PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 174, 28–32 (1983)

Early Action of Prolactin on Ornithine Decarboxylase Activity Is Not Essential for the Subsequent Actions of Prolactin on Casein and Lipid Biosynthesis[1] (41699)

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Abstract. The actions of prolactin (PRL) on casein and lipid biosynthesis in cultured mouse mammary gland explants require the ongoing synthesis of the polyamines. This is supported by the fact that (MGBG) methylglyoxal bis(guanylhydrazone), a drug that inhibits the conversion of putrescine to spermidine, abolishes the effects of PRL on casein and lipid biosynthesis; the inhibitory effects of MGBG are reversed by the addition of spermidine to the culture medium. α-Difluoro methyl ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase activity, reduces the PRL-stimulated ornithine decarboxylase (ODC) activity by more than 95%, and yet does not suppress the effects of PRL on RNA, casein or lipid synthesis. These observations suggest that PRL's early action on ODC activity is not essential for the subsequent actions of PRL on the synthesis of certain of the components of milk.

The involvement of the polyamines in the regulation of metabolic processes in mammary tissues has been suggested by information presented by several laboratories. First of all, intracellular spermidine concentrations of up to 5 m*M* were observed in mammary glands of lactating rats (1). Also, all of the spermidine biosynthetic enzymes including arginase, ornithine decarboxylase (ODC), 5-adenosylmethionine decarboxylase (SAMD), and spermidine synthase are stimulated by one or more of the hormones (insulin, cortisol, and prolactin) that enhance the rate of synthesis of milk components in cultured rat or mouse mammary tissues (2–8). Finally, MGBG [methylglyoxal bis(guanylhydrazone)], a drug that inhibits SAMD, abolishes the action of prolactin on casein synthesis (4, 8). All of these studies, therefore, clearly suggest that the actions of several hormones including prolactin on lactational processes in mammary tissues involve the polyamines.

In general, ODC is believed to be the rate-limiting enzyme for the actions of hormones and other substances on metabolic processes in a variety of cell types. In lactating rats, prolactin deficiency results in a parallel decrease of milk production and ODC activity. Since one of PRL's earliest actions on cultured mouse mammary tissues is a several-fold increase in the activity of ODC (7), the present studies were designed to determine if this effect of PRL is essential for the subsequent actions of PRL on the synthesis of milk components.

Materials and Methods. All experiments used mid-pregnant (10–14 days of pregnancy) Swiss–Webster mice purchased from Harlan Laboratories, Inc. Ovine prolactin (NIH-P-S-14) was a gift from NIAMD and α-difluomethylornithine was a gift from Merrell Dow Pharmaceuticals Inc. Other substances were from the following sources: Cortisol from Charles Pfizer; Nystatin from E. R. Squibb and Sons, Inc.; Medium 199 Earle's salts from K. C. Biol. Inc.; [1-^{14}C]acetate (57.7 mCi/mmole), [5,6-^{3}H]uridine (41.3 Ci/mmole), DL-[1-^{14}C]Ornithine (52.5 mCi/mmole), and [4,5-^{3}H]leucine (59.2 Ci/mmole) from the New England Nuclear Corporation; porcine insulin, penicillin, and streptomycin from the Eli Lilly Company; Methylglyoxal bis-(guanylhydrazone)-dihydrochloride monohydrate from the Aldrich Chemical Company Inc.

Methods used to culture mammary tissues were described earlier (10). Briefly, mice were killed by cervical dislocation and the most caudal pair of mammary glands, the inguinal pair, were removed aseptically and placed in Medium 199 Earle's salts. The glands were cut into pieces weighing 3–5 mg, and four explant pieces, one from each of four animals,

[1] This project was supported by NIH Grant HD 06571 from the National Institute of Child Health and Human Development.

[2] J.A.R. is the recipient of Faculty Research Award FRA-186 from the American Cancer Society.

