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PREVENTION OF HALOTHANE INDUCED CHANGES IN RAT LIVER ENZYMES BY 3-AMINO-1,2,4-TRIAZOLE

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Abstract—It has already been shown that 3-amino-1,2,4-triazole inhibits the biological transformation of halothane in liver. The results of this study indicate that inhibition of the transformation of halothane prevents some of the changes in liver metabolism observed after halothane treatment. Changes in enzyme activities were measured as sensitive indicators. Halothane and its stable metabolite trifluoroacetate greatly increased the activity of the mitochondrial glycerol-3-phosphate oxidase. Pretreatment of rats with aminotriazole inhibited the activity increase caused by halothane, but not by trifluoroacetate. Aminotriazole also prevented the increase in the activity of malic enzyme, NADPH-oxidase, and lactate dehydrogenase produced by halothane treatment. The induction of glycerol-3-phosphate oxidase could not be counteracted by actinomycin D because actinomycin itself increased the activity of glycerol-3-phosphate oxidase.

HALOTHANE, a widely used volatile anesthetic, is comparatively slow to be eliminated from the organism. Experiments with ^{36}Cl-labeled halothane have shown that only 60 per cent of the halothane is exhaled during 30 hr.[1] Therefore, the organism has enough time for the biological transformation of halothane. Cohen injected halothane-2-^{14}C intraveneously into mice and found that the radioactivity in liver increased 3-fold during an 8-hr period.[2] Furthermore, the metabolites formed accumulated in the liver and caused the changes in liver metabolism.

Halothane and trifluoroacetate, the main end product of halothane metabolism, have similar effects on liver metabolism.[3] It was postulated that the alterations were the result of the biological transformation of halothane. Evidence is presented in this article to show that 3-amino-1,2,4-triazole, which blocks the biological transformation of halothane,[4] inhibits changes in liver metabolism occurring after halothane treatment.

Halothane and trifluoroacetate did not cause great alterations in the enzyme pattern. Therefore, the very few changes must be considered characteristic. This applies especially to mitochondrial glycerol-3-phosphate oxidase,[5] which increased in activity to the same extent as under the influence of thyroid hormones, and also to lactate dehydrogenase. The activity of the malic enzyme and microsomal NADPH-oxidase also increased due to halothane treatment, but not to trifluoroacetate.

The alterations in enzyme activities are merely signs of manifold metabolic adaptations. Therefore, these changes provide appropriate parameters for proving that metabolites, and not halothane *per se*, are responsible for the halothane induced alterations in liver metabolism. Adrenalectomized rats were also used in these experiments since microsomal enzyme systems are responsible for both the metab-

olism of steroids and of drugs. Adrenalectomy increased the activity of the enzymes measured, particularly that of glycerol-3-phosphate oxidase.*

MATERIALS AND METHODS

Animals. Male Wistar strain rats weighing 200–300 g were kept in groups of 6 at a room temperature of 22° and a 12 hr light/dark cycle. They received standard laboratory diet ("Altromin", Lage-Lippe, Germany) and water *ad lib.*

Reagents. All chemicals and enzymes were obtained from Merck, Riedel-de Häen, Fluka and Boehringer, Halothane was purchased from Imperial Chemical Industries, trifluoroacetate from Fluka, and actinomycin D from Merck Sharp & Dohme.

Halothane treatment. The rats were exposed to an atmosphere of 1 vol-% halothane–O_2-mixture for 1 hr daily for 14–17 days.

Trifluoroacetate treatment. Trifluoroacetic acid (sodium salt) was diluted in the drinking water to provide about 130 μmoles/100 g body wt at normal water consumption for 14–17 days.The first effects of trifluoroacetate treatment could be measured, however, after 48 hr.[3]

Aminotriazole treatment. 3-Amino-1,2,4-triazole was dissolved in water and injected intraperitoneally at a dose of 75 mg/100 g body wt (corresponding to 0·9 mmoles). In combined treatment with halothane, aminotriazole was injected 2–4 hr prior to halothane narcosis. The animals were killed 1 day after the last halothane or trifluoroacetate treatment.

Bilateral adrenalectomy. Bilateral adrenalectomy was performed under Evipan narcosis; after the operation the normal drinking water was replaced by a solution of 0·9% NaCl; animals were used for experiments one week after the operation.

