THE EFFECT OF BENZO[a]PYRENE ON DNA SYNTHESIS AND DNA POLYMERASE ACTIVITY OF RAT LIVER MITOCHONDRIA

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1. Introduction

The circular, double-stranded, covalently-closed mitochondrial genome has proved to be a target of choice for some carcinogenic drugs. When animal cells are incubated with radioactively labeled benzo[a]-pyrene (B[a]P), metabolites of the drug are found covalently linked to both nuclear and mitochondrial DNA, although the amount of derivative/μg DNA is higher in the organelle genome [1,2]. The cause of the higher mitochondrial DNA labeling, as compared with the nuclear DNA, has been attributed to the presence in the nucleus of proteins able to interact with DNA, thus protecting it against the action of xenobiotics. Mitochondrial DNA is much less complexed to proteins, but a histone-like protein has been described in yeast mitochondria [3].

Considerable interest has been addressed to the problem of the metabolic fate of some carcinogenic drugs once they have entered the animal cell. The most studied site of metabolic activation of these drugs is the endoplasmic reticulum [4,5]. The enzymes involved in the activation of such substances are also found in the nucleus [6], and the mitochondria [7,8] of animal cells.

We are studying the implications of carcinogenic drugs like B[a]P at the level of DNA synthesis. For this purpose we have followed DNA synthesis in isolated rat liver mitochondria of normal and B[a]P-treated animals, and studied the level and properties of the mitochondrial DNA polymerase solubilized from the organelles of animals treated with the drug.

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2. Materials and methods

Calf thymus DNA was purchased from Sigma. DNA activation was performed by mild digestion with pancreatic DNase purchased from Sigma or Boehringer [9]. Synthetic polynucleotides and nucleotides triphosphates were from Sigma or Boehringer. Ethidium bromide, *N*-ethyl maleimide, digitonin and dideoxy TTP (ddTTP) were from Boehringer. Aphidicolin was a kind gift of Dr B. Hesp and A. H. Todd from Imperial Chemicals. Radioactive precursors for DNA synthesis were from New England Nuclear.

2.1. *Animal treatment*

Female mice strain Wistar 35 days old were used. B[a]P at 40 mg/kg animal was injected intraperitoneally in corn oil. Control animals were injected with corn oil. Animals were killed by decapitation 48 h after the injection.

2.2. *Purification of rat liver mitochondria*

About 20 g rat liver were cut with scissors and homogenized in a medium containing: 250 mM sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 3 mM $CaCl_2$ and 0.5 mM phenyl methyl sulphonyl fluoride (PMSF) (medium A). The homogenization was carried out in a Potter (Teflon–glass) system. The crude homogenate was centrifuged at 3000 × *g* for 10 min. The pellet was discarded or used for nuclei purification. The supernatant was centrifuged at 10 000 × *g* for 10 min. The pellet was resuspended in 5 ml buffer B (10 mM Tris–HCl (pH 7.4), 0.1 mM EDTA and 0.1 mM PMSF) in 15% (w/v) sucrose. This suspension was centrifuged over a discontinuous sucrose gradient formed of 10 ml 25% over 10 ml 42% sucrose in buffer B. Centrifugation was carried

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out in a SW-25-2 rotor of the Spinco ultracentrifuge for 45 min at 14 000 rev./min at 4°C. The mitochondrial layer, in the border between the 25 and 42% layers was recovered with a Pasteur pipette, diluted with an equal volume of buffer B in 10% sucrose and centrifuged at 12 000 × *g* for 20 min. The pellet was resuspended in 10% sucrose in buffer B and treated with digitonin (0.3 mg digitonin/mg mitochondrial protein). The treated organelles were centrifuged at 10 000 × *g* for 20 min. The mitochondrial pellet was washed twice by centrifugation under the same conditions and the purified mitochondria were resuspended in buffer B in 10% sucrose and kept at −80°C.

2.3. *DNA synthesis in isolated mitochondria*

The incubation mixture contained the following reagents in a final volume of 0.1 ml: 50 mM Tris–HCl (pH 8.0), 2 mM DTT, 50 μM dCTP, dATP and dGTP each, 4 μCi [^{3}H]TTP 10 μM (spec. act. 500–2000 cpm/pmol), 80 mM KCl, 2 mM $MnCl_2$, 100 μg bovine serum albumin, 1 mM ATP and varying amounts of mitochondrial protein. The reactions were stopped by addition of 0.2 ml 10% trichloroacetic acid plus 100 mM sodium pyrophosphate. After 10 min in ice the precipitates were filtered through nitrocellulose membranes (Scleicher and Schuell), washed with 2% trichloroacetic acid, dried and counted in a PPO, POPOP, toluene scintillation mixture.

