The Effects of Diethylnitrosamine on Ribonucleic Acid and Protein Synthesis in the Liver and Lung of the Syrian Golden Hamster

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1. Syrian golden hamsters were treated with a single subcutaneous dose of 200mg of diethylnitrosamine/kg. In the liver the treatment produced a significant and early inhibition of the incorporation of orotic acid into RNA and of leucine into protein. Diethylnitrosamine also lowered basal and 20-methylcholanthrene-stimulated activities of hepatic aryl hydrocarbon hydroxylase. 2. RNA synthesis, protein synthesis and aryl hydrocarbon hydroxylase activity were also evaluated in the lungs of the same animals. In this organ only protein synthesis was affected by diethylnitrosamine, but not RNA synthesis or aryl hydrocarbon hydroxylase activity. 3. The incorporation of thymidine into DNA was inhibited in both organs early after diethylnitrosamine treatment and increased 2–3 days later. 4. Although diethylnitrosamine, injected subcutaneously, accumulates in liver and lung in toxicologically active amounts, the acute biochemical responses of the two organs are not identical.

In the preceding paper I have demonstrated that biosynthesis *in vivo* of RNA and of protein is very similar in hamster lung and hamster liver (Witschi, 1973a). Pulmonary RNA synthesis proceeds at about one-half of the rate of hepatic RNA synthesis and is susceptible to the action of actinomycin D. Studies with cycloheximide showed that the inhibition of pulmonary protein synthesis follows the same time-response and dose-response patterns in both lung and liver. Although our findings do not strictly prove that biosynthesis of RNA and of protein in lung is identical in all details with the same process in liver, they nevertheless have established that some currently used methods can evaluate RNA and protein synthesis in the lung as efficiently as in the liver. They also established that both organs respond in an identical way to two commonly used metabolic inhibitors, actinomycin D and cycloheximide.

In the present paper I report the effects of diethylnitrosamine on pulmonary RNA and protein synthesis. The acute biochemical effects of the alkylnitrosamines have been most thoroughly studied in the liver, where these compounds acutely produce widespread tissue necrosis and, later, cancer (Magee & Barnes, 1967; Magee & Swann, 1969). In the Syrian golden hamster diethylnitrosamine has, however, yet another highly susceptible target organ: the respiratory tract. Acute exposure to diethylnitrosamine seems to have very little effect, if any, on the pulmonary parenchyma (Greenblatt & Rijhsinghani, 1969), but chronic exposure by any route produces tumours of the respiratory tract (Saffiotti, 1969; Mohr, 1970; Montesano *et al.*, 1970). Therefore a difference exists between liver and lung:

in the liver diethylnitrosamine produces both extensive acute cell death and, later, cancer, whereas in the lung carcinogenesis seems to be the only serious response. This paper provides some evidence that several acute biochemical responses to diethylnitrosamine are found in the liver but not in the lung.

Experimental

Materials

DL-[1-¹⁴C]Leucine (sp. radioactivity 25.4Ci/mol), [6-¹⁴C]orotic acid hydrate (sp. radioactivity 51.6Ci/mol), [2-¹⁴C]thymidine (sp. radioactivity 54.1Ci/mol) and [2-¹⁴C]uridine (sp. radioactivity 54.1Ci/mol) were purchased from New England Nuclear Corp., Waltham, Mass., U.S.A. Special chemicals for the enzyme assays were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Diethylnitrosamine was from Eastman-Kodak Co., Rochester, N.Y., U.S.A. An authentic sample of 3-hydroxybenzo[*a*]pyrene was a gift from Dr. H. V. Gelboin, National Institutes of Health, Bethesda, Md., U.S.A.

Animals

Non-inbred male Syrian golden hamsters (80–100g) were purchased from ARS/Sprague-Dawley, Madison, Wis., U.S.A.

Administration of drugs and radioactive precursors

Diethylnitrosamine was mixed with 0.15 M-NaCl and injected subcutaneously in the interscapular

skin fold at a dose of 200mg/kg. 20-Methylcholanthrene was dissolved in corn oil (10mg/ml) and injected intraperitoneally. The radioactive precursors were dissolved in 0.15M-NaCl and injected intraperitoneally (injection volume 0.2ml).

