COMMUNICATIONS

Norflurazon—An Inhibitor of Essential Fatty Acid Desaturation in Isolated Liver Cells

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ABSTRACT

Norflurazon is a herbicide known to inhibit carotene biosynthesis and linolenic acid biosynthesis in plants. In the present work, the effect of norflurazon on the metabolism of essential fatty acids was studied in isolated rat liver cells and in rat liver microsomes, incubated with [1-^{14}C] labeled linolenic acid (18:3, n-3), dihomogammalinolenic acid (20:3,n-6) and eicosapentaenoic acid (20:5, n-3). Norflurazon (0.1 mM, 1.0 mM) was found to inhibit essential fatty acid desaturation. The Δ6 desaturation is inhibited more efficiently than the Δ5 and Δ4 desaturation. The chain elongation of essential C_{18} fatty acids to their C_{20} and C_{22} homologs was not inhibited by norflurazon.
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INTRODUCTION

Norflurazon (4-chloro-5-methylamino-2-[3-trifluoromethylphenyl]-pyridazin-3[2H] one, SAN 9789) is a commonly used herbicide known to inhibit the carotene biosynthesis (1). Several workers have shown that in plants, norflurazon also inhibits the desaturation of linoleic acid (18:2,n-6) to linolenic acid (18:3,n-3), without affecting the rate of desaturation of oleic acid or stearic acid (2-4).

In animals, dietary linoleic acid and linolenic acid are converted to longer and more desaturated fatty acids, mainly to arachidonic acid (20:4,n-6) and docosahexaenoic acid (22:6,n-3), respectively. The aim of this work was to investigate whether norflurazon inhibits animal fatty acid desaturation.

MATERIALS AND METHODS

Norflurazon (Fig. 1) was from Sandoz A.G., Basel, Switzerland. Labeled fatty acid substrates were purchased from the Radiochemical Center, Amersham, U.K. ([1-^{14}C]linolenic acid) and from New England Nuclear, Boston, Massachusetts ([1-^{14}C]dihomogammalinolenic acid and [1-^{14}C]eicosapentaenoic acid). The specific activity of labeled fatty acid was 7 mCi/mmol. Parenchymal liver cells and liver microsomes were prepared from male weanling rats of the Wistar strain (from Møllegård Laboratory, Denmark). The animals were fed a semisynthetic diet deficient in essential fatty acids (5) with 15 wt% hydrogenated coconut oil for more than six weeks. Isolated liver cells were prepared according to the method of Seglen (6). The concentration of the cells was approximately 5×10^6 cells/ml, and 90-95% were viable, as measured by resistance to uptake of tryphan blue. Cells were incubated in an oxygenated suspension medium (7) with 1.5% (w/v) bovine serum albumin and 10 mM (+)-lactate (from Sigma Chemical Co., St. Louis, Missouri). One ml of the cell suspension (in a total volume of 2 ml) was incubated with 200 nmole of labeled fatty acid. When indicated, the hepatocytes were preincubated for 20 min with norflurazon (dissolved in methanol) or methanol. The concentration of norflurazon was 0.01 mM, 0.1 mM or 1.0 mM, with final concentrations of methanol of 0.1%, 0.2% or 2%, respectively. Microsomes were separated according to the method of Marcell et al. (8) by centrifugation at 105,000 × g for 2 hr. Each labeled substrate fatty acid, bound to bovine serum albumin, was incubated in a concentration of 100 nmoles per ml. Each incubation in 1 ml of a 15 mM Hepes-1 mM EGTA-0.25 M sucrose solution contained in μmoles: ATP, 10; CoA, 0.3; NADH, 1.0; magnesium chloride, 10; phosphate buffer (pH 7.4), 15, and microsomes (5 mg of protein). When indicated, norflurazon (1.0 mM) or methanol was added.

The measurements of radioactive CO_2 and

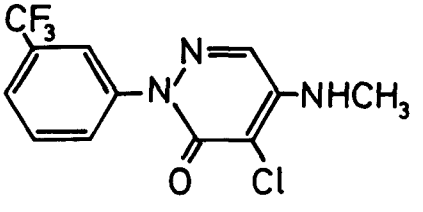

FIG. 1. The chemical structure of Norflurazon.

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TABLE 1

Effect of Norflurazon on the Pattern of ^{14}C-Labeled Fatty Acids in Hepatocytes Incubated with [^{14}C]Linolenic Acid