2.4. *Solubilization of mitochondrial DNA polymerase activity*

To the mitochondrial suspension were added Triton X-100 to 0.5% final conc. and KCl to 100 mM final conc. Sonication was performed in a Braunsonic 1510 sonicator at ~50 W for 3 periods of 15 s with intervals of 45 s in ice. Centrifugation for 10 min at 10 000 × *g* gave a pellet that was discarded. The final supernatant was adjusted to 50% glycerol and ~2 mg protein/ml final conc.

2.5. *DNA polymerase γ assay*

The assay conditions for DNA polymerase γ were used [11], with poly (A–dT_{12}) as template and 50 mM potassium phosphate to inhibit DNA polymerase β activity. When aphidicolin was tested, the drug was solubilized in dimethyl sulphoxide and a control with the same amount of solvent was used. In the experiments with aphidicolin, activated DNA was used as template and the concentration of dNTPs was 10 μM for dCTP, 50 μM for dGTP and dATP and [^{3}H]TTP was used with spec. act. ~2000 cpm/pmol.

Protein was determined by the Lowry method [12] and DNA by the diphenylamine method [13].

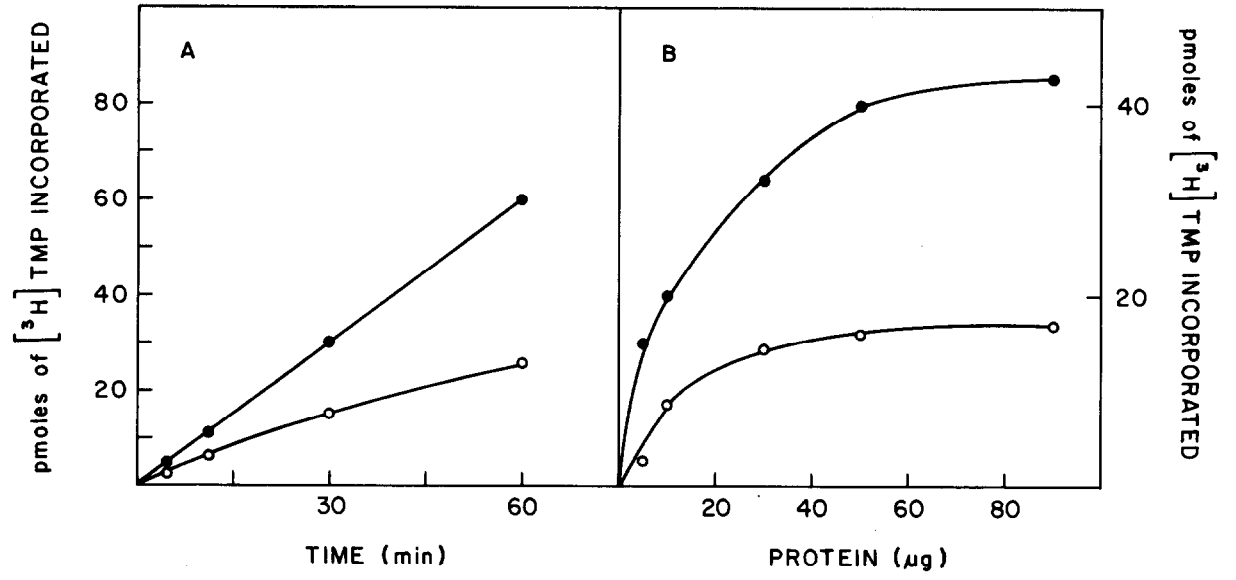

Fig.1. DNA synthesis in whole mitochondria: (A) Kinetics of TMP incorporation in mitochondria from normal (•) and B[a]P-treated rats (○); (B) incorporation of TMP with different amounts mitochondrial protein; (•) normal mitochondria; (○) B[a]P-treated animal mitochondria. Incubation was carried out for 30 min at 37°C as described in the text. The amount of mitochondrial DNA is not affected in B[a]P-injected rats, thus pmol TMP incorporated/mg protein or mg DNA are equivalent.

