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EFFECT OF CLOMIPHENE ON FATTY ACIDS, STEROLS AND MEMBRANE FLUIDITY IN CLAVINE PRODUCING CLAVICEPS PURPUREA STRAINS

Sylvie Pažoutová, Vladimír Křen, Tomáš Řezanka and Přemysl Sajdl

Institute of Microbiology, Czechoslovak Academy of Sciences, Vídeňská 1083, Prague 4, 142 20, Czechoslovakia

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Summary: Clomiphene depressed the growth and enhanced clavine production of Claviceps purpurea strains 129,35 and 59. Mycelial content of 18:2 and 16:0 fatty acids decreased, whereas that of 18:1 and 18:0 acids increased. In the mutant strain 59 clomiphene, triadimefon and ergosterol stimulated the impaired function of chanoclavine cyclase. Their effect was counteracted by plant oil. Clomiphene decreased the content of total lipids (44 %), triglycerides (32 %), sterols (22 %) and sterol/phospholipid molar ratio. The PC/PE ratio was 9x increased. Clomiphene and triadimefon enhanced membrane fluidity of protoplasts, ergosterol and oil reverted their effect. © 1988 Academic Press, Inc.

Chanoclavine cyclase catalyzes the oxidation of chanoclavine to its aldehyde and subsequent cyclization of chanoclavine-I-aldehyde to agroclavine, first tetracyclic clavine. The mutant strain C. purpurea 59 accumulates chanoclavine and aldehyde due to impaired cyclase function. We have observed an enhanced cyclase function after clomiphene addition to submerged cultures of the strain 59 (1). Similar effect was achieved after addition of triazole fungicide triadimefon (2) or ergosterol. Their effect was counteracted by plant oil. Clomiphene lowers the lipid and sterol content in algae (3) and yeasts (4). In this paper we show the effect of clomiphene on lipid metabolism and membrane fluidity of the mutant strain 59 and its ancestor strains 129 and 35 producing tetracyclic clavines.

Materials and Methods

Claviceps purpurea strains 129,35 and 59 were cultivated under submerged conditions in sucrose-citrate synthetic medium on the rotary shaker (1,2). Clomiphene citrate was added as an autoclaved aqueous solution at the beginning of the cultivation.

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Total alkaloids and the composition of the clavine mixture was done by HPLC (1). Lipids and sterols were analyzed in 22-day old mycelium. Total lipid analysis was performed by TLC-FID, sterols and fatty acids were analyzed by GC-MS (2,5). The unsaturation index was calculated according to (6), oxidation and cyclization indices according to (1). For the sterol/phospholipid molar ratio the relative molecular masses 396 and 800 were taken, respectively.

The membrane fluidity was measured as steady state fluorescence anisotropy of the protoplast suspension with DPH and TMA-DPH as fluorescent probes. The preparation of protoplasts and anisotropy measurement with DPH proceeded as in (2). The results in Tab. 1 were obtained using 1-(4-trimethylammonium) phenyl-6-phenyl-1,3,5-hexatriene (TMA-DPH) (7), added immediately before the measurement.

Results

Clomiphene influenced the composition of clavines produced only in the partially blocked mutant strain 59. Both ancestral strains, i.e. 129 and 35, producing mainly tetracyclic clavines, changed only the quantity, not the quality of the clavines produced. Clomiphene diminished the growth in all observed strains, but increased their production activity (Tab. 1).

The changes in the content and composition of fatty acids after clomiphene treatment were similar in all strains (Fig. 1). The amount of stearic and oleic acid increased, the content of palmitic and linoleic acid decreased, which was also manifested by a lower unsaturation index. The index was calculated from the percentage of all fatty acids including minor components not exceeding 1 %.

In producing cultures of the strain 59, clomiphene, triadimefon and ergosterol stimulated both chanoclavine cyclase functions. The effect of clomiphene and triadimefon on oxidation reaction was synergistic, however, cyclization was stimulated to a lower degree than with each agent separately. The same held for clomiphene and ergosterol interaction (Tab. 2). Plant oil reverted the effect of clomiphene on cyclase functions, production and growth. Ergosterol reverted only growth depression, the production activity was lowered.

The membrane fluidity, undirectly proportional to anisotropy, raised after clomiphene and triadimefon treatment in all three strains. In the combination with ergosterol and oil, the liquefying effect of clomiphene was less marked (Tab. 1, 2).

