Effects of oestradiol and progesterone on the metabolism of [U-¹⁴C]glucose by mouse morulae and early blastocysts *in vitro*

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Summary. The addition of progesterone (10^{-7} to 10^{-5} M) and/or oestradiol (10^{-10} M) during 24-h chase culture of pulse-labelled morulae–early blastocysts did not affect the degradation of radiolabelled glycogen or other biochemical fractions. The presence of a high concentration of progesterone (10^{-5} M) during 5-h pulse culture significantly inhibited incorporation of substrate carbon from [U-¹⁴C]glucose into both the acid-soluble and acid-insoluble glycogen fractions, but had no effect on non-glycogen fractions. Catabolic utilization of glucose as estimated by the rate of carbon dioxide and lactate production was not affected by the presence of progesterone (10^{-7} to 10^{-5} M), oestradiol (10^{-10} to 10^{-8} M) or a combination of both. The results indicate that ovarian steroids at expected physiological concentrations do not directly influence embryonic energy metabolism.

Introduction

The hormonal status of the mother changes during the preimplantation period and the balanced production of ovarian hormones after fertilization is essential for the adequate preparation of the maternal reproductive organs to produce optimum conditions for supporting the developing embryo and initiating successful implantation. Investigations using exogenous steroids administered parenterally to ovariectomized animals have shown that both oestradiol and progesterone affect carbohydrate metabolism (Menke & McLaren, 1970; Torbit & Weitlauf, 1974; Nilsson *et al.*, 1980), as well as protein and nucleic acid synthesis (McLaren, 1968; Weitlauf & Greenwald, 1968; Prasad *et al.*, 1968; Smith & Smith, 1971; Lau *et al.*, 1973; Cheng *et al.*, 1985) of preimplantation embryos. The low metabolic activity of delayed implanting blastocysts, as measured by the rate of carbon dioxide (CO₂) production, increases rapidly to near normal levels after termination of dormancy by injecting one or both of the ovarian hormones (Menke & McLaren, 1970; McLaren & Menke, 1971; Menke, 1972; Weitlauf, 1974; Torbit & Weitlauf, 1975). These hormones have also been reported to affect the total glycogen content of mouse blastocysts (Ozias & Weitlauf, 1971; Nilsson *et al.*, 1980) and Edirisinghe & Wales (1984), in an attempt to explain the discrepancy in glycogen content of cultured, as compared to freshly collected, blastocysts (Ozias & Stern, 1973), found that transfer of pulse-labelled embryos to ovariectomized recipients receiving progesterone resulted in an increased degradation of acid-soluble glycogen, an effect antagonized by oestradiol.

Both oestrogen and progesterone cause structural and biochemical changes to the uterine epithelium (Yochim, 1975; Finn, 1977) and influence the chemical composition of uterine fluid (Iritani *et al.*, 1971; Wales & Restall, 1971; Arthur & Daniel, 1972; Tantayaporn *et al.*, 1974; Surani, 1975; Fishel, 1979). Therefore, one possible mode of action for these hormones on embryos is an indirect one resulting from changes to the uterine milieu (Weitlauf & Greenwald, 1968; Roblero & Izquierdo, 1976). On the other hand, ovarian hormones of maternal origin have been found in the fluids of the genital tract during early pregnancy (Cowan *et al.*, 1976; Fowler *et al.*,

1977) and it has been postulated that *in vivo* they could have a direct effect on embryos (Smith, 1968).

The present experiments were undertaken to test for evidence of direct effects of ovarian steroids on the anabolic and catabolic utilization of glucose.