3. Results and discussion

Mitochondria isolated from normal and B[a]P-treated rats were assayed for DNA synthesis. As seen in fig.1, the extent of DNA synthesis, both at varying times of incubation (1A) or at different protein concentrations (1B), was higher in mitochondria from control animals than in those from treated rats. Essentially the same results were obtained using labeled thymidine (not shown) or thymidine triphosphate as precursors. Animal mitochondria possess an efficient system of nucleotide phosphorylation [14]. The lower ability to synthetise DNA of the mitochondrial fraction from rat liver of B[a]P-treated animals may be related to the high degree of modification found in the mitochondrial DNA of animal cells after exposure to carcinogens [1,2]. Mitochondria have one major DNA polymerase activity, the so called DNA polymerase γ [15–17], this enzyme being responsible for the replication of mitochondrial DNA. DNA polymerase γ is strongly inhibited by ddTTP and ethidium bromide, while aphidicolin, a specific inhibitor of DNA polymerase α does not affect the mitochondrial polymerase [10,18,19]. As we have found that nuclear DNA polymerase α is severely decreased by in vivo treatment with B[a]P (unpublished), it seemed interesting to study the effect of the drug on the mitochondrial DNA polymerase. To our surprise we found that the level of DNA polymerase solubilized from mitochondria of in vivo B[a]P-treated rats was much higher than in control mitochondria as can be seen in fig.2. We have also analysed the membrane mitochondrial pellet after sonication to see whether the higher activity solubilized from mitochondria of B[a]P-treated rats could be explained by a different release mediated by sonication, detergent and ionic strength as in section 2. We found that this is not a satisfactory explanation, since the mitochondrial pellet obtained after solubilization of DNA polymerase γ from treated rat mitochondria had also a higher activity than the mitochondria of control animals (not shown).

The effects of ddTTP on mitochondrial DNA synthesis in whole organelles from both, B[a]P and control animals, is shown in fig.3A: the inhibition of DNA synthesis in normal mitochondria was strongly affected by ddTTP, while in mitochondria from treated animals the extent of inhibition was much lower (the extent of incorporation as shown in fig.1 is lower in treated rats). In fig.3B we have studied

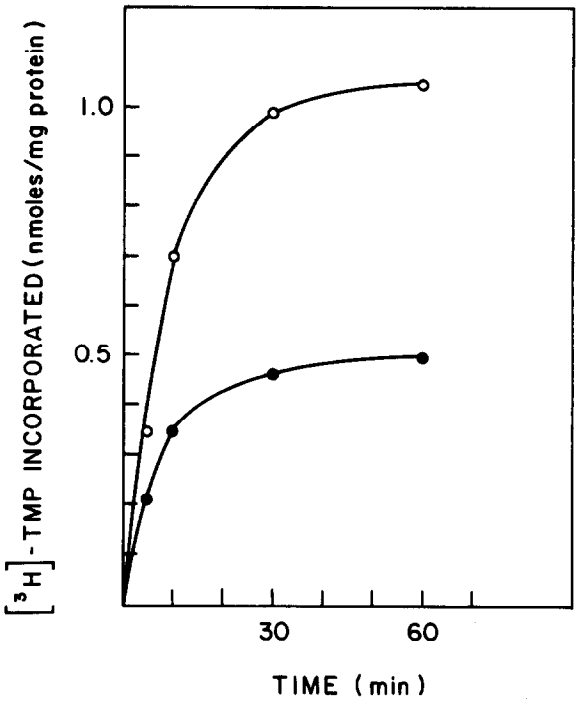

Fig.2. Mitochondrial DNA polymerase activity solubilized from normal (•) and B[a]P-treated rats (○). The assay for DNA polymerase γ was performed as in section 2.

the DNA synthetic capacity of mitochondria from control and treated animals in the presence of the detergent Triton X-100. It can be seen that the results in fig.3A and 3B are very similar, indicating that the permeability of ddTTP was not affected in the organelle of B[a]P-treated animals. The level of phosphatases is undistinguishable in both types of mitochondria (not shown). No effect of aphidicolin on DNA synthesis in whole mitochondria from both types of animals was observed in the presence or absence of Triton X-100 (not shown). Some possible explanation to this observation is that the increased DNA polymerase activity found in the mitochondria of B[a]P-injected rats (fig.2) can be ascribed to other polymerases than polymerase γ, or to a modified form of this polymerase resistant to ddTTP. In order to try to solve this problem we performed the experiment shown in table1 where the action of different inhibitors was assayed on the DNA polymerase activity solubilized from the mitochondria of control or drug-injected rats. As seen in table 1 the pattern of inhibition by ddTTP and ethidium bromide is very similar for the polymerases solubilized from both types of organelles, while