	Additions				
		Norflurazon dissolved in methanol			Control with methanol (2.0%)
	No addition	0.01 mM	0.1 mM	1.0 mM	
Fatty acid in phospholipid + triacylglycerol fraction					
18:3	68.4 ± 6.1	67.2 ± 6.5	85.4 ± 7.1	154.0 ± 0.4	68.7 ± 5.7
18:4	4.9 ± 0.5	4.0 ± 0.7	3.8 ± 0.3	4.2 ± 0.1	9.2 ± 1.1
20:3	1.5 ± 0.5	1.2 ± 0.3	1.5 ± 0.1	2.5 ± 0.2	2.1 ± 0.1
20:4	8.1 ± 0.5	8.2 ± 0.1	8.7 ± 0.3	6.8 ± 0.8	11.1 ± 1.0
20:5	63.1 ± 4.1	67.8 ± 7.0	53.4 ± 5.3	11.7 ± 0.3	64.5 ± 3.2
22:5	9.7 ± 0.3	9.0 ± 0.3	9.6 ± 1.2	6.4 ± 0.2	10.0 ± 1.0
22:6	22.7 ± 0.9	24.1 ± 1.9	21.8 ± 0.3	3.8 ± 0.3	23.6 ± 1.3
Δ6-Desaturase activity	61.6	62.8	53.6	18.7	63.8
Δ5-Desaturase activity	95.1	92.5	86.9	76.3	90.0
Δ4-Desaturate activity	70.1	72.8	69.4	37.3	70.0
Chain elongation of C_{18} to C_{20}	58.9	60.7	51.6	16.1	58.5
Chain elongation of C_{20} to C_{22}	33.9	32.8	37.0	47.0	34.3
Oxidation products	22.1 ± 1.1	19.0 ± 1.2	16.0 ± 0.8	8.7 ± 0.2	23.3 ± 2.3
Phospholipids	82.4 ± 0.6	82.0 ± 0.2	79.1 ± 0.4	51.1 ± 0.8	75.9 ± 0.5
Triacylglycerols	93.5 ± 2.1	96.9 ± 0.7	103.0 ± 0.9	138.4 ± 0.6	96.5 ± 2.2
Free fatty acids	2.1 ± 0.4	2.1 ± 0.2	1.9 ± 0.1	1.7 ± 0.4	2.5 ± 1.0

The incubation conditions were as described in Materials and Methods. Labeled linolenic acid (0.2 mM) and hepatocytes (24.1-24.4 mg of protein) were incubated for 60 min. The results are given as nmol of ^{14}C-labeled fatty acid esterified, oxidized or remaining as free linolenic acid substrate.

Means ± S.D. of two parallel analyses from three different livers are given. Activities of desaturases and chain elongations are given as per cent.

radioactive acid soluble products were performed as described by Christiansen (9). The lipids were extracted by the method of Folch et al. (10) and separated on silicic acid thin layer plates (Stahl H+) (hexane/diethylether/glacial acetic acid, 80:20:1, v/v/v). Aliquots of the total lipid extracts were transmethylated (11) and analyzed by radio gas chromatography (12). Cellular protein was determined according to the method of Lowry et al. (13).

RESULTS AND DISCUSSION

Δ6 Desaturating activity was studied by using [1-^{14}C]linolenic acid (18:3,n-3) as substrate for isolated liver cells from rats fed an essential fatty acid deficient diet. The main metabolites recovered in triacylglycerol and phospholipids were eicosapentaenoic acid (20:5,n-3) and docosahexaenoic acid (22:6,n-3) (Table 1). Nearly all the fatty acid substrate was metabolized, either esterified or oxidized under the conditions used.

With norflurazon (0.1 mM, 1.0 mM) present in the medium, the total amount of desaturated and chain-elongated fatty acids formed decreased markedly compared with the control. Table 1 shows that the activity of Δ6 desaturase, calculated as the sum of 18:4(n-3), 20:4 (n-3), 20:5(n-3), 22:5(n-3) and 22:6(n-3) in per cent of the sum of these five fatty acids and 18:3(n-3), decreased by 70% in the presence of norflurazon (1.0 mM). The fatty acids most markedly reduced were 20:5(n-3) and 22:6(n-3), with a concomitant increase of the amount of unaltered linolenic acid esterified.

Isolated microsomes also were used to study the effect of norflurazon. Table 2 shows that the Δ6 desaturating activity, calculated as 18:4 (n-3) formed from 18:3(n-3), decreased by 67% in the presence of norflurazon (1.0 mM). The solvent alone (methanol) had no effect on Δ6 desaturation.

The present experiments show that norflurazon inhibits desaturation of essential fatty acids in liver cells and in microsomes under the conditions used. The results cannot differentiate between an effect on the Δ6 desaturase itself or an effect on the electron transfer from NADH via cytochrome b_5 to the desaturase.

Several workers have shown that the regulation of Δ5 desaturase activity differs from the

mechanisms regulating the Δ6 desaturase (12, 14). In order to study the effect of norflurazon on Δ5 desaturation, labeled dihomogammalinolenic acid (20:3,n-6) was used as substrate. Table 3 shows that norflurazon (1 mM) decreased the conversion of 20:3(n-6) to arachidonic acid (20:4,n-6) modestly (by 15%). In experiments with 18:3(n-3) as substrate, where the Δ5 desaturation was calculated as the sum of 20:5(n-3), 22:5(n-3) and 22:6(n-3) in per cent of these three and 20:4(n-3) (Table 1), norflurazon decreased the Δ5 desaturating activity, but also in this case less efficiently than observed with Δ6 desaturation.