Materials and Methods

Collection of embryos. Random bred Swiss albino mice of the Quackenbush (Q(s)) strain were induced to super-ovulate and morulae and early blastocyst-stage embryos were collected 96 h after hCG injection, as described previously (Pike & Wales, 1982). The medium used for collection and washing of embryos was a modified Krebs–Ringer–bicarbonate solution containing 25 mM-lactate, 0.25 mM-pyruvate and supplemented with antibiotics and 1 mg bovine serum albumin/ml (Brinster, 1965). The embryos from all the mice used in a particular replicate of an experiment were pooled and assigned at random to treatment groups within 30–40 min of the completion of collection. The number of embryos used per treatment group ranged from 25 to 60. The basic culture medium for the experiments was the modified Krebs–Ringer–bicarbonate solution described above supplemented with a further 4 mg bovine serum albumin/ml. All incubations were carried out at 37°C in a humidified atmosphere of 5% CO₂:95% air.

Hormone-containing media. Stock solutions of 1,3,5[10]-oestratriene-3,17 β -diol 3-benzoate (10^{-2} M) and progesterone (10^{-1} M) (Sigma Chemical Co., St Louis, MO, U.S.A.) were prepared in absolute ethyl alcohol and stored at –20°C. On the day of culture, the ethanol stocks were serially diluted with culture medium to obtain the desired concentrations. All incubation media, including control, had the same final concentration of ethanol (0.01%).

A preliminary study incubating droplets of labelled progesterone under oil showed that most of the steroid had diffused into the oil within 20 min. Therefore, all incubations of embryos with hormones were carried out in glass culture tubes (12 × 100 mm) containing 0.7 ml medium.

Effect of hormones on incorporation of glucose. The incorporation of glucose carbon into various biochemical fractions of embryos was investigated by incubating embryos in tubes for 5 h in basic culture media containing 0.28 mM-[U-¹⁴C]glucose (sp. act. 1.11 MBq/ μ mol) with or without hormones. On completion of culture, all embryos were washed twice and stored at –70°C before fractionation.

Effect of hormones on turnover of glucose. Embryos were pulse labelled by incubating under oil for 2 h in droplets of basic culture medium containing 0.28 mM-[U-¹⁴C]glucose (sp. act. 1.11 MBq/ μ mol). After pulse culture, a sample of washed embryos was collected and stored at –70°C until fractionation to measure incorporation during pulse. The fate of incorporated label in the remaining embryos was determined by transfer to tubes containing 0.7 ml medium $\pm$ hormones for 24-h chase culture. After chase culture, the embryos were washed twice through 2 ml hormone-free medium containing unlabelled glucose and stored at –70°C until fractionation.

Effect of hormones on catabolic utilization of glucose. The production of CO₂ and lactate from glucose was used to assess the catabolic utilization of substrate in the presence or absence of hormones. The rate of CO₂ production was estimated by a modification of the method of Brinster (1967). After collection, embryos were washed 3 times in substrate-free medium and, for each treatment, a sample of 10 washed embryos was incubated in a small glass tube (5 × 25 mm) in 50 μ l medium containing 0.28 mM-[U-¹⁴C]glucose (sp. act. 1.11 MBq/ μ mol) as sole energy substrate. The tube was sealed in a glass scintillation vial together with a second empty tube (9 × 30 mm). The tightly capped scintillation vial was gassed for 1 min with 5% CO₂ in air. After 4 h, the culture was terminated by injection of 100 μ l 1 M-H₂SO₄ through the rubber seal. This was immediately followed by injection of 100 μ l 2.5 M-NaOH into the empty tube to trap CO₂. The vials were left sealed overnight after which the NaOH was recovered and the radioactivity in the trapped CO₂ was assayed following the addition of 1 ml 1 M-Tris-HCl buffer. The counts per minute were converted to pmol CO₂ per embryo per hour after taking into account specific activity of the substrate, machine efficiency and background counts. The incubation medium with H₂SO₄ was stored at –70°C until estimation of lactate production.

