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Short Communication

AMINOTRIAZOLE IS A POTENT INHIBITOR OF α-OXIDATION OF 3-METHYL-SUBSTITUTED FATTY ACIDS IN RAT LIVER

MINNE CASTEELS, KATHLEEN CROES, PAUL P. VAN VELDHOVEN and GUY P. MANNAERTS*

Katholieke Universiteit Leuven, Afdeling Farmacologie, Campus Gasthuisberg, Herestraat 49, B 3000 Leuven, Belgium

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Abstract—The production of CO_2 and formate in isolated rat hepatocytes incubated in the presence of 3-methyl[1-^{14}C]margaric acid was investigated. Production rates of formate were approximately 4-fold lower than those of CO_2. Aminotriazole (3-amino-1, 2, 4-triazole), an irreversible inhibitor of catalase, potently suppressed α-oxidation of 3-methylmargaric acid, whereas β-oxidation of palmitate, 2-methylpalmitate and trihydroxycoprostanic acid and conversion of exogenously added formate to CO_2 were not or only slightly affected. This shows that aminotriazole is not only an inhibitor of catalase, but also of α-oxidation of 3-methyl-substituted fatty acids.

Key words: aminotriazole; peroxisomes; catalase; formate; α-oxidation; phytanic acid

The presence of a methyl group in the 3-position prevents 3-methyl-substituted fatty acids from being degraded via β-oxidation. Therefore, 3-methyl-substituted fatty acids first undergo α-oxidation. This process is thought to consist of a hydroxylation in the 2-position, followed by an oxidative decarboxylation generating CO_2 [1]. The shortened 2-methyl-substituted fatty acid that is formed by α-oxidation is subsequently β-oxidized, mainly in peroxisomes [2–4].

Reports on the subcellular localization of α-oxidation of phytanic acid and other 3-methyl-substituted fatty acids have been somewhat controversial. Some groups claim that α-oxidation is confined to mitochondria [5, 6], to microsomes [7] or to peroxisomes in humans and to mitochondria in rodents [8]. The fact that phytanic acid accumulates in several peroxisomal diseases suggests that in man at least part of the enzymatic process is localized in peroxisomes.

Regardless of the subcellular localization found or the combination of cofactors added, an important discrepancy remains between the rates of α-oxidation in broken cell systems and those obtained in isolated hepatocytes or fibroblasts, whereby the rates in broken cell systems are several-fold lower than those in intact cells [7]. In addition, the existence of a 2-hydroxy-intermediate is not generally accepted. Tsai *et al.* [9] and ten Brink *et al.* [10] demonstrated its presence, but Skjeldal and Stokke were unable to confirm this finding [11].

In a recent paper Poulos *et al.* [12] described the production of formate by human fibroblasts when these are incubated with 3-methyl-substituted fatty acids, suggesting that formate and not CO_2 may be the primary product of α-oxidation. In the present study, we investigated the production of radiolabelled CO_2 and formate in isolated hepatocytes incubated in the presence of 3-methyl-[1-^{14}C]margaric acid (3-methyl[1-^{14}C]heptadecanoic acid). Since catalase is able to convert formate into CO_2 [13], we used hepatocytes from normal rats and from rats treated with aminotriazole, an irreversible inhibitor of catalase when animals are pretreated with intraperitoneal injections of the compound [14, 15].

Materials and Methods

Bovine serum albumin, fraction V, was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and was defatted according to Chen [16]. 3-Amino-1,2,4-triazole (aminotriazole†) was from Janssen Chimica (Beerse, Belgium). Centre wells and rubber caps were obtained from Kontes (Vineland, NJ, U.S.A.). Hionic-Fluor was from Packard Instruments (Groningen, The Netherlands). Collagenase and nucleotides were from Boehringer Mannheim (Mannheim, Germany). Other reagents and solvents (analytical grade) were from Merck (Darmstadt, Germany) or Janssen Chimica (Turnhout, Belgium).

[1-^{14}C]Formate, sodium salt (sp. radioact. 60 Ci/mol) and [1-^{14}C]palmitic acid (sp. radioact. 57 Ci/mol) were from New England Nuclear (Bad Homburg, Germany). 2-Methyl[1-^{14}C]palmitic acid (sp. radioact. 35 Ci/mol), 3-methyl[1-^{14}C]margaric acid (sp. radioact. 54 Ci/mol) and [26-^{14}C]THCA (sp. radioact. 22 Ci/mol) were synthesized as described previously by Vanhove *et al.* [2], Huang *et al.* [7] and Casteels *et al.* [17], respectively.