TABLE 2

The Effect of Norflurazon on the Desaturation of [^{14}C]Linolenic Acid in Rat Liver Microsomes

Addition	μmoles of 18:4/min/mg protein
No addition	21.2 ± 0.5
Norflurazon (1 mM)	7.1 ± 0.5
Methanol	24.2 ± 0.9

The incubation conditions were as described in Materials and Methods. The results are expressed as μmole [^{14}C]18:4 formed per min per mg protein.

Means ± S.D. of three parallel analyses from two different livers are given.

It has been shown previously that the Δ4 desaturase, which converts 22:5(n-3) to 22:6(n-3) and, less efficiently, 22:4(n-6) to 22:5(n-6) is similar to the Δ6 desaturase, in that essential fatty acid deficiency increases the activity of both enzymes (12). This is not the case with Δ5 desaturase (12,14). Δ4 desaturase activity was studied by using eicosapentaenoic acid 20:5(n-3) as substrate which is chain-elongated to 22:5 (n-3) and subsequently desaturated to 22:6(n-3). Table 3 shows that norflurazon (1 mM) caused a moderate reduction of the desaturation of 22:5(n-3) to 22:6(n-3). With 20:3(n-6) as the substrate, norflurazon was found to inhibit the desaturation of 22:4(n-6) to 22:5(n-6) to a similar extent. Only small amounts of 22:4(n-6) and 22:5(n-6) were formed in these experiments.

Norflurazon did not inhibit the chain elongation reactions. The elongation of C_{20} to C_{22} fatty acids even seemed to be stimulated in the presence of norflurazon. Thus with 20:5(n-3) as the substrate the sum of 22:5(n-3) and 22:6 (n-3) (Table 3) increased with norflurazon. Only a minor fraction of the fatty acids used was oxidized in the present experiments. The oxidation of 18:3(n-3) was reduced by norflurazon. The oxidation of 20:5(n-3) was reduced slightly, and the oxidation of 20:3(n-6) seemed to be unaffected by the inhibitor.

TABLE 3

The Effect of Norflurazon on the Pattern of ^{14}C-Labeled Fatty Acids in Hepatocytes Incubated with [^{14}C]Dihomogammalinolenic Acid or [^{14}C]Eicosapentaenoic Acid

	Fatty acid substrate				
	20:3(n-6)			20:5(n-3)	
	No addition	Norflurazon (1.0 mM)		No addition	Norflurazon (1.0 mM)
Fatty acid in phospholipid + triacylglycerol fraction					
20:3	40.9 ± 1.5	53.1 ± 0.6	20:5	114.3 ± 0.5	100.1 ± 1.7
20:4	107.7 ± 0.4	91.0 ± 0.3	22:5	27.5 ± 0.6	39.5 ± 2.5
22:4	2.0 ± 0.1	4.8 ± 0.2	22:6	24.2 ± 0.6	27.6 ± 2.1
22:5	1.4 ± 1.0	2.5 ± 0.2			
Δ5-Desaturase activity	73.0	64.9			
Δ4-Desaturate activity	41.0	34.2		47.0	40.0
Chain elongation of C_{20} to C_{22}	3.0	7.4		31.1	40.1
Oxidation products	48.2 ± 1.5	48.5 ± 0.3		34.3 ± 0.5	30.1 ± 0.2
Phospholipids	121.5 ± 0.5	88.4 ± 0.6		109.1 ± 0.3	76.5 ± 0.2
Triacylglycerols	29.7 ± 0.5	61.7 ± 0.8		59.8 ± 0.2	91.5 ± 0.2
Free fatty acids	1.4 ± 0.1	1.3 ± 0.1		1.7 ± 0.1	1.8 ± 0.1

The incubation conditions were as described in Materials and Methods. Labeled dihomogammalinolenic acid or eicosapentaenoic acid (0.1 mM) and hepatocytes (24.1-24.4 mg of protein were incubated for 60 min.

The results are given as nmol of ^{14}C-labeled fatty acid esterified, oxidized or remaining as free fatty acid substrate.

Means ± S.D. of two parallel analyses from three different livers are given. Activities of desaturases and chain elongations are given as per cent.

Tables 1 and 3 show that parallel to the inhibition of desaturases, norflurazon increased the incorporation of labeled fatty acids in triacylglycerol and reduced the incorporation in phospholipids. This change in distribution of labeled fatty acids between phospholipids and triacylglycerol probably is a consequence of the effects of norflurazon on the desaturation reactions. Thus, the intact 18:3(n-3) substrate is mainly esterified in triacylglycerol, while quantitatively dominating products formed after Δ6 desaturation, 20:5(n-3) and 22:6(n-e), are preferentially esterified in the phospholipids (12). Intact 20:3(n-6) is mainly esterified in triacylglycerol, while arachidonic acid formed by Δ5 desaturation is very efficiently directed to the phospholipids (12). With 20:5(n-3) as substrate, norflurazon stimulates the chain elongation to 22:5(n-3), which is preferentially esterified in triacylglycerol (14). The present experiments show that norflurazon inhibits the desaturation of essential fatty acids in liver cells and in liver microsomes under the conditions used. Δ6 desaturase is inhibited more efficiently than the other two desaturases studied.

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