Biochemical procedures. The liver was removed under slight ether narcosis and homogenized for the determination of enzymes as follows *Catalase*; Liver was homogenized 1:10 in M/150 Sörensen phosphate buffer with an Ultraturrax homogenizer (Fa. Jahnke und Kunkel, Stauffen). Catalase was measured according to the method of Feinstein[6] in 0·1 ml of homogenate. *Malic enzyme, pyruvate kinase, lactate* DH, *malate* DH, *glycerol-3-phosphate*-DH, *glyceraldehyde-phosphate*-DH, *isocitrate* DH, *glutamate-oxaloacetate-transaminase and glucose-6-phosphate* DH: liver was homogenized 1:10 in M/150 Sörensen phosphate buffer and centrifuged at 80,000 *g* for 10 min. The activities of enzymes in the supernatant were determined according to standard methods described in detail in *Hoppe–Seyler's Handbuch der physiologischen Chemie.*[7]

Glycerol-3-phosphate oxidase. Liver was homogenized 1:10 in M/150 Sörensen phosphate buffer with a glass–Teflon Potter homogenizer. The homogenate was centrifuged for 10 min at 80,000 *g*. The supernatant was discarded and the sediment

* *International enzyme nomenclature of the enzymes that were measured:* Glycerol-3-phosphate oxidase = EC 1.1.9.95. L-Glycerol-3-phosphate: cytochrome *c* oxidoreductase; NADPH-oxidase = EC 1.6.99.1. Reduced-NADP:(acceptor)oxidoreductase; Malic enzyme = EC 1.1.1.40. L-malate: NADP oxidoreductase (decarboxylating); Pyruvate kinase = EC 2.7.1.40. ATP: pyruvate-phosphotransferase; Lactate dehydrogenase = EC 1.1.1.27. L-lactate: NAD oxidoreductase; Malate dehydrogenase = EC 1.1.1.37. L-malate: NAD oxidoreductase; Glycerol-3-phosphate dehydrogenase = EC 1.1.1.8. L-Glycerol-3-phosphate: NAD oxidoreductase; Glyceraldehyde phosphate dehydrogenase = EC 1.2.1.12. D-glyceraldehyde-3-phosphate NAD oxidoreductase (phosphorylating); Isocitrate dehydrogenase = EC 1.1.1.42. L-isocitrate: NADP oxidoreductase (decarboxylating); Glutamate-oxaloacetate transaminase = EC 2.6.1.1. L-aspartate-2-oxoglutarate aminotransferase; Glucose-6-phosphate dehydrogenase = EC 1.1.1.49. D-Glucose-6-phosphate: NADP oxidoreductase.

TABLE 1. DATA REPRESENT ENZYMATIC BASE-VALUES FOR SOME DIFFERENT EXPERIMENTAL SERIES IN ORDER TO JUSTIFY THE EFFECTS OF THE BIOLOGICAL TRANSFORMATION OF HALOTHANE

Enzymes (Units × 10^2/g wet wt)	Non-adrenalectomized (Mean ± S.E.M. (n = 12))	Adrenalectomized (Mean ± S.E.M. (n = 12))	Halothane Non-adrenalectomized (Mean ± S.E.M. (n = 7))	Aminotriazole Non-adrenalectomized (Mean ± S.E.M. (n = 7))
Glycerol-3-phosphate oxidase	12·3 ± 5·1	21 ± 7·8	44·3 ± 17·9	5·3 ± 1·1
NADPH-oxidase	13·6 ± 4·2	14·3 ± 3·6	22·2 ± 3·44	9·5 ± 1·3
Malic enzyme	78 ± 24·6	116 ± 45·2	142 ± 25·6	66 ± 31·7
Pyruvate kinase	1320 ± 330	1720 ± 389	1040 ± 130	1540 ± 408
Lactate dehydrogenase	30,800 ± 6780	36,000 ± 4320	45,800 ± 5500	26,400 ± 7660
Malate dehydrogenase	25,700 ± 4880	31,300 ± 6570	32,000 ± 3680	33,300 ± 6660
Glycerol-3-phosphate dehydrogenase	6000 ± 1800	5660 ± 1130	7290 ± 875	6140 ± 1290
Glyceraldehyde phosphate dehydrogenase	8650 ± 1080	7850 ± 1020	7970 ± 1200	5660 ± 1470
Isocitrate dehydrogenase	2040 ± 124	1670 ± 376	1780 ± 230	1460 ± 175
Glutamate-oxaloacetate transaminase	8050 ± 2130	8680 ± 2170	10,300 ± 1340	93,800 ± 2345
Glucose-6-phosphate dehydrogenase	213 ± 50	278 ± 72·3	231 ± 21	170 ± 50·2

n = number of experiments.

