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Hormonal Alterations of the Sensitivity of Amino Acid Transport to Growth Hormone in Muscle of Young Rats (39954)

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Previous reports from this laboratory (1) and by Albertsson-Wikland and Isaksson (2) showed that diaphragm muscle taken from young normal rats will respond to growth hormone (GH) added *in vitro* at concentrations within the physiological range for the rat (3, 4). Maximum responsiveness appears to occur about age 15–19 days, at which time GH approximately doubles the rate of amino acid transport and significantly increases the rate of protein synthesis. After this age, the responsiveness of the transport system diminishes rapidly; responsiveness reappears if the rats are hypophysectomized (1, 5). Hjalmarson and Ahrén (5) have shown that in diaphragm muscle from chronically hypophysectomized rats aged 6–8 weeks, GH has a transient (2 to 3-hr) "insulin-like" action of enhancing amino acid and sugar uptake, and that this is then replaced for 35–45 hr by a period of refractoriness to restimulation by GH. Since short-term (2-day) hypophysectomy does not lead to a fall in the basal rate of amino acid transport (1), the loss of endogenous GH following hypophysectomy seems to result in a decline within the muscle of a long-acting inhibitor of the hormone's insulin-like stimulation of amino acid transport (1, 6). Thus, we hypothesized (1) that shortly after birth the insulin-like response to GH develops, and then after age 15–19 days, the inhibitor progressively becomes dominant. The latter causes the transport system of muscle from normal rats older than 30 days to be relatively unresponsive to additional GH, particularly during the light period (4).

In the studies described below, the ability of GH, prolactin, and adrenal steroids to modify the *in vitro* responsiveness to GH was examined in diaphragms from young rats. In particular, we tested whether GH or prolactin has the ability to induce refractoriness during midlactation (age 11–15 days). Further, since at about age 18–19 days the rat adrenal cortex begins its diurnal secretion of corticosterone (7), which induces many metabolic changes in preparation for weaning (8), we tested whether removal of adrenal glands would delay the onset of the refractoriness and whether injection of cortisone would induce refractoriness prematurely.

Materials and methods. Normal Sprague-Dawley rats were bred and cared for as described previously (1). Unless otherwise noted, they were fasted for 20–26 hr; fasting increases both the magnitude and consistency of the responsiveness to GH of diaphragms from young normal rats (1, 2). Rats adrenalectomized at age 30 days were given 0.9 g/dl of NaCl to drink. Mothers of the rats that were adrenalectomized at age 13 days were given 0.9 g/dl of NaCl to drink. The pups were removed from their mothers 17–18 hr prior to killing and given access to a solution of 5 g/dl of sucrose in 0.9 g/dl of NaCl for the next 7–8 hr, and then just 0.9 g/dl of NaCl for the final 10 hr. Calculations [based on data in Ref. (9)] show that rat milk contains a substantial amount of sodium, approximately 54 m*M*. All experiments were begun between 0900 and 1000 hr, which was 3–4 hr after the onset of the 14-hr light period. To study effects of GH on amino acid uptake, intact, paired hemidiaphragms, or, occasionally, intact quartered diaphragms (10) were prepared and incubated for 1 hr with the nonmetabolizable analog [1-^{14}C]2-aminoisobutyric acid (AIB) (1 m*M*, 0.2 μCi/ml), as described previously (1). The incubation medium for one member of each pair also contained 5 or 25 μg/ml of ovine GH (NIH-GH-S10) or 25 μg/ml of ovine prolactin (NIH-P-S10). When the effects of GH on sugar transport were studied, the same 1-hr

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incubation period was used, but the non-metabolizable analog, 3-*O*-methyl-D-[^{3}H]-glucose(3-OMG) (1 m*M*, 0.5 μCi/ml), was present only for the final 30 min of incubation (11). The remainder of the protocol for each experiment will be described, together with the results, in the next section. The data are expressed as the distribution ratios (intracellular concentration/concentration in the medium), after correcting for isotope present in the extracellular space of the muscle (1).

Statistical analysis of data from paired hemidiaphragms utilized Student's *t* test for the significance of the paired differences and two-factor analysis of variance (ANOVA) with repeated measures (12) to assess both the overall treatment effects and the interaction between two hormone treatments. If the latter analysis showed a significant interaction ($P < 0.05$), then Tukey's honestly significant difference test was used to test for individual differences (12). When quartered diaphragms were used, the analysis was by one-factor ANOVA with repeated measures, followed by the Newmann-Keuls test (12).