Clomiphene lowered total mycelial lipid (Tab. 3) to 44 %, the content of triglycerides to 32 % and sterols to 20 %. The sterol/phospholipid molar ratio decreased 12.6x, on the other

Table 1

Changes in growth, alkaloids, lipids and membrane fluidity after clomiphene treatment of C. purpurea strains

Strain	Dose (mg/l)	Dry mass (g/l)	Total alkaloids (mg/g dry mass)	Anisotropy[a]	U.I.[b]	o_i[c]	c_i
129	0	17.8	161.6	0.243	0.985		
	70	14.6	180.0	ND	1.022		
	300	6.5	352.6	0.220	0.909		
35	0	18.7	152.5	0.250	0.985		
	70	14.1	167.2	ND	1.011		
	300	8.7	280.5	0.240	0.836		
59	0	22.7	164.2	0.220	1.006	1.1	1.4
	70	15.2	184.7	0.184	0.978	1.7	2.3
	300	12.8	209.3	0.181	0.960	4.0	3.3

a) Anisotropy measured with TMA-DPH

b) unsaturation index

c) o_i = (100-% chanoclavine)-1

c_i = % tetracyclic clavines/% chanoclavine-I-aldehyde

hand the PC/PE ratio raised 8.5x. Sterol-decreasing effect of clomiphene appeared also in the cultures with oil; the phosphatidylethanolamine content was lowered similarly. Nevertheless,

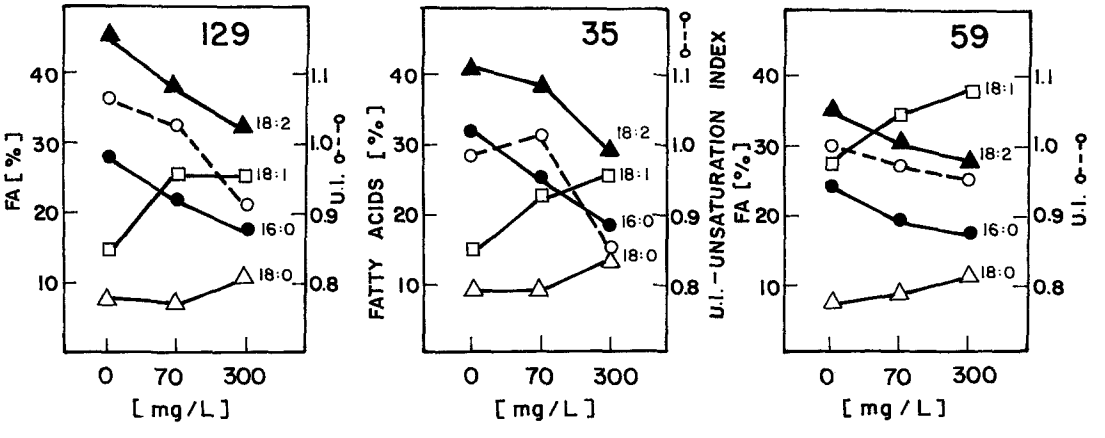

Fig. 1

Effect of clomiphene on the fatty acid composition of mycelial lipids in strains C. purpurea 129, 35 and 59

(●—● palmitic acid; △—△ stearic acid; □—□ oleic acid; ▲—▲ linoleic acid; ○···○ unsaturation index)

Table 2

Effect of clomiphene, ergosterol, triadimefon and plant oil on growth, alkaloid production and membrane fluidity of C. purpurea 59

Culture	Dose (mg/l)	Dry mass (g/l)	Total alkaloids (mg/g dry mass)	Anisotropy[a)]	o_i	c_i
Control	-	15.2	486.5	0.1650	0.92	1.08
Clomiphene	100	8.0	457.3	0.1524	2.88	3.00
Ergosterol	10	11.2	567.2	0.1780	2.70	3.38
Triadimefon	5	4.9	620.3	0.1330	4.65	2.12
Oil	7200	19.0	378.6	0.1651	0.70	0.68
Clomiphene Ergosterol	100 10	12.8	131.4	0.1708	3.56	1.98
Clomiphene Triadimefon	100 5	3.4	388.7		7.73	1.63
Clomiphene Oil	100 7200	16.2	397.9	0.1610	0.75	0.78

a) Anisotropy measured with DPH

the triglyceride content was fully restored in clomiphene-oil cultures.

The sterol demethylation was weakly inhibited by clomiphene (Tab. 4), especially in the C-4 position; also the $\Delta 8 \rightarrow \Delta 7$ iso-

Table 3

Lipid composition in the cultures of C. purpurea 59

Culture	Total[a)] lipids	Free sterols	Phospho-lipids	Triglyce-rides	PC[b)]	PE	PC/PE	S/PL
Control	21.5	0.821	9.95	7.80	9.10	1.42	6.40	0.143
Clomiphene[c)]	9.1	0.166	5.89	2.48	4.78	0.082	54.22	0.054
Oil	49.3	2.420	10.93	13.81	8.32	1.91	4.35	0.440
Clomiphene Oil	20.8	0.603	8.78	9.48	6.68	0.62	10.77	0.138

a) Lipids are expressed as percents of dry mass

b) Abbreviations: PC - phosphatidylcholine; PE - phosphatidylethanolamine; S/PL - sterol/phospholipid molar ratio

c) Doses as in Table 2.

