation of Mitochondria. Livers were washed free of blood clots with ice-cold 0.15 M NaCl and minced with a scissor into 2- to 3-mm slices. The tissue slices were homogenized in mitochondrial isolation buffer (2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5-0.2 M o-mannitol-0.07 M sucrose-1 mM EDTA-bovine serum albumin, 0.5 mg/ml) with 3 strokes of a loosely fitted (0.02-cm clearance) Teflon homogenizer at 1000 to 1200 rpm. The homogenate was made to 10% (w/v) with mitochondrial isolation buffer and centrifuged at 4°C for 15 min at 3000 g. The resulting mitochondrial pellet was washed twice in the isolation buffer, resuspended in the same buffer, and centrifuged at 4°C for 15 min at 3000 g. The mitochondrial pellets were suspended in 0.5 ml of the isolation buffer and stored at -80°C for later use. All operations were carried out at 4°C.

Protein Synthesis with Mitoplasts. The details of the method used for protein synthesis with isolated mitochondria have been described elsewhere (6, 7). Briefly, mitoplasts were suspended in protein synthesis buffer (15 mM Tris-HCl, pH 7.4; 65 mM KCl; 6 mM Mg(CH3COO)2; 5 mM 2-mercaptoethanol; 4 mM potassium phosphate buffer, pH 7.4; and 0.14 M sucrose at a concentration of 10 mg protein per ml and 4% (w/v) yeast RNA (20). The mitoplasts were incubated in a shaking water bath at 31°C for 0.5 hr and 1 hr in the presence of [3H]UTP and [3H]GTP.

Received October 12, 1980; accepted February 8, 1982.

1 Supported by NIH Grant CA-22762.
2 Recipient of a fellowship from the Nigerian Institute for Trypanosomiasis Research. Present address: Nigerian Institute for Trypanosomiasis Research, Vom, Jos, Nigeria.
3 To whom requests for reprints should be addressed.
4 The abbreviations used are: AFB, aflatoxin B1; SDS, sodium dodecyl sulfate.
supplemented with 2 mM ATP; 2 mM GTP; 5 mM creatine phosphate; 4 mM pyruvate; creatine phosphokinase, 0.2 mg/ml; and 100 μM concentrations each of 19 L-amino acids except methionine and cycloheximide, 300 μg/ml. The mixture was incubated for 5 min at 35° with or without added chloramphenicol. Protein synthesis was initiated with [35S]methionine (500 μCi/ml; 1000 Ci/mol), and the labeling was continued for the required length of time. Aliquots of 10 μl were removed at intervals and used for determining the hot CCl4COOH-insoluble counts (6). At the end of incubation, remaining mitoplasts were pelleted at 10,000 x g for 10 min, washed once with mitochondrial isolation buffer and kept frozen at -70° in the presence of 3 μg each of leupeptin and pepstatin as protease inhibitors.

RNA Synthesis with Mitoplasts. The method for RNA synthesis was modified from Avadhani et al. (1). Mitoplasts were suspended in protein synthesis buffer at a concentration of 3 mg protein per ml and supplemented with 2 mM ATP, 2 mM UTP, 0.5 mM GTP, 0.5 mM CTP, 5 mM malate, 5 mM creatine, creatine phosphokinase (400 μg/ml), spermidine (20 μg/ml), and 100 μM concentrations each of 20 amino acids. The mixture was preincubated at 37° for 3 min, and RNA synthesis was initiated with 20-μCi/ml concentrations each of [3H]GTP and [3H]CTP. The incorporation was continued for 40 min. At intervals, 10-μl samples were withdrawn and used for determining cold trichloroacetic acid-insoluble counts (1).

Protein Synthesis with Hepatocytes. Hepatocytes were prepared by perfusion with collagenase buffer, as described by Williamson et al. (30), except that Hanks’ medium was used for perfusion as well as for releasing the cells (10). The details of protein synthesis using [35S]methionine were as described elsewhere (20). Unless otherwise mentioned, protein synthesis was carried out for 120 min in either the presence or the absence of added inhibitors such as chloramphenicol and cycloheximide. Aliquots (10 μl) were taken out at intervals and used for determining the hot CCl4COOH-insoluble cpm as in the case of mitoplasts (6).

Electrophoretic Analysis of Proteins. Mitochondrial samples were dissolved in a lysis buffer containing 4% SDS and 10% 2-mercaptoethanol and dissociated by heating at 90° for 2 min (6). Electrophoresis was carried out on 8 to 16% gradient polyacrylamide slab gels containing 0.2% SDS, using the buffer system of Laemmli (14). The gels were stained with Coomassie blue, destained in 30% methanol-10% acetic acid, and processed for fluorography with En3hance (New England Nuclear).