Evaluation of RNA, DNA and protein synthesis

In all these experiments food was withdrawn from the animals the evening before the incorporation of the precursors was evaluated. The incorporation of orotic acid into RNA, of thymidine into DNA and of leucine into protein was measured as described previously (Witschi, 1970, 1972). All samples were mixed with 10ml of Aquasol (New England Nuclear Corp.) and counted for radioactivity in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer. Quench correction was made by external standardization.

Enzyme assays

The activity of uridine kinase (EC 2.7.1.48) was measured as described previously (Witschi, 1970), except that the incubation mixture contained 2.5 μ mol of ATP. The activity of aryl hydrocarbon hydroxylase was measured by the method of Wattenberg *et al.* (1962) as modified by Nebert & Gelboin (1968). The fluorescence of the NaOH extract containing the polar metabolites of 3-benzo[a]pyrene was measured in a Turner 111 fluorimeter (excitation wavelength max. at 405nm, emission wavelength max. at 525nm). The fluorimeter was calibrated with the authentic 3-hydroxybenzo[a]pyrene and fluorescence units were converted into ng of 3-hydroxybenzo[a]pyrene. In all enzyme assays product formation was linear within the adopted time-period and proportional to the amount of protein in the incubation mixture.

Presentation of results

All results in the tables and figures are given as mean values $\pm$ s.e.m. Significance levels were estimated with a Student's *t* test (Snedecor & Cochran, 1967) and *P* values of <0.05 were considered to be significant.

Results

In all experiments, hamsters were treated with one single subcutaneous injection of 200mg of diethylnitrosamine/kg. All animals survived this dose for at least 3 days; after 1 week about 70% of the diethylnitrosamine-treated animals were still alive. Signs of toxic liver damage, such as increased friability and a pale discoloration of the liver, were noticed as early as 4h after diethylnitrosamine treatment and

were definitely present 8–16h after the treatment. A time-response study on the incorporation of orotic acid into RNA was done. The results are plotted in Fig. 1. A very marked and statistically significant inhibition of hepatic RNA synthesis was observed 4 and 8h after diethylnitrosamine administration. At 16 and 24h incorporation was still lowered and then became higher than in controls 2 and 3 days after diethylnitrosamine treatment. Lung behaved differently: incorporation seemed first to be higher than in controls and then fell slightly below control values and increased again; however, the differences were not statistically significant.

The incorporation of leucine into protein was examined over the same time-period. In this experiment it was found that diethylnitrosamine extensively inhibited both pulmonary and hepatic protein synthesis 4h after administration (Fig. 2). Subsequently, recovery occurred in both organs and reached a peak at 24h. At this time protein synthesis in the lung appeared to be normal again, whereas in the liver it was still lower than in controls. A new decrease in protein synthesis was then seen on about day 2 or 3.

The activity of uridine kinase was measured in liver and lung of animals 4, 16 and 24h after diethylnitrosamine treatment. The results are given in Table 1. The activity of this particular enzyme in the lung

Fig. 1. Effects of diethylnitrosamine on hepatic and pulmonary RNA synthesis in liver and lung

Male hamsters were injected at zero time with a single subcutaneous dose of diethylnitrosamine (200mg/kg). At the times indicated on the abscissa groups of five animals were injected with 6.25 μ Ci of [14 C]orotic acid and killed 15 min later. The specific radioactivities of hepatic and pulmonary RNA were then measured and are plotted as means $\pm$ s.e.m. The region between the upper horizontal bars represents control values for liver and between the lower bars, the control values for lung ($n=20$ for both groups). Values significantly different from controls are designated with an asterisk. ●, Liver; ■, lung.

was not affected by diethylnitrosamine. In liver a small but statistically significant increase in specific activity of the enzyme was observed at 24h after diethylnitrosamine but not before; most probably this increase was a presage of the increase in RNA synthesis observed on day 2 after diethylnitrosamine treatment.

Studies were done to determine if diethylnitrosamine would interfere with cellular regulatory mechanisms in liver or lung. One possible way to study this was to examine the action of diethylnitrosamine on enzyme induction. A suitable model was to examine the effects of diethylnitrosamine treatment on the induction of aryl hydrocarbon hydroxylase by 20-methylcholanthrene. Two possibilities were examined: first, whether a pretreatment time of 24h with diethylnitrosamine would interfere or even block subsequent enzyme induction; secondly, whether diethylnitrosamine would be capable of lowering enzyme content in the lung once it had been increased by prior induction.