was taken up in the initial volume of phosphate buffer and homogenized for 2 × 10 sec with an Ultraturrax. Glycerol-3-phosphate oxidase was estimated as described by Kadenbach[8] in a 1 cm cuvette containing 100 mM K-phosphate buffer, pH 7·4, 5 mM EDTA, 0·1% cytochrome *c*, 1 mM KCN, 10 mM $MgCl_2$, 20 mM glycerol-3-phosphate, and 0·05 ml tissue extract in a final volume of 1 ml; 25°, $\epsilon_{546\,nm} = 9{\cdot}7$ (mM).

NADPH-oxidase. Liver was homogenized with a glass–Teflon Potter homogenizer 1:10 in saccharose buffer (0·25 M saccharose, 20 mM triethanolamine HCl, 1 mM EDTA, pH 7·4). The homogenate was centrifuged for 10 min at 7000 *g*. The supernatant was centrifuged for 10 min at 80,000 *g*. The supernatant of the second centrifugation was discarded and the sediment washed with saccharose medium and again centrifuged for 10 min at 80,000 *g*. The supernatant was again discarded and the sediment was taken up in the initial volume of saccharose medium and homogenized for 2 × 10 sec with an Ultraturrax. NADPH-oxidase activity was measured in a 1 cm cuvette containing 200 mM triethanolamine HCl buffer, pH 7·6, 5 mM EDTA, 0·226 mM NADPH, and 0·1 ml homogenate in a final volume of 1 ml, 25°, $\epsilon_{366\,nm} = 3{\cdot}3$ (mM). Liver samples were also removed with frozen metal clamps[9] for the measurement of substrates.[10] The soluble protein was determined according to the Biuret method,[11] calculating with a factor of 17·5.

RESULTS AND DISCUSSION

Effects of 3-*amino*-1,2,4-*triazole.* Aminotriazole, a frequently used herbicide, is best known as an inhibitor of catalase (with the exception of the catalase contained in erythrocytes).[12,13] It also reduces the activity of liver microsomal drug metabolizing systems, and the content of cytochrome P-450[14] and NADPH-oxidase.

Stier[4] provided evidence that compounds which interfere with the metabolism of hydrogen peroxide in the cell also inhibit the conversion of halothane into trifluoroacetate, the end product of halothane metabolism. Aminotriazole inhibits the biological transformation of halothane by as much as 90 per cent.

Aminotriazole did not cause general or severe toxic changes in rat liver metabolism as substrate couples show (the control values of untreated animals are in parentheses): lactate–pyruvate 16 (11), glycerol-3-phosphate–dihydroxyacetone phosphate 11 (8), and ATP–ADP 2·5 (3·3).

Table 1, which represents the basal values of enzyme activities under different experimental conditions, shows that aminotriazole had very little influence on the activities of enzymes cited in this paper. The exception to this is glycerol-3-phosphate oxidase, the activity of which was decreased by aminotriazole, and NADPH-oxidase as mentioned above.

Glycerol-3-phosphate oxidase. Aminotriazole inhibited the increase in glycerol-3-phosphate oxidase activity induced by halothane. The same inhibitory effect of aminotriazole was also found in adrenalectomized rats even though the activity of this enzyme was increased to 290 per cent by halothane alone or 560 per cent compared with non adrenalectomized control rats.

In contrast to the effect of the combined treatment of halothane and aminotriazole, the combination of trifluoroacetate and aminotriazole increased the activity of glycerol-3-phosphate oxidase, even though aminotriazole itself decreased the activity of this enzyme by 50 per cent as compared with untreated animals.

