Non-involvement of nucleolytic activities in the early effect of dimethylnitrosamine on the content of free mRNA in mouse liver

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1. After dimethylnitrosamine (DMNA) administration to mice, the content of poly(A)-containing RNA decreases rapidly in the postmicrosomal fraction of the liver. We report here that the loss of free mRNA is not a result of increased nucleolytic activity. On the contrary, a decreased activity of microsomal endonuclease, assayed by its effect on polyribosomal mRNA, was demonstrated already 15 min after the administration of DMNA at 37.5 mg/kg body wt. The loss of activity was more pronounced in the rough than in the smooth membranes. Total detergent-released microsomal nucleases, as assayed by use of labelled poly(U) as substrate, showed a less rapid decline. No corresponding increase in enzyme activities was observed in the postmicrosomal fraction. 2. The dimethylnitrosamine effect on the microsomal endonuclease was not accounted for by altered lysosomal contamination of the microsomal fraction. 3. No early effect of dimethylnitrosamine administration was found on the cytoplasmic ribonuclease inhibitor.

The administration of the hepatotoxin DMNA to mice is followed by a rapid depression of protein synthesis in the liver, and a concomitant loss of poly(A)-containing RNA from the postmicrosomal liver fraction (Nygård & Hultin, 1979, 1981). These effects appear within 15 min of DMNA treatment. Later, RNA synthesis is also affected (Villa-Trevino, 1967; Herzog & Farber, 1976), and the content of poly(A)-containing RNA decreases in all cytoplasmic fractions (Hultin *et al.*, 1978).

The cause of the early decrease in postmicrosomal poly(A)-containing RNA is not clear, but the following alternatives have been suggested: (a) a mobilization of the free mRNA pool for polyribosome formation in response to the decreased efficiency of mRNA utilization on pre-existing polyribosomes; (b) increased nucleolytic activity, preferentially acting on uncomplexed mRNA (Nygård & Hultin, 1979). In the preceding paper (Nygård & Hultin, 1981) some indirect evidence was presented in favour of the former alternative. In the experiments described below the latter possibility is examined.

Work on interferon (Kerr *et al.*, 1976) indicates that the activation of mRNA-degrading endoribonucleases may be involved in the inhibition of protein synthesis *in vivo*. In the present experiments the

highly sensitive polyribosome-degradation assay (Davies & Larkins, 1974) was used for determining endoribonuclease activities, including any specific mRNA-degrading enzymes. Poly(A) hydrolase(s) and total RNAase activities were measured by homopolyribonucleotide degradation. In the liver increased RNAase activity may result from the inactivation of a RNAase inhibitor (Roth, 1958), as demonstrated for aminofluorene *in vitro* (Morais *et al.*, 1972). This possibility was also investigated.

No increase in nuclease activities was found in the early phase of DMNA treatment. On the contrary, a decreased endonuclease activity was constantly observed in the microsomal fraction. The RNAase inhibitor was unaffected during the same period. The results seem to rule out RNAase activation as a cause of the early loss of postmicrosomal poly(A)-containing RNA in DMNA-treated mice.

Experimental

Chemicals

DMNA was obtained from Eastman Kodak Co., Rochester, NY, U.S.A. [³H]Poly(A) (76 870 c.p.m./μg) and [³H]poly(U) (30 494 c.p.m./μg) were from Miles Laboratories, Slough, Bucks., U.K. DNA, D-glucose 6-phosphate, β-glycerophosphate, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, *o*-nitrophenyl β-D-galactopyranoside, ribonuclease A (EC 3.1.27.5) and *N*-ethylmaleimide were from Sigma

Abbreviations used: DMNA, dimethylnitrosamine; RNAase, ribonuclease.

Chemical Co., St. Louis, MO, U.S.A. Methyl Green was from E. Merck, Darmstadt, Germany. Sephadex G-15 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Membrane filters (HAWP, 0.45 μ m) were from Millipore Co., Bedford, MA, U.S.A.

Methods

Preparation of subcellular liver fractions. Animals and DMNA treatment were as described in the accompanying paper (Nygård & Hultin, 1981). Treatment was for 15 min, if not otherwise indicated. The livers from each experimental group of mice were homogenized in buffer A (20 mM-triethanolamine/HCl, pH 7.6, 25 mM-KCl, 3 mM-magnesium acetate, 1 mM-dithiothreitol and 0.25 M-sucrose) (2 ml/g of tissue) and centrifuged for 10 min at 15 000 $g_{av.}$. The supernatants were centrifuged for 60 min at 166 000 $g_{av.}$ to give the post-microsomal supernatant and the microsomal pellet. Small samples of the supernatants were rapidly frozen, and stored at -70°C . The microsomal pellets were resuspended in 5.45 ml of buffer A, and 0.55 ml of 10% (w/v) sodium deoxycholate was added before re-centrifugation for 90 min at 107 000 $g_{av.}$. Small batches of the extracts were frozen and stored at -70°C .

