The Effect of Lutropin on Specific Protein Synthesis in Tumour Leydig Cells and in Leydig Cells from Immature Rats

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The amount of ^{35}S incorporated into the various proteins after separation by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels was used as an estimate of their synthesis in the Leydig cells. Increased synthesis of proteins with apparent mol.wts. 27000 and 29000 was observed 3 h after addition of lutropin to tumour Leydig cells. Incubation of Leydig cells from immature rats with lutropin (100 ng/ml) for 2 h or longer resulted in increased synthesis of proteins with apparent mol.wts. 11000, 21000, 27000 and 29000. At higher concentrations (≥ 100 ng/ml) of lutropin there was a decrease in the synthesis of a protein with apparent mol.wt. 13000. The amount of lutropin required for the stimulation of protein synthesis in both types of Leydig cells was similar to that needed for stimulation of steroidogenesis. Lutropin-stimulated specific protein synthesis was not due to increased concentrations of testosterone, however, because (1) addition of testosterone to the cells had no effect on the synthesis of the proteins, and (2) inhibition of steroidogenesis with elipten phosphate (an inhibitor of the cholesterol side-chain-cleavage enzyme complex) did not abolish the effect of lutropin. The stimulation of specific protein synthesis was also not due to contaminating follitropin in the lutropin preparation. Addition of actinomycin D to the cells at the start of the incubation prevented the effect of lutropin on specific protein synthesis, indicating that mRNA synthesis may be needed for this effect of lutropin. Incubation of the cells with cycloheximide for 30 min after labelling of the proteins did not result in a detectable decrease in the amounts of the lutropin-induced proteins, indicating that their half-life is longer than 30 min.

Stimulation of steroidogenesis in rat testis Leydig cells by lutropin involves synthesis of protein and RNA (Cooke *et al.*, 1975a; Mendelson *et al.*, 1975) and it has been postulated that the continuous synthesis of a protein with short half-life (≤ 13 min) is necessary for the stimulation of steroidogenesis (Cooke *et al.*, 1975b).

It has been shown that lutropin stimulates in rat testis Leydig cells the synthesis of a protein with an apparent mol.wt. of 21000 (referred to as lutropin-induced protein); the half-life of this protein is longer than 30 min (Janszen *et al.*, 1976b, 1977). The lutropin stimulation of the synthesis of this protein is mediated by cyclic AMP and probably involves mRNA synthesis (Janszen *et al.*, 1978). In the present study the effect of lutropin on the synthesis of lutropin-induced protein and other proteins has been investigated in two other types of Leydig cells, i.e. those from immature rats and from a Leydig cell tumour, by first labelling the proteins with [^{35}S]methionine and then separating the proteins on sodium dodecyl sulphate/polyacrylamide gels,

followed by fluorography of the dried gels, impregnated with 2,5-diphenyloxazole.

Materials and Methods

Follitropin (NIH-FSH-S12) and lutropin (NIH-LH-S19) were gifts from the Endocrinology Study Section of the National Institutes of Health, Bethesda, MD, U.S.A. [^{35}S]Methionine (280 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Elipten phosphate [α -ethyl- α -(*p*-aminophenyl)glutarimide] was a gift from Ciba, Basle, Switzerland. The Leydig-cell tumour was obtained from the Mason Research Institute Tumour Bank, Worcester, MA, U.S.A., and originated from R. Huseby, American Medical Center at Denver, Spivak, CO, U.S.A. The tumour originally arose spontaneously in Fischer male rats. The tumour was grown and sustained in castrated 90-day-old male Fischer rats. A cell suspension of the tumour was prepared by incubation of small pieces of the tumour in 7 ml of Krebs–Ringer bicarbonate buffer, pH 7.4