RESULTS

To determine the direct and indirect effects of AFB, on mitochondrial biosynthetic and genetic processes, we have carried out experiments with intact hepatocytes and with isolated mitochondrial particles. As shown in Chart 1, the hepatocyte system used in these studies is highly active in protein synthesis. Addition of cycloheximide (300 μg/ml) inhibits the [35S]methionine incorporation by about 87%. Addition of cycloheximide and chloramphenicol together results in >98% inhibition. In separate experiments reported elsewhere (7), we have shown that the cycloheximide-resistant and chloramphenicol-sensitive incorporation, corresponding to about 8 to 12% of total cell synthesis in rat hepatocytes, represents true mitochondrial-specific protein synthesis.

The system for RNA and protein synthesis with isolated mitochondria involves the incubation of digitonin-washed mitoplasts in a hypotonic medium, which permits efficient incorporation (8). The kinetics of [35S]methionine incorporation by mitoplasts isolated from control liver has been presented in Chart 2A. Addition of chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, results in a near-total inhibition of incorporation. Similarly, the system incorporates [3H]GTP and [3H]CTP linearly up to about 40 to 60 min (Chart 2B), and the incorporation is sensitive to low amounts (0.5 μg/ml) of ethidium bromide as reported for other mitochondrial systems (17, 23).

The patterns of mitochondrial-specific translation and transcription after various time intervals of AFB (6 mg/kg) are presented in Chart 3. Experiments with isolated mitoplasts show a progressive inhibition of mitochondrial translation up to 60 hr after AFB administration. The extent of translational inhibition observed with the mitoplast system is nearly identical to the cycloheximide-resistant incorporation, which is characteristic of mitochondrial-specific translation intact hepatocytes (Chart 3). Similarly, the mitochondrial transcription activity as estimated by [3H]GTP and [3H]CTP incorporation by isolated mitoplasts resembles the pattern of translation inhibition following AFB administration. A previous report from this laboratory showed that nuclear transcription and cytoplasmic
translation activities in intact hepatocytes following a drug dose of 6 mg/kg are progressively inhibited up to about 6 to 9 hr (10). It was also observed that both of these activities are recovered to control level by about 12 hr and reach a near 150 to 220% level by 24 hr (10). Thus, in contrast to the recovery process seen in the nuclear and cytoplasmic activities between 9 and 24 hr, the mitochondrial biosynthesis processes remain inhibited at least up to 60 hr after carcinogen administration.

To determine the nature of AFB,-induced inhibition, the 35S-labeled mitochondrial translation products were analyzed by electrophoresis on 8 to 16% polyacrylamide gels under dissociating conditions. The autoradiogram presented in Fig. 1 shows that rat liver mitochondria synthesize about 20 polypeptides in the size range of about 8 x 10^3 to 13 x 10^4 daltons. The pattern of mitochondrial translation, 6 hr after AFB,-administration, shows several qualitative differences. Some unusual products in the size range of 10, 4.5, and 1.8 x 10^4 daltons are noticeable. The 12-hr pattern shows marked inhibition of several products, and the inhibition appears to be general. Although not shown here, the electrophoretic patterns of proteins after 24 and 58 hr of AFB,-treatment show pronounced inhibition.

To determine if the AFB,-induced changes in mitochondrial translation pattern are due to extramitochondrial effects or direct effects on mitochondrial genetic and biosynthetic systems, we have carried out 2 types of experiments.

In the first set of experiments, the effects of AFB,- on the labeling of mitochondrial polypeptides contributed by the cytoplasmic translation system were studied. As shown in Fig. 2, hepatocytes from rats treated with AFB,- (6 mg/kg) were labeled with [35S]methionine in the presence of chloramphenicol, and the mitochondrial particles isolated from these cells were electrophoresed on 8 to 16% gradient gels under highly dissociating conditions. The pattern of cytoplasmic translation inhibition by AFB,- appears to be general. Although not shown here, the electrophoretic patterns of proteins after 24 and 58 hr of AFB,- treatment show pronounced inhibition.

In the second set of experiments, we have determined the inhibitory effect appears to be recovered by 12 hr (Fig. 2B), and the 24-hr pattern (Fig. 2C) compares with the control pattern (Fig. 2D) both qualitatively and quantitatively.