Rough and smooth microsomal membranes were prepared as described by Dallner (1974, 1978). The isolated membrane fractions were treated with 1% (w/v) sodium deoxycholate and re-centrifuged as described above. The extracts were stored in small portions at -70°C .

N-Ethylmaleimide treatment. Samples (0.5 ml)

of the fractions to be treated were passed through a 5 ml (1.1 cm $\times$ 4.5 cm) Sephadex G-15 column equilibrated with buffer B (40 mM-Tris/HCl, pH 8.5, 20 mM-KCl and 10 mM-MgCl₂). The gel-filtered material was treated with 1 mM-N-ethylmaleimide for 10 min at 20°C , and the excess reagent was inactivated by the addition of dithiothreitol (final concn. 2 mM).

Preparation of polyribosomes. The livers from starved mice were homogenized in 2 vol. of buffer A. The homogenates were centrifuged for 10 min at 15 000 $g_{av.}$. The lipid layer was carefully removed. The supernatant was collected and treated with 0.1 vol. of 10% (w/v) sodium deoxycholate. Samples (3 ml) were layered on to 2 ml 'cushions' of 1.46 M-sucrose in 50 mM-Tris/HCl, pH 7.6, containing 25 mM-KCl, 5 mM-magnesium acetate and 1 mM-dithiothreitol. The material was re-centrifuged for 90 min at 189 000 $g_{av.}$ in a Beckman SW 50.1 rotor. Ribosomal pellets were quickly frozen in a solid- CO_2 /ethanol bath and stored at -70°C until use.

Assay of RNAase activities. Endonuclease activity was analysed by the polyribosome-degradation assay of Davies & Larkins (1974). Polyribosomes (approx. 3.9 A_{260} units) were incubated for 30 min at 0°C with different amounts of nuclease-containing liver fractions. The ion composition of the reaction mixture was as in buffer B. The 0.3 ml incubation mixtures were analysed by centrifugation in 0.29–1.46 M-sucrose gradients as previously described (Nygård & Hultin, 1979) (Fig. 1). The decrease in the number of inter-ribosomal bonds (Vassart *et al.*, 1970) was used to quantify the endonucleolytic effect.

Fig. 1. Endonuclease activity of mouse liver microsomal fractions as determined by polyribosome-degradation assay. Mouse liver polyribosomes (approx. 3.9 A_{260} units) were incubated in buffer B with protein released from mouse liver microsomal fractions by sodium deoxycholate treatment. After 30 min at 0°C , the 0.3 ml reaction mixtures were placed on 11 ml 0.29–1.46 M-sucrose gradients containing 20 mM-Tris/HCl, pH 7.6, 20 mM-KCl, 2 mM-magnesium acetate and 1 mM-dithiothreitol. The gradients were centrifuged for 2 h at 200 000 $g_{av.}$ and monitored at 260 nm in a recording spectrophotometer equipped with a flow cell. (a) No protein added; (b) microsomal protein (20 μ g) from control mice; (c) microsomal protein (20 μ g) from mice treated for 15 min with DMNA (37.5 mg/kg body wt.).

Fig. 2. Endonucleolytic activity of postmicrosomal supernatants

Postmicrosomal fractions from (●) control and (▲) DMNA-treated mice were incubated for 60 min with mouse liver polyribosomes as described in Fig. 1. (a) Untreated postmicrosomal fractions; (b) postmicrosomal fractions treated with 1 mM-*N*-ethylmaleimide. In this and subsequent Figures, '-Δ Inter-ribosomal bonds' is as used by Davies & Larkins (1974).

Poly(A) hydrolase and total RNAase activities were determined by degradation of [³H]poly(A) and [³H]poly(U). The 120 μl reaction mixtures contained 30 mM-Tris/HCl, pH 8.5, 25 mM-KCl, 7.5 mM-MgCl₂, 0.1 μg of [³H]poly(A) or 0.4 μg of [³H]poly(U), and protein as indicated. The samples were incubated at 37°C for different periods. After incubation, 2 ml of cold 10% (w/v) trichloroacetic acid was added, and the tubes were placed on ice for 20 min. Precipitated material was collected on cellulose nitrate filters, dried and counted for radioactivity as described by Nygård & Hultin (1981), but at an efficiency of 25%. Protein was measured as described by Nygård & Hultin (1981).

Assay of lysosome-associated enzymes. Acid deoxyribonuclease (EC 3.1.22.1) was determined by use of the Methyl Green method (Kurnick, 1962). The activities of β-galactosidase (EC 3.2.1.23) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) were determined as described by Sellinger *et al.* (1960). Glucose 6-phosphatase (EC 3.1.3.9) activity was measured as described by de Duve *et al.* (1955). The activity of acid phosphatase (EC 3.1.3.2) was measured by the method of Gianetto & de Duve (1955) by using tartrate inhibition (Barrett, 1969).