The incubation medium was neutralized by the addition of an equimolar amount of barium acetate and 50 μ l medium were applied to a strip of Whatman No. 1 chromatography paper and chromatographed for 16 h using butanol:water:acetic acid (4:5:1, by vol.) as solvent. Dried chromatograms were serially cut into 1 cm lengths and radioactivity was estimated by liquid scintillation spectrometry. The lactate peak was identified by the *R_f* value and its comigration with authentic lactate. Counts per minute were converted to picomoles as described under CO₂ production.

Fractionation of embryos. Labelled embryos obtained from the turnover and incorporation studies were processed to recover major biochemical fractions using the fractionation procedure described previously (Edirisinghe *et al.*, 1984). The radioactivity in these fractions was then determined using scintillation spectrometry. The glucose carbon incorporated (in pg atoms per embryo) was calculated from the disintegrations per minute and the specific activity of [U-¹⁴C]glucose.

Statistical analyses. The statistical significance of the data was tested by standard analyses of variance after logarithmic transformation of the data to equalize variances. The levels of significance are quoted in the text.

Results

Experiment 1

As shown in Table 1, the presence of 10^{-5} M-progesterone during 5-h culture significantly reduced the amount of label incorporated into the acid-soluble glycogen ($P < 0.01$) and the acid-insoluble glycogen ($P < 0.05$) fractions, the reduction in label being more marked in the former than the latter fraction. Lower concentrations of progesterone had no significant effect on incorporation into either glycogen fraction and the accumulation of glucose carbon in the other biochemical fractions of embryos was unaffected by the presence of progesterone at any of the concentrations tested.

Experiment 2

As shown in Table 2, oestradiol alone had no effect on incorporation of glucose carbon. As in Exp. 1, 10^{-5} M-progesterone reduced incorporation into acid-soluble glycogen to 50% of control but a 100-fold rise in oestradiol concentration did not antagonize the effects of progesterone. In this experiment progesterone tended to lower the incorporation of glucose into the acid-insoluble glycogen pool but the differences did not attain statistical significance, unlike those in Exp. 1. The other biochemical fractions isolated were unaffected by any of the treatments imposed.

Table 1. Effect of progesterone on the incorporation of glucose carbon into various biochemical fractions of morulae-early blastocysts cultured for 5 h in a medium containing 0.28 mM-[U- 14 C]glucose, 25 mM-lactate and 0.25 mM-pyruvate

Progesterone conc. in medium (M)	Incorporation of glucose carbon (pg atoms/embryo) into:			
	Acid-soluble fraction		Acid-insoluble fraction	
	Glycogen	Non-glycogen	Glycogen	Non-glycogen
0	15.87 $\pm$ 0.65	21.11 $\pm$ 4.80	7.69 $\pm$ 0.73	7.99 $\pm$ 0.57
1×10^{-7}	15.16 $\pm$ 1.66	16.15 $\pm$ 3.14	7.72 $\pm$ 1.11	7.77 $\pm$ 0.31
1×10^{-6}	16.57 $\pm$ 2.33	17.60 $\pm$ 3.20	7.19 $\pm$ 0.43	8.32 $\pm$ 0.47
1×10^{-5}	8.55 $\pm$ 2.12**	17.45 $\pm$ 2.28	5.72 $\pm$ 0.74*	7.50 $\pm$ 0.88

Values are mean $\pm$ s.e.m. for three replicates.

Significant effect of progesterone; * $P < 0.05$; ** $P < 0.01$.

Table 2. Effects of oestradiol with and without the addition of progesterone (1×10^{-5} M) on the incorporation of glucose carbon into morulae-early blastocysts cultured for 5 h in a medium containing 0.28 mM-[U- 14 C]glucose, 25 mM-lactate and 0.25 mM-pyruvate

