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SHORT COMMUNICATIONS

Delayed biochemical effects of *N*-nitrosodiphenylamine in rat liver and brain

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The nitrosoamines have an important place in the production of rubber as retardants in the vulcanization [1]. Because of this and other uses waste water may contain varying amounts of unreacted nitrosoamines [2]. The mutagenic potential of *N*-nitrosoamines has been emphasized in recent studies [3] while only a few investigations have been published on its organotypic toxicity and dose-related mechanisms in mammals.

Metabolic studies indicate that *N*-nitrosodiphenylamine is reductively denitrosated and that the microsomal cytochrome P-450 complex may participate in the process [4]. This reaction represents a true detoxification mechanism as the parent compound is an uncoupler of oxidative phosphorylation already at 0.2 mM concn [5].

With that background, we injected rats intraperitoneally with *N*-nitrosodiphenylamine and compared the biochemical effects in liver and brain with the actual concn of the chemical and its primary metabolite, diphenylamine.

Materials and methods

Fifteen male Wistar rats (300–350 g) were dosed intraperitoneally with *N*-nitrosodiphenylamine (1 g/kg body wt in olive-oil). The animals were decapitated in groups of five 4, 24 or 48 hr later. Five control rats were injected with olive-oil. Brain, liver and kidneys were taken at autopsies. Liver and kidney *N*-nitrosodiphenylamine and diphenylamine concns were analyzed as follows. The samples (0.5 g) were extracted twice with 1 ml acetonitrile using an Ultraturrax homogenizer. The organic phases were recovered after centrifugation at 2500 *g* for 10 min, and 0.5 ml water was added to 0.5-ml aliquot. The *N*-nitrosodiphenylamine and diphenylamine concns were analyzed by liquid chromatography using a u.v. detector at 275 nm. The apparatus was equipped with a reverse-phase column (Shandon ODS-Hypersil, 5 μm, 12.5 × 0.5 cm), and an isocratic separation mode was used. The eluent consisted of an acetonitrile–water–acetic acid mixture (50:50:1, v/v/v). The structure of the eluted compounds was confirmed by mass spectrometry. The recovery of the method in the concn range 10–100 μg per sample was, for the *n*-nitroso compound, 93 ± 6% (± S.D.) and for diphenylamine 94 ± 4%. The detection limits were 0.5 and 0.2 μg/g, respectively. The degradation of the *N*-nitroso compound was negligible during the 2–3 hr while a longer storage caused conversion to diphenylamine.

The liver nonprotein sulfhydryl groups (glutathione) [6], glutathione peroxidase [7] and succinate dehydrogenase activities [8] were determined in the homogenate, and microsomes were isolated [9]. They were assayed for cytochrome P-450 and b_5 concns [10] and for ethoxycoumarin *O*-deethylase [11], and epoxide hydrolase [12] and cytochrome *c* reductase activities [13].

Cerebral homogenates were analyzed for glutathione concn [14], and succinate dehydrogenase [8] and glutathione peroxidase activities [7].

Statistical evaluation of the results was made with the Student's *t*-test.

Results

Two of the injected rats died after between 24 and 48 hr. The intestines of treated rats were darkly stained and dilated after 48 hr. The livers had a greenish discolouration

Table 1. Liver and kidney *N*-nitrosodiphenylamine and diphenylamine concns

Time after injection (hr)	Liver (μg/g)		Kidney (μg/g)	
	N-Nitrosodiphenylamine	Diphenylamine	*N*-Nitrosodiphenylamine	Diphenylamine
4	116 ± 50	35 ± 11	85 ± 10	17 ± 2.7
24	2.6 ± 2.0	3.2 ± 3.2	4.5 ± 1.5	2.2 ± 1.5
48	1.5 ± 0.5	0.6 ± 0.2	< 0.5	< 0.2

Each figure is the mean of five rats ± S.D. except that $N = 3$ for 48 hr.

Table 2. Biochemical effects of *N*-nitrosodiphenylamine injection in liver and brian homogenates

Time after injection (hr)	Non-protein sulfhydryls (μmoles/g)		Glutathione peroxidase (nmoles/min × mg protein)		Succinate dehydrogenase (nmoles/min × mg protein)	
	Liver	Brain	Liver	Brain	Liver	Brain
4	3.2 ± 1.3*	0.7 ± 0.07	474 ± 44‡	34.5 ± 2.7‡	1.5 ± 0.08*	3.1 ± 0.09
24	4.8 ± 0.9†	0.6 ± 0.04†	412 ± 37†	37.2 ± 6.2‡	1.3 ± 0.07*	2.9 ± 0.11
48	10.9 ± 1.9*	0.6 ± 0.06†	336 ± 18*	37.5 ± 4.9†	1.4 ± 0.13†	2.8 ± 0.11‡
Control	6.5 ± 0.4	0.8 ± 0.07	556 ± 65	30.8 ± 3.1	1.9 ± 0.17	3.0 ± 0.16

Each figure is the mean of five rats ± S.D. except that N = 3 for 48 hr.
* $P < 0.001$.
† $P < 0.01$.
‡ $P < 0.05$.