Results

Endoribonuclease activity

The endonuclease activities of the postmicrosomal liver fractions from DMNA-treated and control mice were determined by the polyribosome-degradation assay (Fig. 2). Because of the presence of RNAase inhibitor (Roth, 1958), no polyribosome degradation was obtained with either preparation in the native state, even at very high protein concentrations (Fig.

Fig. 3. Effect of DMNA treatment on the endonuclease activity of mouse liver microsomal fractions

Incubation was as described in Fig. 1, with different concentrations of sodium deoxycholate-released microsomal protein from (●) control mice or mice treated with DMNA (37.5 mg/kg body wt.) for (▲) 15 min or (■) 120 min.

2a). After inactivation of the inhibitor with *N*-ethylmaleimide, the polyribosomes were rapidly degraded (Fig. 2b). No increase, but rather a slight gradual decrease, in specific endonuclease activity was observed in the *N*-ethylmaleimide-treated postmicrosomal fractions after DMNA administration.

The endonuclease activity released from the microsomal pellets by sodium deoxycholate treatment is shown in Fig. 3. After DMNA administration the activity decreased progressively. Already after 15 min it was decreased by 20%. The decrease was more pronounced in the rough than in the smooth membranes (Fig. 4). *N*-Ethylmaleimide

Fig. 4. Endonuclease activity of smooth and rough endoplasmic membranes
Incubation was as described in Fig. 1. The sodium deoxycholate-released proteins were obtained from (a) smooth and (b) rough endoplasmic membranes, from (●) control or (▲) DMNA-treated (15 min, 37.5 mg/kg body wt.) mice.

treatment markedly increased the endonuclease activity of the microsomal extracts, but the difference between the DMNA-treated and control mice was still very pronounced (results not shown). It should be noted that the total endonuclease activity was much smaller in the microsomal extracts than in the *N*-ethylmaleimide-treated post-microsomal fractions.

Poly(A) hydrolase activity

The poly(A)-degrading activities of the post-microsomal fractions and microsomal extracts are shown in Table 1. The DMNA treatment had very little effect on the activity and distribution of the enzyme(s). After 2 h a slight increment was noticed in the microsomal extract, but not in the post-microsomal fraction. Thus the poly(A) hydrolase determinations failed to account for the rapid decrease in postmicrosomal poly(A) in the early period after DMNA administration (Nygård & Hultin, 1979, 1981).

Poly(U)-degrading activities

For measuring the total endo- and exo-nuclease activities of the microsomal and postmicrosomal fractions, [^3H]poly(U) was used as substrate (Table 1). In this assay appreciable activity was observed in both fractions without *N*-ethylmaleimide treatment. No increase in poly(U)-degrading activity was observed after DMNA treatment. In the post-microsomal fraction the activity was essentially the same even after 2 h. In the microsomal extracts there was a progressive decrease in activity.

Activity of the RNAase inhibitor

Liver RNAase inhibitor is susceptible to inactivation by electrophilic reagents (Roth, 1958), and is therefore a potential target of reactive DMNA

Table 1. Poly(A)- and poly(U)-degrading activities of postmicrosomal and microsomal mouse liver fractions

The reaction mixtures contained 30 mM-Tris/HCl, pH 8.5, 25 mM-KCl, 7.5 mM-MgCl₂, 0.1 μg of [^3H]poly(A) (76 870 c.p.m./ μg) or 0.4 μg of [^3H]poly(U) (30 494 c.p.m./ μg) and 170 μg of protein from the postmicrosomal fraction or 15 μg of sodium deoxycholate-released microsomal proteins. Incubation was at 37°C for 20 min [poly(A) degradation] and 5 min [poly(U) degradation]. After incubation, the undegraded material was precipitated with cold 10% (w/v) trichloroacetic acid and collected on Millipore filters. Values in parentheses represent percentages of controls.

Fraction	Treatment	Polynucleotide degraded ($\mu\text{g}/\text{mg}$ of protein)	
		Poly(A)	Poly(U)
Supernatant	Control	0.20 (100)	2.04 (100)
	DMNA 120 min	0.20 (99)	1.87 (92)
Microsomal	Control	0.50 (100)	13.4 (100)
	DMNA 15 min	0.51 (102)	12.0 (90)
	DMNA 120 min	0.57 (114)	7.1 (53)

metabolites (cf. Morais *et al.*, 1972). As shown in Fig. 5, the inhibitor in the postmicrosomal fraction was only partially saturated by the endogenous endoribonucleases. The excess capacity could be measured by adding increasing amounts of RNAase A to the assay (Fig. 5). No decrease in the inhibitor capacity was observed during the early phase of DMNA treatment, concomitant with the rapid loss of poly(A)-containing RNA from the post-microsomal fraction (Nygård & Hultin, 1979, 1981).