Results. As observed previously, the response of the amino acid transport system to GH declined after age 15 days, and no significant responses were obtained in rats 30–40 days old. (Table I). A similar age-related decline in sensitivity to GH was seen in the monosaccharide transport system of rat muscle. The 3-OMG distribution ratios at age 23 days were: control = 0.14, GH = 0.28, mean difference ± SE = +0.14 ± 0.03, $N = 9$, $P < 0.005$; and at age 31 days were: control = 0.10, GH = 0.08, mean difference ± SE = −0.02 ± 0.02, n = 8).

Adrenal steroids. Adrenalectomy at an age (30 days) when the rat is largely refractory to the insulin-like actions of GH did not permit GH responsiveness to return by age 35 days (Experiment 1, Table I). This differs from the effect of hypophysectomy (1). A second hypothesis tested was that at about 18–19 days after birth, a brief exposure to adrenal steroids induces a permanent change in the muscle, so that after this age the long-lived inhibitory substance is synthesized in response to GH, and thus the refractoriness response predominates. This hypothesis was tested in two ways. First, rats were injected with cortisone acetate (1 mg/100 g body weight, sc) at ages 11 and 12 days, and tested at age 15 days

TABLE I. EFFECT OF ADRENAL HORMONES ON GH RESPONSIVENESS OF HEMIDIAPHRAGMS FROM NORMAL RATS.[a]

In vivo treatment	Age at testing (days)	AIB distribution ratio (mean ± SE)			
		In vitro treatment		Difference	Change (%)
		Control	GH		
Experiment 1 (fed *ad libitum*)					
None	30	0.57 ± 0.02	0.55 ± 0.03	−0.02 ± 0.02	↓4
Adrenalectomy at 30 days	35	0.80 ± 0.05	0.83 ± 0.05	+0.03 ± 0.03	↑3
None	40	0.40 ± 0.01	0.41 ± 0.02	+0.01 ± 0.02	↑3
Experiment 2					
Saline; fasted 20 hr	15	0.76 ± 0.02	1.36 ± 0.09	+0.60 ± 0.08*	↑79
Cortisone (1 mg/100 g body wt) at ages 11 and 12 days; fasted 20 hr	15	0.81 ± 0.10	1.88 ± 0.08	+1.06 ± 0.09*, **	↑130
Experiment 3					
Sham adrenalectomy at 13 days; fasted 10 hr	23	0.54 ± 0.01	0.78 ± 0.03	+0.24 ± 0.03*	↑44
Adrenalectomy at 13 days; fasted 10 hr	23	0.66 ± 0.05***	0.94 ± 0.06***	+0.28 ± 0.06*	↑42

[a] Paired hemidiaphragms were incubated for 1 hr in Krebs-Ringer bicarbonate buffer containing 10 m*M* glucose and 1 m*M* [^{14}C]AIB. One member of each pair was also exposed to ovine GH (25 μg/ml). $N = 9$–11 pairs.

* Effect of GH *in vitro* was significant at the $P = 0.005$ level or less.

** GH effect was significantly greater ($P < 0.002$ by ANOVA) in diaphragms from cortisol-treated rats than in muscle from saline-treated rats.

*** Adrenalectomy significantly increased the AIB distribution ratio ($F = 7.328$, $df = 1, 21$, $P = 0.013$).

to see if the ability of GH to stimulate AIB transport acutely had been lost. Approximately this dose of glucocorticoid has been shown to induce premature formation of certain hepatic enzymes during the suckling period (13, 14). However, as shown in Experiment 2 of Table I, the administration of cortisone in this manner did not reduce either the basal rate of AIB transport or the increase in AIB uptake produced by GH. If anything, the response to GH was enhanced ($P < 0.002$ by ANOVA). In a second experimental approach, it was found that adrenalectomy at age 13 days did not postpone the reduction in responsiveness to GH usually observed by age 23 days (Experiment 3, Table I). In this experiment, muscle from the adrenalectomized animals had a slightly higher rate of AIB uptake than did muscle from sham-operated animals ($P < 0.02$ by ANOVA). In Experiment 1, adrenalectomy also seemed to increase the rate of amino acid transport, since the AIB distribution ratios for both control and GH-treated diaphragms from 35-day-old adrenalectomized rats were much higher than those of intact rats aged 30 or 40 days. However, a firm conclusion regarding the effects of adrenalectomy on 35-day-old rats was not possible since no age-matched, sham-operated animals were tested in that experiment.

Prolactin. Prolactin did not affect GH actions during this period. We previously reported (1) that ovine prolactin did not stimulate either AIB transport or protein synthesis at age 4 or 15 days. In additional *in vitro* experiments, prolactin, at a concentration equal to or five times greater than that of GH neither synergized with nor antagonized the insulin-like action of GH on AIB uptake in diaphragms from young normal rats (unpublished observations). Further, as shown in Table II, administration of 50 μg of ovine prolactin every 12 hr for 2 days did not induce refractoriness to GH *in vitro* in muscle from 15 to 16-day-old normal rats.