The first possibility was examined as follows: hamsters were injected with 200mg of diethylnitrosamine/kg; an equal number of control animals

received 0.15M-NaCl. After 24h one-half of the treated and one-half of the control animals were injected with 100mg of 20-methylcholanthrene/kg dissolved in corn oil; the remaining animals received corn oil alone. Then 24h after this second injection the animals were killed and aryl hydrocarbon hydroxylase activity was measured in liver and lung. Control animals induced with 20-methylcholanthrene had higher enzyme activities in both liver and lung than did controls given corn oil alone. The injection of diethylnitrosamine 24h before the methylcholanthrene seemed to decrease enzyme induction in liver. However, diethylnitrosamine had substantially lowered the basal enzyme activities in the livers of rats treated with corn oil alone. Therefore the inducing effect of methylcholanthrene in liver (increase of induced activity over basal activity) was at least as large in the animals treated with diethylnitrosamine as it was in the animals injected with NaCl. On the other hand, no significant effects of diethylnitrosamine on basal or induced enzyme activities were observed in the lungs (Table 2).

In a second experiment methylcholanthrene or corn oil alone was injected intraperitoneally into two groups of hamsters. After 24h one-half of the animals from the induced group and one-half of the animals from the corn oil group were injected subcutaneously

Fig. 2. Effects of diethylnitrosamine on hepatic and pulmonary protein synthesis in liver and lung

Male hamsters were injected at zero time with a single subcutaneous dose of diethylnitrosamine (200mg/kg). At the times indicated on the abscissa groups of five animals were injected with 7 μ Ci of [14 C]leucine and killed 20min later. The specific radioactivities of hepatic and pulmonary protein were then measured and plotted as means $\pm$ S.E.M. The region between the upper horizontal bars represents control values for liver and between the lower bars, the control values for lung ($n=14$ for both groups). Values significantly different from controls are designated with an asterisk. ●, Liver; ■, lung.

Table 1. Activity of uridine kinase in hamster liver and lung after diethylnitrosamine pretreatment

Male hamsters (80–100g) were injected subcutaneously with 200mg of diethylnitrosamine/kg; control animals were injected with 0.15 M-NaCl. The animals were killed 4, 16 and 24h later and the activity of uridine kinase was measured in the 100000g supernatants of liver and lung as described in the Experimental section. The results are given as means $\pm$ S.E.M. and the numbers of animals are given in parentheses.

Treatment	Uridine phosphorylated (nmol/min per mg of protein)	
	Liver	Lung
Controls	0.42 $\pm$ 0.03 (6)	1.25 $\pm$ 0.11 (6)
Diethylnitrosamine (4h)	0.39 $\pm$ 0.03 (6)	1.22 $\pm$ 0.18 (6)
Controls	0.45 $\pm$ 0.03 (6)	1.10 $\pm$ 0.07 (6)
Diethylnitrosamine (16h)	0.46 $\pm$ 0.04 (5)	0.83 $\pm$ 0.10 (6)
Controls	0.45 $\pm$ 0.03 (6)	1.05 $\pm$ 0.11 (6)
Diethylnitrosamine (24h)	0.76 $\pm$ 0.04 (6)*	0.83 $\pm$ 0.14 (6)

* The difference between treated and control animals was statistically significant ($P < 0.05$).

Table 2. *Effects of diethylnitrosamine on hepatic and pulmonary aryl hydrocarbon hydroxylase*

Male hamsters (80–100g) were given the pretreatment indicated. The injection volumes and doses were: 20-methylcholanthrene, 100mg/kg in 1.0ml of corn oil; corn oil (oil) alone, 1.0ml; diethylnitrosamine, 200mg/kg (0.2ml subcutaneously); 0.15 M-NaCl (0.2ml subcutaneously). The animals were then killed and hepatic and pulmonary aryl hydrocarbon hydroxylase were measured as described in the Experimental section. The results are means $\pm$ S.E.M. and the numbers of animals are given in parentheses.