TABLE 2. CHANGES IN ENZYME ACTIVITIES IN RAT LIVERS WITH REGARD TO THE INHIBITION OF HALOTHANE TRANSFORMATION BY AMINOTRIAZOLE

Treatment (n = 7–12)	Enzymes (Units × 10^2/g wet wt)							
	Glycerol-3-phosphate oxidase (Mean ± S.E.M.)		Malic enzyme (Mean ± S.E.M.)		Lactate dehydrogenase (Mean ± S.E.M.)		NADPH-oxidase (Mean ± S.E.M.)	
Nonadrenalectomized								
Untreated	12·3 ± 5·1	(100%)	78 ± 24·6	(100%)	30,800 ± 6700	(100%)	13·6 ± 4·2	(100%)
Halothane	44·3 ± 17·9	(+260%)	142 ± 25·6	(+82%)	45,800 ± 5500	(+50%)	22·2 ± 3·44	(+63%)
Trifluoroacetate	36·0 ± 5·4	(+192%)	88 ± 16·7	(+12%)	35,400 ± 6730	(+15%)		
Aminotriazole (AT)	5·3 ± 1·1	(−57%)	66 ± 31·7	(−15%)	26,400 ± 7660	(−14%)	9·5 ± 1·3	(−30%)
Halothane + AT	9·0 ± 0·8	(−27%)	73 ± 15·3	(−6%)	30,100 ± 4520	(−2%)	11·6 ± 2·3	(−15%)
Trifluoroacetate + AT	20·1 ± 6·6	(+63%)	97·5 ± 7·7	(+25%)	39,000 ± 4880	(+27%)	11·0 ± 3·7	(−19%)
Adrenalectomized								
Untreated	21 ± 7·8	(100%)	116 ± 45·2	(100%)	36,000 ± 4320	(100%)		
Halothane	81·5 ± 45·6	(+288%)	237 ± 19·7	(+104%)	51,000 ± 6120	(+42%)		
Trifluoroacetate	75 ± 12·8	(+257%)	139 ± 27·8	(+20%)	40,300 ± 6580	(+12%)		
Aminotriazole (AT)	8·6 ± 2·6	(−59%)	69 ± 13·8	(−41%)	26,200 ± 4720	(−27%)		
Halothane + AT	8·0 ± 3·0	(−62%)	69 ± 26·9	(−41%)	28,400 ± 6250	(−21%)		
Trifluoroacetate + AT	37·3 ± 11·6	(+78%)	145 ± 54	(+25%)	42,500 ± 3100	(+18%)		

n = Number of experiments; AT = aminotriazole.

Taking the enzyme activity under the influence of aminotriazole alone as a control value, trifluoroacetate in combination with aminotriazole increased the enzyme activity by 280 per cent in intact rats and by 340 per cent in adrenalectomized animals. Consequently, the effect of the halothane metabolite, trifluoroacetate, upon glycerol-3-phosphate oxidase was not counteracted by simultaneous addition of aminotriazole (Table 2).

Malic enzyme. Similar to the alterations in glycerol-3-phosphate oxidase activity, halothane increased the activity of the malic enzyme, especially after adrenalectomy. Trifluoroacetate had almost no effect on this enzyme.

Pretreatment with aminotriazole completely inhibited the increase in activity of the malic enzyme. This effect was also evident in adrenalectomized rats, indicating that the increase in enzyme activity caused by halothane is directly related to the metabolism of the anesthetic, since trifluoroacetate itself had no significant effect. Other intermediates, formed during the biological transformation of halothane must be responsible for the effect. These were not isolated or further characterized, but there is no doubt that chlorine and bromine atoms are consecutively split off from halothane. The metabolites produced by this process are probably unstable and consequently short-lived.[4] It has been shown indirectly that these products are responsible for fatty livers in rats and mice after chronic halothane treatment.[5]

Some authors have associated malic enzyme with fat metabolism,[15] and it may be connected with biological transformation.

NADPH-oxidase. To a large extent the description of malic enzyme also applies to NADPH-oxidase. The activity of this enzyme was also increased by halothane and pretreatment of the animals with aminotriazole inhibited the increase in activity. Trifluoroacetate had no influence on NADPH-oxidase activity so other metabolites must be responsible for the effect on microsomal metabolism.

Enzyme induction or activation? Glycerol-3-phosphate oxidase activity in particular is greatly increased by both halothane and trifluoroacetate. The question whether halothane or trifluoroacetate was involved in enzyme induction or activation could not be answered.

The interval between the addition of the compound and the increase in enzyme activity indicates induction but experiments using actinomycin D, usually employed to decide this point, did not give an answer because actinomycin D itself increased the activity of glycerol-3-phosphate oxidase by about 70 per cent. In this context it should be mentioned that we, like Miller,[16] were also unable to substantiate the data provided by Tarentino[17] and Sellinger.[18] The induction of glycerol-3-phosphate oxidase by triiodothyronine was not completely counteracted by actinomycin D (see further details in Ref. 19).

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