GH-induced refractoriness. Finally, a series of experiments was conducted to see if refractoriness to GH is demonstrable in muscle from young normal rats, and, if so, whether the time course for the onset and decline in refractoriness after a single exposure to GH differs from that observed in muscle from older, chronically hypophysectomized rats (5). For this, the insulin-like response to GH (AIB transport) was measured *in vitro* in diaphragms from fasted 15-day-old rats, using the standard 1-hr test

TABLE II. EFFECTS OF GH AND PROLACTIN (PRL) *in vivo* ON THE SENSITIVITY OF DIAPHRAGM MUSCLE OF YOUNG FASTED RATS TO GH *in vitro*.[a]

In vivo treatment	No. of rats	AIB distribution ratio (mean ± SE)			
		In vitro treatment			
		Control	GH	Difference	*P* vs control
Experiment 1, Rats tested 4 hr after injection					
Saline, 4 hr	9	0.73 ± 0.05	1.07 ± 0.07	+0.33 ± 0.09	<0.01
GH, 10 μg, 4 hr	9	0.81 ± 0.04	0.85 ± 0.04	+0.04 ± 0.06	NS
Experiment 2, Rats tested 12 hr after last injection					
Saline, 2 days	12	0.81 ± 0.05	1.15 ± 0.07	+0.34 ± 0.08	<0.005
GH, 50 μg q 12 hr for 2 days	9	0.75 ± 0.08	0.82 ± 0.07	+0.07 ± 0.08	NS
PRL, 50 μg q 12 hr for 2 days	10	0.74 ± 0.06	1.25 ± 0.10	+0.51 ± 0.11	<0.005
Experiment 3, Rats tested 48 hr after last injection					
Saline, 2 days	7	0.84 ± 0.12	1.36 ± 0.17	+0.51 ± 0.12	<0.005
GH, 50 μg q 12 hr for 2 days	8	0.81 ± 0.07	1.23 ± 0.09	+0.42 ± 0.10*	<0.005

[a] All rats were 15–16 days old when sacrificed and had been fasted for 22–26 hr prior to killing. *In vitro* incubations were performed as in Table I.

* Not significantly less (by two-factor ANOVA) than the GH response obtained in diaphragms from saline-treated rats.

conditions, at intervals following the ip injection of ovine GH or saline *in vivo* (Table II). As expected, saline-treated rats responded to GH *in vitro*. But when 10 μg of GH was given 4 hr prior to killing, it blocked the stimulation of AIB transport by GH *in vitro*. The basal rate of AIB uptake was not elevated 4 hr after the injection of GH, suggesting that *in vivo* the transient insulin-like effect had declined by 4 hr and that the muscle was in its refractory phase.

To see how long the refractory period lasts *in vivo*, GH was given to rats in four doses of 50 μg each at 12-hr intervals over a 2-day period. When the final dose was given 12 hr prior to killing, the muscle failed to respond to additional GH *in vitro* (Experiment 2, Table II). However, the muscle did respond to GH *in vitro* when the last injection was given 48 hr previously.

Discussion. The results reported here show that muscle from young normal rats, aged 15–16 days, has a biphasic response to GH. Acutely, GH enhances the uptake of certain amino acids and sugars. However, 4 hr after *in vivo* administration of GH, the rate of AIB uptake is no longer increased and the amino acid transport system of the muscle will not respond to additional GH *in vitro*. This period of refractoriness persists for at least 12 hr, but less than 48 hr, after a moderately high dose of GH (50 μg/rat, approximately 2 mg/kg). Thus, the time courses for the responses of young normal rats to GH *in vivo* appear to be similar to those obtained in experiments of a similar design, performed on older, hypophysectomized rats [Ref. (5) and unpublished observations by D. F. Nutting and L. J. Coats].

The latter conclusion would seem to differ from that of Albertsson-Wikland and Isaksson (2). However, these investigators used an entirely *in vitro* design to study the biphasic response in diaphragms from 18-day-old rats. They reported that all phases of GH action on transport processes in the diaphragm were more rapid in young normal rats than in chronically hypophysectomized rats aged 6–8 weeks: The insulin-like actions began earlier (<10 min versus 20–30 min) and disappeared sooner (<90 min versus 2–3 hr), and the ensuing refractoriness was much shorter (only about 1 hr versus 35–45 hr). There are several possible explanations for the difference between our results and those of Albertsson-Wikland and Isaksson concerning the length of the refractory period. It may be that the effects of the rather large doses of GH used by us persisted for a very long time. In this regard, Hjalmarson and Ahrén (5) have shown that the magnitude of the response of muscle from hypophysectomized rats to GH *in vitro* is inversely proportional to the size of an *in vivo* dose of GH given 3.5 hr previously. On the other hand, the explanation may lie in the difference in experimental design; the GH used to produce refractoriness was given *in vivo* in the experiments reported here, but *in vitro* at the start of a long preincubation period by Albertsson-Wikland and Isaksson. Perhaps the more rapid rate of turnover of muscle protein *in vitro* than *in vivo* (15) might partially account for the shorter period of refractoriness seen with the *in vitro* design, since refractoriness may be mediated by an inhibitory protein (6).