Male Wistar rats weighing between 120 and 150 g were maintained on a standard laboratory diet and a constant light–dark cycle. The experiments were carried out with animals that were fasted overnight. The rats were injected intraperitoneally with 0.9% (w/v) NaCl (sham-injected controls), or with aminotriazole [20% (w/v) in 0.9% NaCl] at a dose of 1 g aminotriazole per kg body weight, 16 hr and subsequently 1 hr before sacrifice [15]. Isolated rat hepatocytes were prepared as described previously [18]. Cells 1.25×10^6 were incubated in 0.5 mL Krebs–Henseleit buffer pH 7.4, containing 10 mg of defatted albumin, 20 mM HEPES buffer and the appropriate substrates in a final concentration of 0.1 mM for THCA (sp. radioact.

* Corresponding author. Tel. 32 16 345813; FAX 32 16 345699.

† Abbreviations: aminotriazole, 3-amino-1,2,4-triazole; ASM, acid soluble material; THCA, trihydroxycoprostanic acid, 3α, 7α, 12α-trihydroxy-5β-cholestan-26-oic acid.

Table 1. Conversion of formate into CO_2 and oxidation of 3-methylmargaric acid by isolated rat hepatocytes

Substrate	Sham-injected			Aminotriazole		
	CO_2	Formate	ASM	CO_2	Formate	ASM
	oxidation products (nmol/min/10^8 cells)					
Formate	32.6 ± 4.0			22.7 ± 3.5		
3-Methylmargaric acid	17.4 ± 1.4	4.8 ± 0.6	14.2 ± 1.3	2.8 ± 0.5*	0.9 ± 0.1*	4.4 ± 0.2*

Rates of oxidation were measured in isolated hepatocytes from sham-injected rats (left column) and from rats treated with aminotriazole (right column). Substrate concentrations of ^{14}C-labelled 3-methylmargaric acid and formate were 0.2 mM and the incubations were stopped after 20 min for 3-methylmargaric acid and after 10 min for formate. ASM includes formate. Results are expressed as means ± SEM for five experiments. Statistical significance was determined by means of Student's *t*-test (*: statistically different from controls with $P < 0.05$).

1.8 Ci/mol) and 0.2 mM for the other substrates (sp. radioact. were 0.9, 0.9, 1.0 and 0.2 Ci/mol for palmitic acid, 2-methylpalmitic acid, 3-methylmargaric acid and formate, respectively). Reactions were terminated with 0.25 mL of 6% (w/v) $HClO_4$ after 20, 15 and 10 minutes for 3-methylmargaric acid, THCA and the other substrates, respectively. The released CO_2 was trapped on a paper strip (positioned in a center well and soaked in 10 N KOH) by shaking for 1 hr at 37° and the paper strip was counted for radioactivity after the addition of Hionic-Fluor [3].

^{14}C-Labelled ASM produced by the oxidation of [^{14}C]-THCA was measured as described previously [17]. ^{14}C-Labelled acid soluble oxidation products from 3-methyl[1-^{14}C]margaric acid, [1-^{14}C]palmitate and 2-methyl[1-^{14}C]palmitate were measured as follows. After acidification with $HClO_4$ as described above, the contents of the vials were transferred to plastic tubes and denatured proteins were removed by centrifugation. The supernatants were diluted with 0.5 mL 2 M acetate buffer, pH 4 containing 5 mM propionic acid, and extracted twice with 5 mL petroleum ether (bp 60–80°). An aliquot of the lower phase was counted for radioactivity. One hundred per cent of the produced formate was extracted with ASM (data not shown).

After acidification and trapping of CO_2, parallel samples were used for measuring formate production as described by Yang [19], but mercuric acetate was used instead of mercuric chloride. The $^{14}CO_2$ originating from the formate produced was trapped as described above. Recovery of ^{14}C-formate in the form of $^{14}CO_2$ was measured in each experiment and was between 88 and 95%.

Residual catalase in the cells was measured after sonication as described previously [20]. Protein was measured according to Peterson [21].