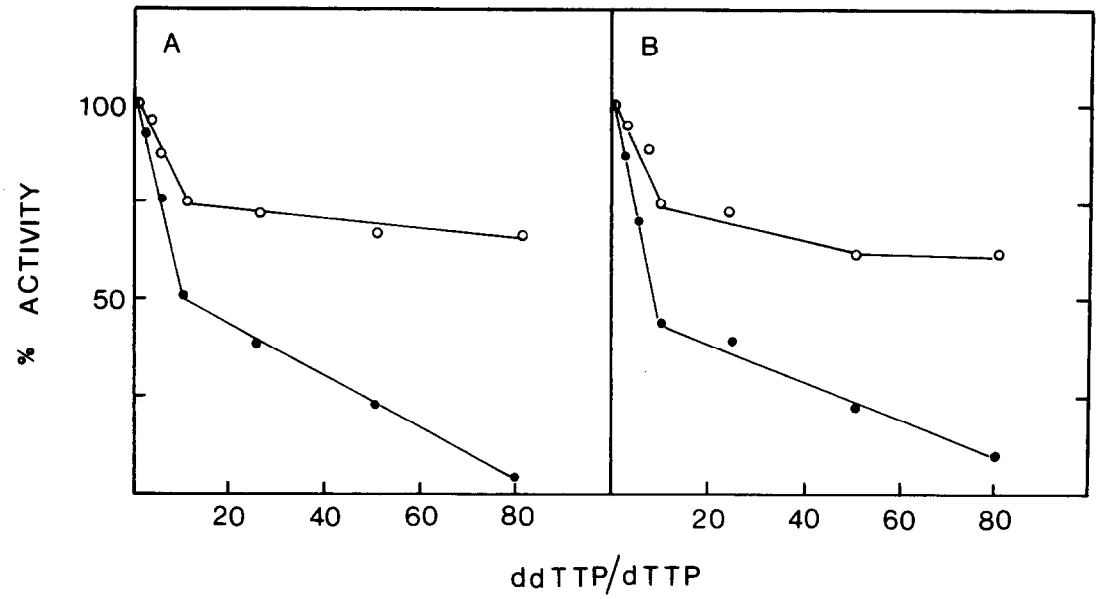

Fig.3. Inhibition of DNA synthesis by ddTTP in whole mitochondria of normal (•) and B[a]P-treated rats (○). (A) The assay was carried out for 30 min as in section 2. The incorporation of TMP corresponding to 100% activity was 35 pmol for normal mitochondria and 9 pmol for B[a]P-treated rat liver mitochondria. (B) Assay performed in the presence of Triton X-100 to 0.5% final conc. Conditions as in (A). 100% activity was 27 pmol in controls and 7 pmol in organelles of B[a]P-treated rats.

Table 1
Effect of inhibitors on the DNA polymerase activity solubilized from rat liver mitochondria of normal and B[a]P-treated rats

Inhibitor	Conc. (μM)	ddTTP / dTTP	% Enzyme inhibition	
			Control	B[a]P-treated
None	–	–	0	0
ddTTP	4	0.4	16	38
ddTTP	10	1	44	60
ddTTP	20	2	78	67
ddTTP	40	4	80	73
Aphidicolin	2	–	4	2
Aphidicolin	4	–	0	8
Aphidicolin	15	–	3	0
Aphidicolin	25	–	0	4
Ethidium bromide	4	–	65	80
Ethidium bromide	10	–	82	82

The incubation mixtures used for each inhibitor are described in section 2. The incorporation of [^{3}H]TMP in the absence of inhibitor (0% inhibition) was 25 pmol/30 min with the enzyme solubilized from normal mitochondria and 38 pmol/30 min for the polymerase from B[a]P-treated rats

aphidicolin does not affect the mitochondrial DNA polymerase activity from normal or B[a]P-treated animals at very high doses. They may indicate that the higher level of solubilized activity from the organelles of treated animals corresponded to polymerase γ and not to the entrance into the organelles of other types of polymerases. Besides the effect of inhibitors shown in table 1 we have found other properties of both types of DNA polymerases that seem to be identical: the preferred utilisation of poly(A–dT_{12}) template, the optimum manganese concentration and the strong stimulatory effect of KCl on DNA polymerase activity (not shown). Thus, the reason for the differential effect of ddTTP on DNA synthesis in both types of mitochondria remains to be established. We can only speculate that the carcinogen treatment can change the interactions of the enzyme inside the organelle leading to a DNA replication complex resistant to ddTTP. Once solubilized by sonication, ionic strength and detergents, both types of polymerases behave similarly with the different inhibitors we have used (table 1).

Moreover we have determined by mixing experiments (with mitochondrial extracts from control and treated animals) that no inhibitory or stimulatory substances are involved in the increased DNA polymerase activity released from mitochondria of B[a]P-treated animals (not shown).

Assuming that DNA polymerase γ is the only mitochondrial polymerase able to copy DNA templates, this enzyme should be responsible for both, the replication and repair of the mitochondrial genome. DNA polymerase β is the only enzyme activity that could account for the strong stimulation of DNA synthesis after ultraviolet irradiation of animal cells: this enzyme has been assigned a role in nuclear DNA repair [20]. Similarly, it can be postulated that DNA polymerase γ is increased in mitochondria from B[a]P-treated rats as a response to the high degree of modification of the mitochondrial genome by the drug. A striking resemblance with our results can be found in the reported induction of *Tetrahymena pyriformis* mitochondrial DNA polymerase after ultraviolet irradiation [21]. Work is in progress to ascertain whether the increase of DNA polymerase activity we have found in B[a]P-treated rat liver mitochondria is due to a higher synthesis of the polymerase or to a more efficient entrance of the enzyme into the organelles.

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