0037-9727/83 $1.50

were placed in each vial on siliconized lens paper floating on 2 ml Medium 199 Earle's salts. All incubations were carried out in polypropylene vials maintained in a 37°C water bath in an atmosphere of 95% O_2–5% CO_2. Explants were incubated in media containing insulin (1 μg/ml) plus cortisol (10^{-7} M) for 24 hr. Prolactin and/or other agents were then added and incubations were continued for 2 hr (ODC experiments), 6 hr (RNA experiments), 16 hr (lipid experiments), or 16 hr (casein experiments). In the experiments designed to measure RNA synthesis, 0.5 μCi/ml [^{3}H]uridine was added to the culture medium for the final 1 hr of incubation and the specific activity of the ^{3}H in the RNA was subsequently assessed by the methods of Munro and Fleck (11) with minor modifications (10). In experiments designed to measure casein synthesis [^{3}H]leucine (0.5 μCi/ml) was added to the culture medium for the final 2 hr of incubation and the extent of [^{3}H]leucine incorporated into phosphoproteins which are isoelectrically precipitable at pH 4.6 was determined (12). In experiments designed to measure lipid synthesis the tissues were exposed to 0.5 μCi/ml [^{14}C]acetate for the terminal 2 hr of incubation. The tissues were then weighed, homogenized in 2 ml distilled water, and the lipids were extracted by the method of Bligh and Dyer (13). Radioactivity in the organic layer was then determined and the results expressed as disintegrations per minute per milligram (dpm/mg) wet tissue weight. For the determination of ODC activity, the tissues were homogenized in 50 mM Tris buffer (pH 7.4) subsequent to the incubations. ODC activity was then determined using a modification (7) of the methods described by Jänne and Williams-Ashman (14). ODC activity was expressed as picomoles CO_2 produced/30 min/10 mg tissue. Statistical comparisons were made with Student's t test.

Results. Tables I and II show the effect of MGBG, an inhibitor of the conversion of putrescine to spermidine, on the prolactin stimulation of casein and lipid synthesis. MGBG at 20 μM (Table I) abolishes the prolactin stimulation of [^{3}H]leucine incorporation into casein, and this inhibition is reversible by the addition of 0.5 or 1.0 mM spermidine. It is also noteworthy that 1 mM spermidine, by itself, had a small but significant stimulatory effect on the rate of [^{3}H]leucine incorporation; we have also observed this effect in other experiments which are not presented. Table II shows that the prolactin stimulation of [^{14}C]acetate incorporation into lipids is abolished by 200 μM MGBG. MGBG concentrations of 100 μM and less did not abolish this effect of PRL (data not presented). The inhibition of the PRL stimulation of lipid synthesis was reversed by the addition of 5 mM spermidine. Since 200 μM DFMO reduced the basal rate of [^{14}C]acetate incorporation, however, this experiment does not allow us to conclude that 5 mM spermidine completely reversed the DFMO inhibition of the PRL effect. The results in Tables I and II along with experiments published earlier (4, 8) provide convincing evidence that ongoing spermidine synthesis is essential for PRL to manifest its

TABLE I. EFFECT OF MGBG ON THE RATE OF [^{3}H]LEUCINE INCORPORATION INTO CASEIN*

Additions		[^{3}H]Leucine incorporation into casein (dpm/mg wet tissue wt)		
MGBG (μM)	Spermidine (mM)	Control	Plus prolactin	P
—	—	277 ± 12**	514 ± 29	<0.05
20	—	284 ± 13	272 ± 16	N.S.
20	0.5	279 ± 14	445 ± 27	<0.05
20	1.0	279 ± 17	434 ± 18	<0.05
—	0.5	318 ± 22	562 ± 29	<0.05
—	1.0	339 ± 15	481 ± 25	<0.05

* Explants were initially cultured for 24 hr in media containing 1 μg/ml insulin plus 10^{-7} M cortisol. MGBG, spermidine, and/or prolactin were then added to certain flasks and incubation continued for 16 hr. [^{3}H]Leucine (0.5 μCi/ml) was present in the culture media for the terminal 2 hr of incubation.

** Values represent the means ± SE of six observations.

TABLE II. EFFECT OF MGBG ON THE RATE OF [^{14}C]ACETATE INCORPORATION INTO LIPIDS*

Additions		[^{14}C]Acetate incorporation into lipids (dpm/mg wet tissue wt)		
MGBG (μM)	Spermidine (mM)	Control	Plus prolactin	P
—	—	2800 ± 161**	4996 ± 193	<0.05
200	—	1930 ± 105	2206 ± 147	N.S.
200	0.5	2410 ± 56	2908 ± 261	N.S.
200	1.0	2266 ± 139	2605 ± 125	N.S.
200	5.0	1734 ± 124	3392 ± 194	<0.05

* Explants were cultured initially for 24 hr with media containing 1 μg/ml insulin plus 10^{-7} M cortisol. MGBG, spermidine, and/or prolactin (1 μg/ml) were then added to certain flasks and culture continued for 16 hr. [^{14}C]Acetate was present in the culture media for the terminal 2 hr of incubation.