Oestradiol conc. (M)	Addition of progesterone	Incorporation of glucose carbon (pg atoms/embryo) into:			
		Acid-soluble fraction		Acid-insoluble fraction	
		Glycogen	Non-glycogen	Glycogen	Non-glycogen
0	—	21.55 $\pm$ 1.36	17.99 $\pm$ 2.55	6.79 $\pm$ 0.52	7.28 $\pm$ 0.25
1×10^{-10}	—	21.67 $\pm$ 2.42	15.01 $\pm$ 1.16	7.20 $\pm$ 2.24	9.09 $\pm$ 1.23
1×10^{-8}	—	18.23 $\pm$ 3.79	17.90 $\pm$ 1.25	5.67 $\pm$ 0.81	8.66 $\pm$ 0.51
1×10^{-10}	+	10.39 $\pm$ 0.63**	15.26 $\pm$ 2.78	4.97 $\pm$ 0.16	7.85 $\pm$ 0.13
1×10^{-8}	+	11.59 $\pm$ 0.94**	14.61 $\pm$ 1.95	5.50 $\pm$ 0.65	8.32 $\pm$ 0.33

Values are mean $\pm$ s.e.m. for 3 replicates.

**Significant effect of hormone, $P < 0.01$.

Experiment 3

The effects of progesterone and oestradiol on the turnover of glucose carbon incorporated into various biochemical fractions of embryos during a 2-h pulse culture was determined by chase culturing the labelled embryos for 24 h in media with or without hormones. Three concentrations of progesterone (10^{-7} , 10^{-6} and 10^{-5} M) were tested either alone or in combination with oestradiol (1×10^{-10} M) (Table 3). The amount of label in both the glycogen and non-glycogen acid-soluble fractions decreased significantly during chase culture ($P < 0.01$). However, the small reduction in the

Table 3. The distribution of glucose carbon in morulae-early blastocysts after pulse culture in medium containing 0.28 mM-[U- 14 C]glucose, 25 mM-lactate and 0.25 mM-pyruvate and after chase culture in medium with and without the addition of progesterone and oestradiol

Treatment	Hormone conc. (M)		Incorporation of glucose carbon (pg atoms/embryo) into:		
			Acid-soluble fraction		Acid-insoluble fraction
			Glycogen	Non-glycogen	
Pulse 2 h	0	0	7.33 $\pm$ 0.40	17.40 $\pm$ 1.97	12.88 $\pm$ 1.35
Chase 24 h	0	0	5.44 $\pm$ 1.14	7.79 $\pm$ 0.30	10.98 $\pm$ 0.58
	1×10^{-7}	0	5.20 $\pm$ 0.93	9.28 $\pm$ 0.38	10.71 $\pm$ 0.56
	1×10^{-6}	0	4.06 $\pm$ 0.37	7.19 $\pm$ 0.26	10.30 $\pm$ 0.72
	1×10^{-5}	0	4.23 $\pm$ 0.71	7.93 $\pm$ 1.09	10.36 $\pm$ 0.22
	0	1×10^{-10}	4.94 $\pm$ 0.69	8.68 $\pm$ 1.05	11.21 $\pm$ 0.52
	1×10^{-7}	1×10^{-10}	4.30 $\pm$ 0.66	9.99 $\pm$ 1.82	10.99 $\pm$ 1.66
	1×10^{-6}	1×10^{-10}	5.86 $\pm$ 0.76	8.15 $\pm$ 0.60	11.57 $\pm$ 0.80
	1×10^{-5}	1×10^{-10}	4.25 $\pm$ 0.79	7.27 $\pm$ 1.22	9.81 $\pm$ 0.05

Values are mean $\pm$ s.e.m. for 3 replicates.