In the second set of experiments, we have determined the levels of bound AFB,- to mitochondrial and nuclear DNA at
Effects of AFB<sub>i</sub> on Mitochondrial Translation

Various times after the carcinogen administration. As shown in Table 1, nuclear DNA contains about 3 AFB<sub>i</sub> adducts/10<sup>7</sup> daltons after 3 hr, whereas the mitochondrial genome at this time period contains about 9 adducts/10<sup>7</sup> daltons DNA. The number of AFB<sub>i</sub> adducts in the nuclear DNA declines steadily to reach about 0.3/10<sup>7</sup> daltons DNA at 24 hr. In contrast, the level of bound AFB<sub>i</sub> in the mitochondrial DNA remains nearly the same even after 24 hr. Results of these experiments together suggest that prolonged inhibition of mitochondrial translation and altered mitochondrial polypeptide pattern may result from the direct effects of AFB<sub>i</sub> on the mitochondrial genetic system, although some indirect effects involving the nuclear gene products cannot be overruled.

DISCUSSION

Recent studies in our laboratory showed that nuclear transcription and cytoplasmic translation activities following a single dose of AFB<sub>i</sub> exhibit typical inhibitory and recovery patterns (10). After nearly 80% inhibition of both of the processes at 6 to 9 hr, the activities reach a peak of about 220% level at 24 hr after AFB<sub>i</sub> administration (10). In view of this, the patterns of mitochondrial transcription and translation activities following AFB<sub>i</sub> administration are markedly different (Chart 3), in that both of these activities remain inhibited without any apparent recovery up to about 24 to 60 hr.

The analysis of mitochondrial translation products on gradient SDS-polyacrylamide gels under highly dissociating conditions shows that control mitochondria synthesize about 20 to 22 polypeptides including some in the range of 13 x 10<sup>4</sup> daltons. Recently, we have shown that all of these polypeptide species represent true mitochondrial translation products, although some may be the precursors of smaller polypeptides (6). The polypeptides synthesized after 6 hr of AFB<sub>i</sub> treatment contain several unusual species not seen in the control samples (Fig. 1C). These polypeptides may be originated from altered processing of mitochondrial transcripts or are probably due to defective processing of the precursor polypeptides. At longer duration of AFB<sub>i</sub> treatment, however, there is increasing inhibition of several products. The inhibition appears to be general and may result from the inhibition of mitochondrial transcription process.

It is now reasonably well established that most of the mitochondrial proteins are imported from the cytoplasm (24). These imported proteins are believed to be coded by the nuclear genes (24, 26). The mitochondrial genetic system, on the other hand, contributes to only ~10% of the proteins, which are highly essential for the assembly of mitochondrial membranes and their function (24, 26). Although not conclusively proven, it is widely believed that most, if not all, of mitochondrially translated proteins are coded for by the mitochondrial genome (26). The observed inhibition of mitochondrial translation products may therefore be due to the direct attack on mitochondrial genetic system. Alternatively, the inhibition may be due to decreased cytoplasmic synthesis of mitochondrial biosynthetic enzymes, which are known to be imported from the cytoplasm (24). Continued inhibition of mitochondrial activity even after full recovery of cytoplasmic translation at 24 hr (Chart 3; Ref. 10, 21) suggests that this is a less likely possibility. This view is further supported by experiments showing a qualitative and quantitative recovery in the labeling patterns of cytoplasmically imported mitochondrial proteins at 24 hr after carcinogen administration (Fig. 2, C and D). Support for the direct involvement of mitochondrial genetic system comes from experiments on the levels of binding of AFB<sub>i</sub> to nuclear and mitochondrial DNA (Table 1). As shown for a number of carcinogens (16), the level of bound AFB<sub>i</sub> in the nuclear DNA reduces gradually to reach 0.3 adduct/10<sup>7</sup> daltons DNA at 24 hr. Thus, the recovery in the nuclear transcription and cytoplasmic translation may reflect upon the removal of AFB<sub>i</sub> adducts and the repair of DNA (15). Contrary to this, the level of bound AFB<sub>i</sub> in the mitochondrial genome remains nearly constant even up to 24 hr, which is possibly due to the lack of repair in this organelle system (4).

The authors are thankful to Nina Leinwand for helping with the preparation of the manuscript and to Joann Shepard and Aruna Bhat for technical assistance.

ACKNOWLEDGMENTS

The authors are thankful to Nina Leinwand for helping with the preparation of the manuscript and to Joann Shepard and Aruna Bhat for technical assistance.

REFERENCES

1. Avadhani, N. G., Batula, N., and Rutman, R. J. Messenger ribonucleic acid...
Inhibition of Mitochondrial Protein Synthesis during Early Stages of Aflatoxin B₁-induced Hepatocarcinogenesis


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/42/5/1876

---

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.