Pretreatment		3-Hydroxybenzo[a]pyrene (ng/min per mg of protein)	
48h before death	24h before death	Liver	Lung
NaCl	20-Methylcholanthrene	42.5 $\pm$ 2.4 (6)	1.37 $\pm$ 0.13 (6)
Diethylnitrosamine	20-Methylcholanthrene	12.2 $\pm$ 1.5 (6)*	1.25 $\pm$ 0.23 (6)
NaCl	Oil	28.9 $\pm$ 1.7 (6)	0.71 $\pm$ 0.13 (6)
Diethylnitrosamine	Oil	5.4 $\pm$ 0.4 (6)*	0.38 $\pm$ 0.03 (6)
20-Methylcholanthrene	NaCl	54.2 $\pm$ 1.3 (6)	1.98 $\pm$ 0.21 (6)
20-Methylcholanthrene	Diethylnitrosamine	25.4 $\pm$ 2.4 (6)*	1.43 $\pm$ 0.08 (6)
Oil	NaCl	35.4 $\pm$ 4.6 (6)	0.48 $\pm$ 0.05 (6)
Oil	Diethylnitrosamine	11.3 $\pm$ 1.9 (5)*	0.36 $\pm$ 0.04 (6)

* The diethylnitrosamine-treated animals were significantly different from the NaCl-treated controls.

Fig. 3. *Effects of diethylnitrosamine on the incorporation of thymidine into DNA in liver and lung*

Male hamsters were injected at zero time with a single subcutaneous dose of diethylnitrosamine (200mg/kg). At the times indicated on the abscissa groups of five animals were injected with 1.5 μ Ci of [14 C]thymidine and killed 60min later. The specific radioactivities of pulmonary and hepatic DNA were measured as described previously (Witschi, 1970). The values are plotted as means $\pm$ S.E.M. The region between the upper horizontal bars represents control values for liver and between the lower bars, control values for lung ($n = 26$ for both groups). Values significantly different from control values are indicated with an asterisk. ●, Liver; ■, lung.

with 200mg of diethylnitrosamine/kg. The rest of the animals were given 0.15M-NaCl. Another 24h later the animals were killed and enzyme activity was measured. It was found that diethylnitrosamine had lowered the enzyme activities in the livers of both the induced and non-induced hamsters significantly, but had no effect on aryl hydrocarbon hydrolase activity in lung. Diethylnitrosamine therefore produced quite extensive alterations in enzyme activity only in the liver.

The incorporation of thymidine into DNA was examined at seven time-periods ranging from 4h to 7 days after diethylnitrosamine administration. Incorporation of thymidine is primarily a biochemical indication of cell multiplication and proliferation. The results are plotted in Fig. 3. In both organs an initial decrease in the incorporation of thymidine into DNA occurred. This was followed later by a significantly increased incorporation.

Discussion

Most of the available evidence suggests that the dialkyl nitrosamines are essentially stable compounds; however, they can be decomposed enzymically in several tissues. A correlation apparently exists between breakdown and acute toxic effects, such as cell death (Magee & Barnes, 1967; Magee & Swann, 1969). A most important finding was the observation by Montesano & Magee (1971) that slices from hamster lung are as active as slices from hamster liver in the production of $^{14}\text{CO}_2$ on incubation with [^{14}C]diethylnitrosamine. This could imply that hamster lung has a similar capacity to that of liver

for metabolizing diethylnitrosamine. It was therefore not unreasonable to expect that diethylnitrosamine would produce essentially the same acute biochemical effects in both organs.

This was, however, not entirely the case: RNA synthesis was affected differently by diethylnitrosamine in the two organs. In liver an early and very significant decrease in the incorporation of orotic acid into RNA was seen; later, recovery occurred, and 2 and 3 days after diethylnitrosamine some overcompensation was observed. This was preceded by a rise in the activity of hepatic uridine kinase. In lung no significant changes were detected. Earlier data had shown that pulmonary and hepatic RNA synthesis respond identically to the inhibitory action of various doses of actinomycin D (Witschi, 1973a), and there is no reason to believe that the lack of response of the lung in the experiments with diethylnitrosamine could be explained by differences in the biochemical processes underlying the incorporation of orotic acid into RNA. On the other hand, the incorporation of leucine into protein and of thymidine into DNA was affected by diethylnitrosamine in a similar way in both organs. These data show that diethylnitrosamine accumulates in both liver and lung in toxic amounts at about the same time after a subcutaneous injection. It remains to be established whether in both liver and lung diethylnitrosamine directly disturbed the synthesis of the macromolecules or whether the observed decreases in incorporation of radioactivity were rather due to changes in specific radioactivity and pool sizes of the precursors used.