Table 4. Effects of oestradiol and progesterone on carbon dioxide and lactate production from glucose by mouse morulae-early blastocysts incubated for 4 h in media containing 0.28 mM-[U- 14 C]glucose

Hormone conc. (M)		Carbon dioxide produced (pmol/embryo h ⁻¹)	Lactate accumulated (pmol/embryo h ⁻¹)
Progesterone	Oestradiol		
<i>Experiment A</i>			
0	0	3.62 ± 0.21 (6)	2.81 ± 0.41 (6)
1 × 10 ⁻⁷	0	3.74 ± 0.44 (6)	1.86 ± 0.19 (6)
1 × 10 ⁻⁶	0	3.43 ± 0.47 (6)	1.87 ± 0.17 (6)
1 × 10 ⁻⁵	0	3.54 ± 0.40 (6)	2.77 ± 0.38 (6)
<i>Experiment B</i>			
0	0	5.44 ± 0.46 (5)	2.50 ± 0.32 (5)
0	1 × 10 ⁻¹⁰	5.57 ± 0.42 (5)	2.59 ± 0.22 (5)
0	1 × 10 ⁻⁹	6.23 ± 0.32 (5)	2.65 ± 0.30 (5)
0	1 × 10 ⁻⁸	4.63 ± 0.50 (5)	2.27 ± 0.16 (5)
<i>Experiment C</i>			
0	0	5.29 ± 0.32 (5)	2.54 ± 0.23 (5)
1 × 10 ⁻⁷	1 × 10 ⁻¹⁰	5.67 ± 0.41 (5)	2.60 ± 0.27 (5)
1 × 10 ⁻⁶	1 × 10 ⁻¹⁰	5.69 ± 0.36 (5)	3.25 ± 0.39 (5)
1 × 10 ⁻⁵	1 × 10 ⁻¹⁰	4.85 ± 0.52 (5)	3.18 ± 0.17 (5)

Values are the means $\pm$ s.e.m. (no. of observations).

amount of label in the acid-insoluble fraction during 24-h chase was not significant. The presence of the hormones during chase culture had no significant effect on the amount of label retained in the acid-soluble glycogen or the other fractions isolated.

Experiment 4

Three concentrations each of progesterone and oestradiol were used to test the effects of the ovarian steroids on the catabolic utilization of [U- 14 C]glucose during 4-h incubation (Table 4). Overall, there was no effect of progesterone, oestradiol or combinations of the two hormones on CO₂ and lactate production. However, there was some suggestion that high levels of steroid may marginally reduce CO₂ production and stimulate lactate accumulation when both hormones are present in the medium.

Discussion

It has been reported that ovarian steroids of maternal origin are present in genital fluids (Cowan *et al.*, 1976; Fowler *et al.*, 1977) and therefore some form of direct action of these hormones on embryos might occur. However, in the absence of data on the concentration of ovarian steroids in the uterine lumen of the mouse, blood hormone levels during early pregnancy probably provide the best indication of the possible physiological levels of these hormones to which the preimplantation embryo may be exposed *in utero*. On Day 4 of pregnancy in the mouse, progesterone is present at 45 ng/ml (1.5×10^{-7} M) in serum (Virgo & Bellward, 1974; Murr *et al.*, 1974), a level well within the range of concentrations tested in the present experiments. McCormack & Greenwald (1974) measured a peak of 21 pg oestradiol-17 β /ml (0.8×10^{-10} M). Whilst this is similar to the level tested in most of the experiments reported here, much higher concentrations were used in some tests to see if any response to the hormone *per se* could be elicited and whether high concentrations of this hormone might antagonize the effects of progesterone *in vitro*.

While there is a body of experimental evidence (see 'Introduction') on the effects of maternal hormones on the metabolism of embryos *in utero*, previous studies of the direct effects of ovarian hormones on preimplantation mouse embryos have been inconclusive and contradictory. Whitten (1957) showed that progesterone at 4 μ g/ml (1.3×10^{-5} M) inhibited cleavage, a finding which was later confirmed by Kirkpatrick (1971). However, Roblero & Izquierdo (1976) failed to find any effect of this concentration of progesterone on cleavage rate of mouse embryos and reported that this concentration of progesterone when combined with high levels of serum (15%) enhanced the cleavage rate of embryos, suggesting that a protein fraction in serum serves as a progesterone carrier. Smith (1968) claimed that mouse blastocysts bind oestradiol but did not confirm whether it was transported into blastomeres or was merely bound to the zona pellucida or plasma membrane. Kirkpatrick (1971) reported inhibition of cleavage of mouse embryos by oestradiol but the concentration of hormone used was so high (10^{-4} M) that the results are of questionable physiological significance. Harrer & Lee (1973) observed an increased uptake of [3 H]uridine by mouse blastocysts in the presence of oestradiol but Warner & Tollefson (1977) concluded that mouse embryos were refractory to modulation by exogenous oestradiol. Torbit & Weitlauf (1975) preincubated normal and delayed implanting blastocysts with oestradiol-17 β and/or progesterone and found that the hormones did not directly stimulate or inhibit CO₂ production by these embryos.