Results and Discussion

In human fibroblasts incubated with 3-methyl-substituted fatty acids formate is the major water soluble oxidation product and its rates of formation are approximately 10-fold higher than those of CO_2 [12]. Table 1 shows that isolated rat hepatocytes also produce formate when incubated with 3-methylmargaric acid. In the hepatocytes, however, the production of formate was approximately 4-fold lower than that of CO_2 and formate amounted to only one-third of the total acid-soluble oxidation products. A possible explanation for the discrepancy between the results obtained in human fibroblasts and rat hepatocytes might be that formate is much more rapidly converted to CO_2 in rat hepatocytes than in human fibroblasts. Two pathways for formate metabolism to CO_2 have been described in the rat: the peroxidative catalase pathway and the folate-dependent one-carbon pathway [13]. In an attempt to suppress the first pathway, rats were injected with aminotriazole, an irreversible inhibitor of catalase, and 3-methylmargaric acid oxidation was studied in hepatocytes isolated from the treated rats. In comparison with hepatocytes from sham-injected rats, catalase activity was inhibited by 97% in the hepatocytes from the aminotriazole-treated rats (0.429 ± 0.021 U/mg of protein versus 0.015 ± 0.001 U/mg of protein; N = 5). The production of CO_2 from formate however, exogenously added to the cells, was only slightly (30%) decreased. This suggests that

Table 2. β-Oxidation of palmitate, 2-methylpalmitate and THCA by isolated rat hepatocytes

Substrate	Sham-injected			Aminotriazole		
	CO_2	Formate	ASM	CO_2	Formate	ASM
	oxidation products (nmol/min/10^8 cells)					
Palmitate	15.2 ± 1.5	9.7 ± 1.0	123 ± 10	14.3 ± 1.6	5.8 ± 0.5*	78.5 ± 1.9*
2-Methylpalmitate	57.5 ± 2.8	1.2 ± 0.04	49.7 ± 0.9	47.9 ± 3.5	0.9 ± 0.2	38.3 ± 4.9
THCA	22.8 ± 2.0	1.3 ± 0.2	12.1 ± 1.2	16.9 ± 1.6	0.7 ± 0.1*	8.7 ± 0.8*

Rates of β-oxidation were measured in isolated hepatocytes from sham-injected rats (left column) and from rats treated with aminotriazole (right column). Substrate concentrations of ^{14}C-labelled palmitate and 2-methylpalmitate were 0.2 mM and the incubations were stopped after 10 min. Concentration of ^{14}C-labelled THCA was 0.1 mM and the incubations were stopped after 15 min. Results are expressed as means ± SEM for five experiments. Statistical significance was determined by means of Student's *t*-test (*: statistically different from controls with $P < 0.05$).

in rat liver formate may be metabolized mainly via the one-carbon pathway or that the residual activity of catalase, which is highly concentrated in peroxisomes, was still sufficient to metabolize significant amounts of formate.

Despite the fact that aminotriazole treatment did not seem to affect the conversion of formate to CO_2 to a major extent, treatment with the inhibitor drastically suppressed CO_2 formation from 3-methylmargaric acid. That this suppression was not the result of an inhibition of the conversion of formate to CO_2 is illustrated by the fact that the production of formate and total acid-soluble oxidation products was also seriously decreased (by 82 and 67%, respectively). The results, therefore, indicate that aminotriazole is an inhibitor not only of catalase but also of α-oxidation of 3-methyl-substituted fatty acids. Whether the inhibition of α-oxidation is linked to the inhibition of catalase remains unknown.

In a subsequent series of experiments we investigated whether aminotriazole treatment would also suppress β-oxidation (Table 2). The oxidation of palmitate, which is oxidized for its major portion in mitochondria [18], the oxidation of 2-methylpalmitate, oxidized for its major portion in peroxisomes [3, 4] and the oxidation of THCA, oxidized exclusively in peroxisomes [22], were not or only slightly affected. β-Oxidation of each of the three substrates also gave rise to the production of some formate.

Conclusions

As in human fibroblasts [12], the α-oxidation of 3-methyl-substituted fatty acids in isolated rat hepatocytes results in the formation of formate but our experiments do not allow us to conclude whether formate or CO_2 is the primary end-product of α-oxidation. Our results further show that aminotriazole, considered to be a specific inhibitor of catalase, is also a potent inhibitor of α-oxidation. Whether the inhibition of α-oxidation is related to the inhibition of catalase requires further investigation.

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