(Umbreit *et al.*, 1964) containing 0.2% glucose and 1mg of collagenase/ml (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) for 50min at 37°C in a 40ml plastic tube under an atmosphere of O₂/CO₂ (19:1) with continuous shaking. After incubation, 25ml of 0.9% NaCl was added and the tube was inverted ten times. The tubes were left for 10min at room temperature (approx. 22°C) to sediment cell clumps. The supernatant was syphoned off and filtered through nylon gauze (60µm pore size). The cells were then sedimented by centrifugation for 10min at 100g. The sedimented cells were washed once by centrifugation for 10min at 100g in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose and 0.1% albumin (fraction V). Histochemical detection of 3β-hydroxy steroid dehydrogenase activity (Janszen *et al.*, 1976a) showed that almost all isolated cells formed formazan precipitates. Leydig-cell suspensions from immature rats were prepared from testes of Wistar rats substrain R-Amsterdam, 21–25 days old, as previously described and purified by centrifugation through a Ficoll solution (Janszen *et al.*, 1976a). The cell suspension was washed once by centrifugation for 5min at 100g through Krebs–Ringer bicarbonate solution, pH 7.4, containing 0.2% glucose and 0.1% albumin (fraction V). The cells were incubated in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose and 0.1% albumin (fraction V) and an amino acid mixture lacking methionine under an atmosphere of O₂/CO₂ (19:1). Proteins were labelled, extracted, separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and detected by fluorography as previously described (Janszen *et al.*, 1977). Molecular weights of proteins were estimated by electrophoresis on a 10–20% discontinuous gradient sodium dodecylsulphate/polyacrylamide gel, with phosphorylase *a* (mol.wt. 93000), catalase (mol.wt. 60000), ovalbumin (mol.wt. 43000), chymotrypsinogen (mol.wt. 25700) and cytochrome *c* (mol.wt. 11700) as markers. When testosterone production was measured, the isolated cells were incubated as described above for 2h in the presence or absence of added lutropin, and testosterone was extracted and determined as described by Verjans *et al.* (1973). For statistical analysis the Student's *t* test for correlated data was used. Differences were considered significant when *P* < 0.05 (one-tailed).

Results

Tumour Leydig cells

Incubation of tumour Leydig cells with lutropin (100ng/ml) for 4h followed by incubation for 1h in the presence of [³⁵S]methionine to label the proteins resulted in increased ³⁵S incorporation into the

Fig. 1. ³⁵S incorporation into proteins of tumour Leydig cells, incubated in the presence of various hormones

Tumour Leydig cells were incubated for 4h in the presence of the following compounds: (a) none, (b) lutropin (100ng/ml), (c) testosterone (100ng/10⁶ cells), (d) oestradiol-17β (100ng/10⁶ cells), (e) pregnenolone (100ng/10⁶ cells), (f) follitropin (100ng/ml), (g) none and (h) lutropin (100ng/ml); followed by incubation for 1h in the presence of [³⁵S]methionine. Incubations (g) and (h) were continued for 30min in the presence of cycloheximide (25 µg/ml). The proteins were separated by electrophoresis on 10–15% discontinuous polyacrylamide gel in the presence of sodium dodecyl sulphate. Only the 15% part of the gel is shown.

two proteins with apparent mol.wt. 27000 and 29000 (referred to as protein 27000 and protein 29000 respectively) (Fig. 1b). The increased ³⁵S incorporation into these proteins compared with the control (incubated Leydig cells without added lutropin) was statistically significant 2–3h after the addition of lutropin to the cells (Figs. 2a and 2b) and during a 4h incubation period maximum stimulation was not observed. The lowest dose of lutropin needed for a significant stimulation of testosterone production was 100ng/ml, and a maximum testosterone response was obtained with approx. 1000ng/ml (Table 1) (testosterone production increased from 1.0 ± 0.7 in control cells to 4.6 ± 1.1 ng/2h per 10⁶ cells in the presence of 1000ng of lutropin/ml; mean ± s.d., *n* = 4). The lowest dose of lutropin to give a significant response in ³⁵S incorporation in proteins 27000 and 29000 was also 100ng/ml and a maximum response was obtained with 1000ng/ml (Table 1).

To investigate whether the increase in ³⁵S incorporation into proteins 27000 and 29000 was due to increased concentration of steroids in the lutropin-stimulated Leydig cells, testosterone, oestradiol-17β and pregnenolone (100ng/10⁶ cells) were added separately to the cell preparations instead of lutropin and incubated for 4h followed by incubation with [³⁵S]methionine for 1h. No change in ³⁵S incorpora-

Fig. 2. Time course of lutropin stimulation of the synthesis of protein 27000 (a) and protein 29000 (b) in tumour Leydig cells. Tumour Leydig cells were incubated with (▲) or without (●) added lutropin (100 ng/ml) for different time periods followed by incubations of the cells with [³⁵S]methionine for 1 h. Specific protein synthesis is expressed as percentage of that after 4 h stimulation with lutropin (mean ± s.d., *n* = 3). Abbreviations: N.S., no significant difference between stimulated and control protein synthesis (*P* ≥ 0.05); S, significant difference between stimulated and control protein synthesis (*P* < 0.05).