The present results on the rate of CO₂ and lactate production suggest that aerobic glycolysis and glucose oxidation *in vitro* are not affected to any extent by ovarian hormones. The involvement of maternal oestradiol and progesterone *in vivo* on the ability of embryos to oxidize glucose (Menke & McLaren, 1970; Torbit & Weitlauf, 1974) is therefore unlikely to be due to the direct effects of these hormones on the embryo. Overall, the rate of CO₂ and lactate production in this study, although lower, compares favourably with other reports (Brinster, 1967; Wales, 1969; Menke & McLaren,

1970). The lower rates of glucose turnover in this study are most probably due to the lower concentration of glucose used (0.28 mM) compared to that in the reports above (5.56 mM). Pike & Wales (1982) reported that a reduction in glucose concentration in the medium from 5.56 to 0.28 mM resulted in a 50–80% decrease in the amount of glucose carbon incorporated into mouse morulae.

The results of the experiments to study the effects of hormones on incorporation of glucose carbon indicate that oestradiol had no effect on embryonic metabolism of glucose but progesterone in high concentration (10^{-5} M) significantly inhibited incorporation into the acid-soluble glycogen and, to a lesser extent, the acid-insoluble glycogen fraction. This influence of progesterone on incorporation was not inhibited even by high concentrations of oestradiol. Although the response to progesterone was seen only with concentrations of the hormone higher than those likely to be encountered by embryos *in vivo*, it is still pertinent to ask if the response is a physiological one, or a pathological one resulting from an overdose of the hormone (Menke & McLaren, 1970; Uehara *et al.*, 1983). The work of Roblero & Izquierdo (1976) suggests that ovarian hormones may require some binding factors (available *in utero*) for direct action on the embryo and that absence of such factors *in vitro* results in poor binding and so the physiological effects of these hormones *in vitro* are only apparent at a high concentration of the hormone. If the effect of progesterone seen in the present study is indeed physiological, the results indicate that any reduction in the accumulation of glucose carbon incorporated as a result of direct hormone action arises as a result of decreased incorporation rather than increased turnover of the polymer.

The finding that there is no effect of exogenous progesterone or oestradiol on the turnover of glucose carbon of pulse-labelled embryos during 24-h chase culture suggests that the earlier reports of increased degradation of acid-soluble glycogen in pulse-labelled embryos transferred to ovariectomized mice receiving progesterone (Edirisinghe & Wales, 1984) cannot be attributed to the direct action of ovarian hormones on embryos. A more likely mechanism for the action of maternal ovarian hormones on embryos *in vivo* is therefore an indirect one, induced by changes in the uterine milieu. The precise mode by which these hormones could affect embryo metabolism through changes in the uterine environment remains unknown. Some possibilities are: (i) changes in the micro-environment of embryos through changes in the composition of uterine secretion (Tantayaporn *et al.*, 1974), (ii) growth and proliferation of the uterine epithelium (Cheng *et al.*, 1985), (iii) secretion of specific uterine factors (Edirisinghe & Wales, 1984), (iv) synthesis of prostaglandins (Ham *et al.*, 1975) and (v) changes in intrauterine oxygen tension via alterations in uterine vasculature (Mitchell & Yochim, 1968). Some of these possibilities are being investigated.

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