** Values represent the means ± SE of six observations.

actions on both casein and lipid biosynthesis in cultured mouse mammary gland explants.

Since (a) ODC is generally believed to be the rate limiting enzyme in the synthesis of the polyamines, and (b) PRL has a several-fold effect on ODC activity in cultured mammary tissues, the effect of DFMO, an irreversible inhibitor of ODC activity (15), on the metabolic actions of PRL was determined. The efficacy of DFMO in inhibiting ODC activity was tested both by adding DFMO to homogenates of mammary tissues (Table III), and by adding it to the media bathing the mammary gland explants (Table IV). In view of the potency of DFMO in inhibiting ODC activity in the broken cell preparations, i.e., 0.01 mM DFMO inhibited ODC activity by about 95% (Table III), it was essential to extensively wash explants exposed to DFMO prior to determining ODC activity; otherwise, the DFMO present in the extracellular spaces of the tissues would be expected to abolish ODC activity in homogenates of these tissues. The efficacy of the wash procedure in removing extracellular DFMO was tested by initially exposing tissues to media at 0°C containing 0, 1, or 5 mM DFMO, and then washing the tissues at 0°C for 30 min. as described in the legend to Table IV. ODC activities in the tissue exposed to 0, 1, or 5 mM DFMO, respectively, were 17.2 ± 1.4, 14.0 ± 0.7, and 12.5 ± 0.5 pmole/30 min/10 mg wet tissue weight. The wash procedure therefore effectively removes most of the extracellular DFMO. The inhibitory effects of DFMO on ODC activity in tissues cultured with DFMO at 37°C as shown in Table IV thus likely represents the intracellular actions of DFMO. There is a dose–response inhibition of ODC activity and 5 mM DFMO reduced both control and PRL-stimulated ODC activities by about 98%.

TABLE III. ODC ACTIVITY WITH DFMO ADDED DIRECTLY TO ENZYME REACTION MIXTURE*

DFMO added (mM)	Enzyme activity (pmole/30 min/10 mg wet tissue wt)
0	28.4 ± 7.3**
0.01	1.9 ± 0.3
0.1	0.20 ± 0.04
1	0

* Details of the enzyme reaction are in the Materials and Methods.

** Means ± SE of six observations.

TABLE IV. EFFECT OF DFMO ON ORITHINE DECARBOXYLASE ACTIVITY*

DFMO concentration (mM)	Ornithine decarboxylase activity (pmole/30 min/10 mg wet tissue wt)		
	Control	Plus prolactin	P
0	9.27 ± 1.79**	28.4 ± 7.3	<0.05
0.1	2.10 ± 0.12	3.37 ± 0.43	<0.05
0.5	0.62 ± 0.12	2.14 ± 0.42	<0.05
1.0	0.60 ± 0.06	1.51 ± 0.18	<0.05
5.0	0.22 ± 0.06	0.67 ± 0.14	<0.05

* Explants were cultured initially for 24 hr with 1 μg/ml insulin plus 10^{-7} M cortisol. DFMO and/or 1 μg/ml prolactin were then added to the culture medium and incubation continued for 2 hr. After washing the tissues for 30 min at 0°C with five 3-ml volumes of media which were changed every 5 min, the tissues were homogenized, and ODC activity was determined.

** Numbers represent the means ± SE of six observations.

TABLE V. EFFECT OF DFMO ON THE RATE OF [^{3}H]URIDINE INCORPORATION INTO RNA*

DFMO concentration (mM)	[^{3}H]Uridine incorporation into RNA (dpm/μg RNA)		
	Control	Plus prolactin	P
0	138 ± 10**	213 ± 8	<0.05
0.1	147 ± 10	216 ± 16	<0.05
0.5	144 ± 9	222 ± 15	<0.05
1.0	154 ± 13	211 ± 8	<0.05
5.0	158 ± 6	245 ± 15	<0.05

* Explants were cultured initially for 24 hr in media containing 1 μg/ml insulin and 10^{-7} M cortisol. DFMO was then added to certain flasks and incubation continued for 2 hr. Finally, prolactin (1 μg/ml) was added to certain flasks and incubation continued for 6 hr. [^{3}H]Uridine (0.5 μCi/ml) was added for the terminal 1 hr of incubation.