Table 1. Effect of different doses of lutropin on the synthesis of testosterone and ³⁵S incorporation into protein 27000 and protein 29000 in tumour Leydig cells

Results are expressed as percentages of the value obtained in the presence of 100 ng of lutropin/ml. Means ± s.d. are given, with the numbers of experiments in parentheses. Abbreviation: N.S., not significant.

Dose of lutropin (ng/ml)	Testosterone production		Protein 27000		Protein 29000	
	(%)	Effect of LH	(%)	Effect of LH	(%)	Effect of LH
0	26 ± 14 (4)		42 ± 27 (4)		31 ± 21 (3)	
1	27 ± 10 (4)	N.S.	36 ± 11 (4)	N.S.	21 ± 24 (4)	N.S.
10	54 ± 26 (4)	N.S.	60 ± 18 (3)	N.S.	76 ± 50 (3)	N.S.
10 ²	100 (4)	<i>P</i> < 0.005	100 (4)	<i>P</i> < 0.005	100 (4)	<i>P</i> < 0.005
10 ³	126 ± 50 (4)	<i>P</i> < 0.01	127 ± 24 (4)	<i>P</i> < 0.01	163 ± 50 (4)	<i>P</i> < 0.005
10 ⁴	145 ± 41 (3)	<i>P</i> < 0.01	128 ± 21 (4)	<i>P</i> < 0.005	143 ± 29 (4)	<i>P</i> < 0.025

tion into proteins 27000 or 29000 was obtained (Figs. 1c–1e) compared with the controls. In other experiments elipten phosphate (300 μg/ml) was added to lutropin-stimulated tumour cells to prevent steroid synthesis (El Safoury & Bartke, 1974). The lutropin-stimulated ³⁵S labelling of the proteins was not inhibited under these conditions. To investigate whether the increase in ³⁵S incorporation was due to contamination of the lutropin preparation with follitropin, tumour cells were incubated with follitropin (100 ng/ml) instead of lutropin; no increase in ³⁵S incorporation into proteins 27000 and 29000 was observed (Fig. 1f).

Addition of actinomycin D (46 μM) to the tumour Leydig cells at the start of the incubation prevented the lutropin stimulation of ³⁵S incorporation into both proteins. Cooke *et al.* (1975a) showed that addition of this amount of actinomycin D to Leydig cells inhibited RNA synthesis by 89–93%. In other experiments cycloheximide (25 μg/ml) was added to the cells after labelling of the proteins with [³⁵S]methionine, and the incubation was continued for 30 min. No effect of this procedure was observed on the amount of ³⁵S incorporated into these two proteins (Figs. 1g and 1h), indicating that their half lives are longer than 30 min.

Fig. 3. ^{35}S incorporation into proteins of Leydig cells from immature rats, incubated in the presence or absence of added lutropin

Leydig cells from immature rats were incubated with (b) or without (a) lutropin (100 ng/ml) for 4 h followed by incubation with [^{35}S]methionine for 1 h: the proteins were separated on 10–15% discontinuous polyacrylamide gels in the presence of sodium dodecyl sulphate. Only the 15% part of the gel is shown.

Leydig cells from immature rats

Incubation of Leydig cells from immature rats with lutropin (100 ng/ml) for 4 h followed by incubation of the cells with [^{35}S]methionine for 1 h resulted in an increase in ^{35}S incorporation in proteins with apparent mol.wts. 11 000, 21 000, 27 000 and 29 000 (referred to as protein 11 000, protein 21 000, protein 27 000 and protein 29 000) (Fig. 3). The first increases in ^{35}S incorporation into these proteins could be observed 1–2 h after the addition of lutropin to the cells (Figs. 4a, 4b and 4c). At the start of the incubation period, the synthesis of protein 27 000 was high in both control and lutropin-treated cells. In the control cells the synthesis of this protein decreased, whereas in the lutropin-treated cells the synthesis of protein 27 000 remained elevated. The lowest amount of lutropin that resulted in a significant increase in ^{35}S incorporation was 1 ng/ml for protein 21 000 and 10 ng/ml for protein 29 000 (Table 2). With 10 ng of lutropin/ml an increase in ^{35}S incorporation into protein 11 000 was always observed (Fig. 4). Decreased ^{35}S incorporation into a protein with apparent mol.wt. 13 000 was found with 100–10 000 ng of lutropin/ml (Fig. 5, Table 3). In contrast with this observation there was an increase in ^{35}S incorporation into protein 13 000 with a low dose of lutropin (Table 3). No change in specific protein synthesis was obtained when the Leydig cells were incubated in the presence of follitropin (100 ng/ml) or with testosterone (100 ng/