Lysosome-associated enzymes in the microsomal fraction

The possibility was considered that the endo-

Table 2. Activity of lysosomal and microsomal enzymes in the microsomal fraction
DMNA treatment was for 15 min at 37.5 mg/kg body wt. Units of enzyme activity were as defined by (a) Gianetto & de Duve (1955), (b) Sellinger *et al.* (1960), (c) Kurnick (1962) and (d) de Duve *et al.* (1955). Values for DMNA treatment in brackets are percentages of control values. Data represent mean values $\pm$ S.D. for three experiments.

Enzyme	Activity (units/mg)	
	Control	DMNA treatment
Acid phosphatase (a)	$1.00 (\pm 0.03) \times 10^{-2}$	$1.02 (\pm 0.01) \times 10^{-2}$ [100]
β -Galactosidase (b)	$4.1 (\pm 0.1) \times 10^{-4}$	$3.7 (\pm 0.1) \times 10^{-4}$ [90]
β -N-Acetylglucosaminidase (b)	$4.4 (\pm 0.1) \times 10^{-3}$	$3.8 (\pm 0.2) \times 10^{-3}$ [86]
Acid deoxyribonuclease (c)	$3.5 (\pm 0.2) \times 10^{-3}$	$3.2 (\pm 0.1) \times 10^{-3}$ [91]
Glucose 6-phosphatase (d)	$6.11 (\pm 0.08) \times 10^{-1}$	$5.92 (\pm 0.04) \times 10^{-1}$ [97]

Fig. 5. Effect of DMNA treatment on the activity of the cytoplasmic ribonuclease inhibitor
Incubation was as described in Fig. 1. Each incubation contained 240 μ g of postmicrosomal protein from (●) control or (▲) DMNA-treated (15 min, 37.5 mg/kg body wt.) mice, and increasing amounts of RNAase A as indicated.

nuclease activity in the microsomal fraction could be of lysosomal origin, and that the rapid decrease after DMNA administration (Figs. 3 and 4) might reflect a decreased contamination of the fraction with lysosomal material, e.g. as a consequence of lysosome enlargement (Ganote & Rosenthal, 1968).

As shown in Table 2, the activities of a number of enzymes, currently used as lysosome markers (Barrett, 1969; Tappel, 1969), failed to show the rapid fall after DMNA treatment characteristic of the microsomal endonuclease. The activity of microsomal glucose 6-phosphatase also decreased less rapidly than the endonuclease activity (cf. Hendy & Grasso, 1975). The data strongly suggest that the microsomal endonuclease was not derived from contaminating lysosomes, and that the early effects of DMNA (Nygård & Hultin, 1979, 1981) were not related to a redistribution of lysosomal components.

Discussion

DMNA produces morphological and functional

alterations in liver microsomal fractions and lysosomes, i.e. in cell organelles known to contain nucleolytic enzymes (Mukherjee *et al.*, 1963; Slater & Greenbaum, 1965; Smuckler *et al.*, 1967; Hendy & Grasso, 1972). The possible involvement of nucleases in the DMNA-induced cell damage has been discussed previously (Plapp & Chiga, 1973). In comparison with the earlier observations, the present experiments were concerned with a more initial period of DMNA treatment.

In the experiments described here, special interest was focused on mRNA-degrading endoribonucleases and poly(A) hydrolases in the microsomal and postmicrosomal fractions. No increase in these activities, or in poly(U)-degrading nucleases, could be demonstrated during the early period after DMNA administration characterized by decreasing protein chain initiation and rapid loss of post-microsomal poly(A)-containing RNA (Nygård & Hultin, 1979, 1981). On the contrary, the microsomal extracts showed a marked decrease in endonuclease activity, as determined by the poly-ribosome-degradation assay. No corresponding increase could be demonstrated in the postmicrosomal fraction, irrespective of whether or not the soluble RNAase inhibitor had been inactivated by *N*-ethylmaleimide treatment.

The possibility was considered that the early fall in endonuclease activity in the microsomal extracts might reflect DMNA-induced alterations in the average size or mechanical stability of lysosomes of consequence for the integrity of the free mRNA pool. This assumption was not supported by the analyses of lysosome-associated enzymes in the microsomal fraction, which failed to reveal any effects suggestive of intracellular redistribution. In addition, lysosomes are characterized by an acid RNAase (Slater & Greenbaum, 1965), resistant to the liver RNAase inhibitor. A release of this enzyme would have been detectable by the sensitive endonuclease assay in the absence of *N*-ethylmaleimide.

In conclusion, the experiments disfavour an increased nucleolytic activity as the cause of the early loss of postmicrosomal poly(A)-containing RNA in the livers of DMNA-treated mice.

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