The induction of enzymes by drugs and other chemicals is a complex biochemical event and requires an unimpaired functioning of the cell. Studies on the effects of various toxic agents on enzyme induction have occasionally helped to pinpoint the site of biochemical lesions (Shank, 1968; Wogan & Friedman, 1968; Magus & Fouts, 1968). To study the effects of diethylnitrosamine on the induction of pulmonary aryl hydrocarbon hydroxylase seemed to provide an additional approach to evaluate eventual lesions in cellular metabolism. In several experiments diethylnitrosamine failed to either prevent enzyme induction or to lower significantly basal or stimulated enzyme activities in lung. In the liver diethylnitrosamine interfered substantially with aryl hydrocarbon hydroxylase activity. Therefore these experiments provided no evidence that diethylnitrosamine would disturb the regulation of cell metabolism in lung.

Another interpretation of the data on the activity of hepatic and pulmonary aryl hydrocarbon hydroxylase has also to be considered. Diethylnitrosamine, like many other dialkyl nitrosamines, causes widespread liver necrosis (Magee & Barnes, 1967). Loss of viable hepatic cells is often preceded

and/or accompanied by loss of enzyme activities. Therefore the decreased aryl hydrocarbon hydroxylase activity in the livers of diethylnitrosamine-treated hamsters reflects the loss of viable parenchyma. In the lungs of the same animals aryl hydrocarbon hydroxylase activities also seemed to be decreased somewhat, but the differences between treated and control animals were not significant. These findings do not allow one to exclude completely the loss of some viable lung parenchyma after diethylnitrosamine treatment. On the other hand, agents known to have widespread acute and/or chronic toxic effects on lung parenchyma decrease the activity of aryl hydrocarbon hydroxylase quite substantially; examples are ozone in hamster (Palmer *et al.*, 1971) and nickel carbonyl (Sunderman, 1967), beryllium (Jacques & Witschi, 1973) and paraquat (Witschi, 1973b) in rat. The data on aryl hydrocarbon hydroxylase therefore corroborate morphological studies in which it was reported that in hamsters diethylnitrosamine produced massive liver necrosis apparently without affecting the pulmonary parenchyma (Greenblatt & Rijhsinghani, 1969).

The decrease in the incorporation of thymidine into DNA observed within the first few hours after diethylnitrosamine treatment might be a consequence of the inhibition of protein synthesis produced by diethylnitrosamine. Inhibition of protein synthesis is known to stop DNA synthesis almost immediately (Bennett *et al.*, 1964; Verbin *et al.*, 1969). After this initial fall in DNA synthesis an increased incorporation of thymidine into DNA was observed. In liver this probably reflects the replacement of necrotic tissue by a regenerating cell population. In lung the observation is not so easy to interpret since there is no evidence, as discussed above, that much cell death occurs. It has, however, been reported that diethylnitrosamine stimulates DNA synthesis in various tissues, not all of them susceptible to the carcinogenic effects of diethylnitrosamine (Schaude *et al.*, 1970).

The main conclusion to be drawn from my data seems to be that the extensive biochemical changes observed in liver after diethylnitrosamine treatment were most probably an expression of the widespread necrosis produced in this organ. Biochemical changes in lung were not entirely similar to the ones found in the liver. Despite apparently identical capabilities to metabolize diethylnitrosamine (Montesano & Magee, 1971), liver and lung respond differently to this compound. This could provide in the future an opportunity to investigate what biochemical alterations are of importance for the development of the chronic effects of diethylnitrosamine in the respiratory tract. The present investigation does not provide definite clues. Stewart & Magee (1971, 1973) found significant and early changes in renal synthesis of RNA and DNA under conditions in which one single

dose of dimethylnitrosamine produces a 100% incidence of kidney tumours. It is possible to increase the incidence and shift the localization of diethylnitrosamine-induced tumours of the respiratory tract by a concomitant treatment with benzo[a]pyrene and/or Fe_2O_3 (Montesano *et al.*, 1970). A biochemical analysis of this effect might reveal more specific alterations in pulmonary nucleic acid metabolism and thus provide more information about the biochemical mechanisms underlying respiratory carcinogenesis in hamsters.

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