Fig. 4. Time course of lutropin stimulation of the synthesis of protein 21 000 (a), protein 27 000 (b) and protein 29 000 (c) in Leydig cells from immature rats

Leydig cells from immature rats were incubated in the absence (●) or presence (▲) of lutropin (100 ng/ml) for different time periods followed by incubation with [^{35}S]methionine for 1 h. Specific protein synthesis is expressed as percentage of that stimulated with lutropin for 4 h (mean $\pm$ S.D., $n = 3$; * mean $\pm$ range, $n = 2$). Abbreviations: N.S., no significant difference between stimulated and control specific protein synthesis ($P \geq 0.05$); S, significant difference between stimulated and control specific protein synthesis ($P < 0.05$).

Table 2. Effect of different doses of lutropin on the ³⁵S incorporation into protein 21 000, protein 27 000 and protein 29 000 in Leydig cells of immature rats

Results are expressed as percentage of ³⁵S incorporation into the proteins in the presence of 100 ng of lutropin/ml. Means ± s.d. are given, with the numbers of experiments in parentheses: for two experiments the means ± ranges are given. Abbreviation: N.S., not significant.

Dose of lutropin (ng/ml)	Protein 21 000		Effect of LH	Protein 27 000		Effect of LH
	(%)			(%)		
0	24 ± 19 (3)			40 ± 28 (2)	29 ± 4 (3)	
1	38 ± 22 (3)		N.S.	46 ± 21 (2)	49 ± 13 (3)	P < 0.025
10	88 ± 37 (3)		P < 0.05	75 ± 22 (2)	77 ± 29 (3)	P < 0.025
10 ²	100 (3)		P < 0.025	100 (2)	100 (3)	P < 0.005
10 ³	99 ± 13 (3)		P < 0.025	92 ± 2 (2)	97 ± 8 (3)	P < 0.005
10 ⁴	103 ± 3 (2)			102 ± 8 (2)	113 ± 20 (2)

Fig. 5. ³⁵S incorporation into proteins of Leydig cells from immature rats, incubated in the presence of different doses of lutropin

Leydig cells were incubated in the presence of different doses of lutropin for 4 h followed by incubation with [³⁵S]methionine for 1 h. Proteins were separated on a 10–20% continuous polyacrylamide gel containing sodium dodecyl sulphate.

10⁶ cells) instead of lutropin. Prevention of steroidogenesis by addition of elipten phosphate (300 µg/ml) to the lutropin-treated Leydig cells did not prevent the lutropin stimulation of ³⁵S incorporation

Table 3. Effect of different doses of lutropin on the ³⁵S incorporation into protein 13 000 in Leydig cells of immature rats

Results are expressed as percentage of ³⁵S incorporation into protein 13 000 in the presence of 100 ng of lutropin/ml (means ± s.d. for the numbers of experiments in parentheses). Abbreviation: N.S., not significant.

Dose of lutropin (ng/ml)	Incorporation	
	(%)	Effect of LH
0	122 ± 10 (3)	
1	143 ± 10 (3)	P < 0.05 ↑
10	145 ± 24 (3)	N.S.
10 ²	100	P < 0.025 ↓
10 ³	89 ± 23 (3)	P < 0.05 ↓
10 ⁴	61	

into the four proteins. Addition of actinomycin D (46 µM) to the Leydig cells from immature rats at the start of the incubation prevented the lutropin stimulation of ³⁵S incorporation into all four proteins. Incubation of the cells in the presence of cycloheximide (25 µg/ml) for 30 min after labelling of the proteins with [³⁵S]methionine did not result in a decrease in the amount of ³⁵S incorporated into any of the four proteins, indicating that their half-life is probably longer than 30 min.