Table 3. *N*-Nitrosodiphenylamine injection-induced effects in liver microsomes

Time after injection (hr)	Cytochrome P-450 (nmoles/mg protein)	Ethoxycoumarin *O*-deethylase (nmoles/min × mg protein)	Cytochrome b_5 (nmoles/mg protein)	Epoxide hydrolase (nmoles/min × mg protein)	Cytochrome *c* reductase (nmoles/min × mg protein)
4	0.45 ± 0.05*	0.53 ± 0.06†	0.42 ± 0.04	10.0 ± 0.7	84.5 ± 9.1
24	0.63 ± 0.05	0.86 ± 0.25	0.38 ± 0.03	16.9 ± 4.8‡	115.3 ± 16.3‡
48	0.67 ± 0.13	0.63 ± 0.15	0.33 ± 0.01‡	28.3 ± 3.4*	98.0 ± 6.7
Control	0.71 ± 0.05	0.70 ± 0.11	0.39 ± 0.03	11.2 ± 1.5	89.1 ± 16.5

Each figure is the mean of five rats ± S.D. except that N = 3 for 48 hr.
* $P < 0.001$.
† $P < 0.01$.
‡ $P < 0.05$.

simultaneously. Kidneys and brain were macroscopically similar to controls.

N-Nitrosodiphenylamine was rapidly removed from liver and kidneys with diphenylamine as an important degradation product (Table 1). The disappearance rates were rather similar in both tissues.

The injections caused decreased glutathione concns in liver after 4 and 24 hr while an increased concn was seen after 48 hr (Table 2). Glutathione peroxidase activity was increasingly inhibited during the follow-up period together with succinate dehydrogenase activity (Table 2).

Liver microsomal cytochrome P-450 and the related ethoxycoumarin *O*-deethylase activity were decreased after 4 hr and restored to control ranges later on (Table 3). Cytochrome b_5 was below control after 48 hr while cytochrome *c* reductase activity increased transiently above the control range after 24 hr (Table 3). Epoxide hydrolase activity increased time-dependently above the respective control ranges (Table 3).

Cerebral glutathione concn was below the control range after 24 hr (Table 2). Succinate dehydrogenase activity was smaller than in controls after 48 hr while glutathione peroxidase activity was above the control range throughout the study (Table 2).

Discussion

Our analyses for the *N*-nitrosodiphenylamine burden indicate a rapid transformation to diphenylamine. Its metabolism by the cytochrome P-450 complex could explain the decrease in the cytochrome concn as a nitroso complex with the prosthetic haem is formed in the process leading to destruction of the haemochrome [4].

The mitochondrial succinate dehydrogenase activity was analyzed to shed light on the consequences of the postulated uncoupling of oxidative phosphorylation by the nitrosamine. Succinate dehydrogenase is coupled to the respiratory chain [15], and the decrease in its activity might reflect mitochondrial damage. For unknown reasons, this process seems to be aggravated at later follow-up times with low *N*-nitrosodiphenylamine concns.

Glutathione is not known to participate in the primary denitrosation. However, an unidentified secondary product or an event related to the mitochondrial effects could have contributed to the decrease in the nonprotein sulfhydryl groups. In this respect, it is interesting to note that epoxide hydrolase activity increased in the liver microsomes in a later follow-up, a finding associated, for example, with structural changes in the membrane [16].

The decrease in the hepatic glutathione peroxidase activity and the enhanced activity in the brain remain largely unexplained. However, cyanide anion is known to inhibit the enzyme by catalyzing the removal of the prosthetic combine with organic selenide, and, furthermore, the glutathione peroxidase activity decreased in liver after 48 hr with minimal amounts of *N*-nitrosodiphenylamine still present.

In conclusion, rats dosed with a large intraperitoneal dose of *N*-nitrosodiphenylamine showed delayed biochemical effects including early inhibition of hepatic drug-oxidizing activity followed by considerable mitochondrial and microsomal membrane effects 48 hr after the dose with almost no nitrosamine or metabolite detectable any more.

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Androstenedione stimulation of ouabain-sensitive $^{86}Rb^+$ influx into human red blood cells *in vitro* [$(Na^+–K^+)$ATPase *in situ*]

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Most of the steroid hormones tested either do not change or inhibit the *in vitro* influx of $^{86}Rb^+$ into human RBC, which is an index of $(Na^+–K^+)$ATPase activity *in situ* [1]. Only some testosterone preparations display stimulating activity caused [2] by an impurity with an R_f of 0.67 in TLC (the testosterone R_f is 0.48). In the present study the