** Values represent the means ± SE of six observations.

TABLE VII. EFFECT OF DFMO ON THE RATE OF [^{14}C]ACETATE INCORPORATION INTO LIPIDS*

DFMO concentration (mM)	[^{14}C]Acetate incorporation into lipids (dpm/mg wet tissue wt)		
	Control	Plus prolactin	P
0	3678 ± 130**	4904 ± 434	<0.05
0.05	2991 ± 197	5053 ± 681	<0.05
0.1	3108 ± 325	4509 ± 576	<0.05
1.0	2911 ± 252	3820 ± 192	<0.05
5.0	3267 ± 273	4632 ± 539	<0.05

* Explants were cultured initially for 24 hr with media containing 1 μg/ml insulin plus 10^{-7} M cortisol. DFMO and/or prolactin (1 μg/ml) were then added to certain flasks and culture continued for 16 hr. [^{14}C]Acetate was present in the culture media for the terminal 2 hr of incubation.

** Values represent the means ± SE of six observations.

Tables V, VI, and VII, respectively, show the effect of DFMO on the PRL actions on RNA, casein, and lipid biosynthesis in cultured mammary gland explants. At concentrations up to 5 mM, DFMO had no effect on the PRL stimulation of RNA, casein, or lipid biosynthesis. This was true even though the tissues in certain experiments were precultured for 2 hr prior to the addition of PRL in order to ensure that ODC activity was virtually abolished at the time of addition of PRL (Tables V and VI).

Discussion. The results of these studies confirm and support earlier studies (4, 8) in which it was suggested that ongoing spermidine synthesis is required for PRL to manifest its action on casein synthesis in cultured mammary tissues. This conclusion is based on the fact that MGBG, a drug that inhibits putrescine conversion to spermidine, abolishes the PRL stimulation of casein synthesis; and this inhibition is reversed by the addition of spermidine to the culture medium. Our results suggest that ongoing spermidine synthesis is apparently similarly required for the action of PRL on lipid biosynthesis since MGBG abolished this effect of PRL and the MGBG inhibition is reversed by adding spermidine to the culture medium. We cannot, however, presently explain why a 10-fold higher concentration of MGBG is required to abolish the effect of PRL on lipid biosynthesis as compared to the PRL effect on casein biosynthesis. Perhaps it is related to the extent of spermidine depletion from the PRL target cells.

TABLE VI. EFFECT OF DFMO ON THE RATE OF [^{3}H]LEUCINE INCORPORATION INTO CASEIN*

DFMO concentration (mM)	[^{3}H]Leucine incorporation into casein (dpm/mg wet tissue weight)		
	Control	Plus prolactin	P
0	440 ± 11**	824 ± 57	<0.05
0.1	415 ± 29	726 ± 39	<0.05
0.5	483 ± 33	764 ± 40	<0.05
1.0	484 ± 34	819 ± 43	<0.05
5.0	490 ± 7	805 ± 50	<0.05

* Explants were cultured initially for 24 hr in media containing 1 μg/ml insulin plus 10^{-7} M cortisol. DFMO was then added to certain flasks and incubation continued for 2 hr. Finally, prolactin (1 μg/ml) was added to certain flasks and incubation continued for 16 hr. [^{3}H]Leucine (0.5 μCi/ml) was added for the terminal 2 hr of incubation.

** Values represent the means ± SE of six observations.

Since ODC is generally believed to be the rate-limiting enzyme for the synthesis of the polyamines, and since PRL has a several-fold effect on enhancing ODC activity in cultured mammary gland explants, it was logical to predict that DFMO, an irreversible inhibition of ODC activity, would attenuate or abolish the effects of PRL on the synthesis of milk components. That this did not occur is clear from the data in Tables V–VII. Despite the fact that control and PRL-stimulated ODC activities were inhibited by 5 mM DFMO to the extent of about 98%, the magnitude of the PRL responses were not different from those

observed in the absence of 5m*M* DFMO. These observations allow us to conclude that the high levels of ODC activity which are observed 2–4 hr after exposing mammary gland explants to PRL is not of critical importance for PRL to express its subsequent actions on RNA, casein, and lipid biosynthesis. However, it is obvious from the MGBG experiments that spermidine levels must be maintained in order for prolactin to express its actions on casein and lipid biosynthesis. Precisely how spermidine levels are maintained in the virtual absence of ODC activity is not known. Possibilities include (a) that ODC is not rate limiting for the synthesis of the polyamines and accordingly either low levels of ODC are adequate for polyamine formation or there may be an alternate biochemical pathway for polyamine formation, or (b) that spermidine formation from spermine is adequate to maintain spermidine levels.

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Received April 4, 1983. P.S.E.B.M. 1983, Vol. 174.