Discussion

From the present results it may be concluded that lutropin stimulates the synthesis of proteins with apparent mol.wts. 27 000 and 29 000 in tumour Leydig cells, and of proteins with apparent mol.wts. 11 000, 21 000, 27 000 and 29 000 in Leydig cells from immature rats. The stimulatory effect of lutropin on the

synthesis of these proteins is specific for lutropin and is not due to increased steroid production or to contaminating amounts of follitropin in the lutropin preparation. In the tumour Leydig cells and in Leydig cells from immature rats (Van Beurden *et al.*, 1976) stimulation of specific protein synthesis was obtained with approximately the same amount of lutropin needed for the stimulation of steroidogenesis. From the results obtained in the experiments with cycloheximide it may be concluded that none of the specific proteins have a short half-life of the order of that proposed for the postulated regulator protein, which has been shown to play a role in the stimulation of adrenal steroidogenesis (Garren *et al.*, 1965).

In a previous study with Leydig cells from mature rats it was demonstrated that lutropin stimulates the synthesis of a protein with an apparent mol.wt. of 21000 (lutropin-induced protein) (Janszen *et al.*, 1976b, 1977). Table 4 summarizes data on the effect of lutropin on specific protein synthesis in Leydig cells from immature and mature rats and in rat tumour Leydig cells. The agreement in molecular weights of lutropin-stimulated proteins in Leydig cells from different sources may suggest that these proteins are identical, but conclusive evidence can only be obtained by further characterization of these proteins. In tumour Leydig cells the synthesis of a protein with an apparent mol.wt. of 21000 was observed. However, in contrast with the observations with Leydig cells from immature and mature rats, synthesis of this 21000-mol.wt. protein in tumour Leydig cells was not affected by the presence of lutropin. Only further characterization of this protein may reveal whether or not it is identical with the 21000-mol.wt. protein from normal Leydig cells.

None of the lutropin-sensitive proteins detected in this study have molecular weights identical with

those of proteins synthesized or secreted by other steroid-producing tissues. For example, the four proteins reported by Rubin *et al.* (1974) and Laychock & Rubin (1974), which are secreted by the cat adrenal in the presence of corticotropin, have apparent mol.wts. of 70000, 58000, 48000 and 12500 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Grower & Bransome (1970) reported that in the supernatant of rat adrenal, corticotropin increased the [3 H]leucine incorporation into one protein but at the same time the [3 H]leucine incorporation into another protein was decreased. The effects observed by these authors occurred within 30 min after addition of corticotropin and disappeared after approx. 60 min. However, no further characteristics of the two corticotropin-sensitive proteins have been reported. In the Leydig cells from immature rats with increasing doses of lutropin the synthesis of protein 13000 first increased and then decreased. This decrease may be caused by a post-translational modification, e.g. splitting off of part of the molecule, so that protein 11000 could be the ultimate product after modification of protein 13000. The difference in effects of lutropin on the stimulation of specific proteins in the Leydig cells obtained from different sources may reflect differences in the lutropin regulatory mechanisms in these Leydig cells. Several other differences between Leydig cells from immature and mature rats have been reported, e.g. in steroid metabolism (Van Beurden *et al.*, 1976; Van der Molen *et al.*, 1975; Sowel *et al.*, 1974; Wiebe, 1976) and in the effect of hypophysectomy on lutropin-stimulated steroid synthesis (Van Beurden *et al.*, 1976). Tumour Leydig cells were also different from mature Leydig cells; e.g. in the present study, testosterone only accounted for less than 10% of the total steroid production by the tumour Leydig cells (F. H. A. Janszen, B. A. Cooke, M. J. A. van Driel & H. J. van der Molen, unpublished work). Such differences between the different Leydig cells make it difficult to extrapolate results, also for the effect of lutropin on specific proteins, from one type of Leydig cell to another.

Table 4. *Effect of lutropin on the synthesis of specific proteins in Leydig cells from immature and mature rats and in tumour Leydig cells*

Symbols: +, stimulation of the synthesis; 0, no effect of lutropin on the synthesis.

	Protein 11 000	Protein 21 000	Protein 27 000	Protein 29 000
Leydig cells from immature rats	+	+	+	+
Leydig cells from mature rats	0	+	0	0
Tumour Leydig cells	0	0	+	+

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