Table 4
Sterol composition of C. purpurea 59

Sterol	RRT	Control	Clomiphene
8,24-lanostadienol	1.52	0.1	1.2
24-methyl-8,24(28)-lanostadienol	1.75	0.0	0.7
24-methyl-8-lanostenol	1.78	0.0	0.1
4,4-dimethyl-8,24-ergostadienol	1.83	0.2	1.3
4-methyl-7,24-ergostadienol	1.87	0.7	0.4
4-methyl-8,24(28)-cholestadienol	1.62	4.2	0.8
4-methyl-8(14)-cholestenol	1.48	0.7	3.2
4-methyl-7-ergostenol	1.66	0.3	0.5
8,24(28)-ergostadienol(fecosterol)	1.38	7.6	14.3
7-ergostenol	1.46	1.2	0.8
5-ergostenol	1.29	0.0	0.1
ergostanol	1.33	0.3	0.7
8(14)-cholestenol	1.13	1.1	2.7
5,7,22,24(28)-ergostatetraenol	1.34	0.9	0.1
7,22-ergostadienol	1.28	10.3	14.3
5,8,22-ergostatrienol	1.19	6.3	17.1
5,24(28)-ergostadienol	1.27	0.8	0.9
5,22-ergostadienol(brassicasterol)	1.12	14.1	15.3
5,7,22-ergostatrienol(ergosterol)	1.26	48.5	22.3

merisation was affected. On the other hand, the isomerisation of $\Delta 8 \rightarrow \Delta 14$ and $\Delta 5$ reduction were stimulated. In control cultures, 89.7 % of functional planar sterols was found in total sterol content, whereas in clomiphene-treated cultures it was 85.2 %. Clomiphene effect consists rather of an inhibition of total sterol biosynthesis than of an interference with single biosynthetic steps.

Discussion

The effect of clomiphene on growth and alkaloid production in submerged Claviceps cultures is due mainly to its impact on lipid metabolism. The mechanism of this effect is not known. The common intermediate of sterol and fatty acid biosynthesis is acetyl coenzyme A, but the depression of its formation could impair also the alkaloid biosynthesis from tryptophan and dimethylallyl pyrophosphate. Nevertheless, this was not observed.

The membrane fluidity was increased by clomiphene, probably due to the decrease in the sterol content. As a compensation for sterol shortage, the unsaturation index decreased. Ergosterol addition removed the clomiphene effect on membrane fluidity.

Clomiphene effects on the cultures of the strain 59 resembled those of triadimefon. Both agents lowered lipid and sterol content, and increased the membrane fluidity. The growth inhibition caused by clomiphene was reverted by ergosterol, whereas in triadimefon-treated cultures this was not manifested. This indicates that apart from the common effect on lipids there are further different effects of triadimefon and clomiphene on the cells. The morphology of treated cultures was also different. Triadimefon-treated cultures contained irregular large cells, often with germ tubes, whereas clomiphene elicited the formation of rounded cells in chains.

The comparison of sterol content and the amount of alkaloids produced up to the 22nd day of cultivation shows that the untreated cultures of the strain 59 invest 15x more of mevalonate into alkaloid biosynthesis, than into sterols. Triadimefon and clomiphene treated cultures build into sterols only 1 ‰ of cellular mevalonate. The cells of Claviceps do not accumulate sterols as yeasts; it seems, however, that almost the whole hydroxymethylglutaryl CoA reductase capacity serves to the purpose of alkaloid production (8). Thus, in the case of impaired sterol synthesis, the isoprenoid units can be easily channeled to alkaloid production.

Stimulation of chanoclavine cyclase function in a partially blocked strain 59 is not connected with the change of membrane fluidity. Cultures with higher fluidity caused by clomiphene and triadimefon and low-fluidity, ergosterol treated cultures produced more of tetracyclic clavines than controls. A common feature of these cultures was the low triglyceride content. Plant oil supplemented these triglycerides and suppressed chanoclavine cyclase activity. At present, the connection between triglyceride content and the activity of chanoclavine cyclase cannot be explained. One possibility is a deposition of alkaloids in lipid inclusions, which can affect the reaction equilibrium of biosynthetic steps.

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