Prolactin failed to stimulate AIB uptake or protein synthesis in diaphragm muscle from young normal rats (1). Similarly, prolactin preparations that had GH contamination removed did not stimulate AIB transport in diaphragms from hypophysectomized rats (16). As reported here, prolactin, given either *in vivo* or *in vitro*, also had no influence on the stimulatory actions of GH in muscle from normal suckling rats. Thus, although (a) prolactin is very similar structurally to GH (17, 18), (b) prolactin and GH share some biological activities, particularly in the more primitive species (19), and (c) immunoreactive prolactin secreted by the maternal pituitary is transferred to the plasma of suckling pups via the milk (20), prolactin does not seem to influence either muscle growth or the actions of GH in muscle of suckling rats.

After the midlactation period, the GH-responsiveness of muscle taken from normal rats falls. This decline does not appear to be due to the onset of the adult pattern of glucocorticoid secretion by the adrenals. Adrenalectomy at age 13 days did not delay

the fall in sensitivity. Premature administration of a high dose of cortisol also failed to hasten the decline in sensitivity; on the contrary, cortisol enhanced the response to GH at age 15 days. This unexpected response to cortisol may be related to the catabolic effect of the hormone on muscle proteins (21), perhaps by enhancing the turnover of the small amount of the refractoriness-producing protein (6) that may be present in muscle of the 15-day-old rats, due to the actions of endogenous GH. We have suggestive evidence for a similar effect of thyroxine in the dwarf mouse (22).

Adrenalectomy significantly increased the rate of AIB transport, in both the presence and absence of GH *in vitro*. This observation is in accord with previous reports that both adrenal glucocorticoids and mineralocorticoids, given either *in vivo* or *in vitro*, decrease the uptake of AIB by muscle from normal and adrenalectomized rats (23–26). However, in contrast to these reports and to the observations made here, others have found that adrenalectomy either had no effect on (25) or decreased (26, 27) the accumulation of AIB by diaphragms. Except for the ages of the rats tested, there is no readily apparent explanation for these differences. In diaphragms taken from intact and adrenalectomized rats, more than 2 hr are required after adrenal steroids are administered *in vivo* or *in vitro* for them to inhibit AIB transport (23, 24). The duration of this inhibitory effect has not been investigated, although it would appear from the data reported here (Experiment 2, Table I) that in 12- to 15-day-old rats, the inhibitory effect of cortisone on the basal rate of AIB uptake lasts less than 48 hr.

The results presented here and previously (1, 2) indicate that in diaphragm muscle taken from normal rats during the first few weeks after birth GH can stimulate the transport of amino acids and monosaccharides. Therefore, although GH can also cause refractoriness at this age, this refractoriness must not be as dominant as it is after weaning. Perhaps during the suckling period the pulsatile secretory episodes of endogenous GH are insufficient (in magnitude, frequency, and/or duration) to produce a prolonged state of refractoriness in muscle. Whether the levels of GH at this age are sufficient even to stimulate transport processes *in vivo* remains to be established. If they are sufficient, then in the suckling rat GH may assist rapid growth by stimulating the peripheral uptake and utilization of nutrients at a time when nourishment is both plentiful and continuously available. Later on, when a feeding–fasting regimen is followed, refractoriness may be the dominant influence. Additional experiments are needed to characterize the *in vivo* time courses for both the secretion of GH and its several metabolic actions in muscle of rats at various stages of development.

Summary. The ability of growth hormone *in vitro* to stimulate amino acid and sugar uptake by diaphragm muscle from normal rats declines from age 15 days to age 30 days. In 15-day-old intact rats, as in older, hypophysectomized rats, GH has a biphasic effect on amino acid transport: a transient stimulatory phase, followed by a period of refractoriness to stimulation by additional GH. It appears that after age 15 days the refractoriness phase becomes progressively dominant. GH actions are not affected by prolactin or by adrenalectomy. However, during midlactation, exogenous glucocorticoid may reduce refractoriness, thus enhancing the response to additional GH.

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1. Nutting, D. F., Endocrinology **98,** 1273 (1976).
2. Albertsson-Wikland, K., and Isaksson, O., Metabolism **25,** 747 (1976).
3. Tannenbaum, G. S., and Martin, J. B., Endocrinology **98,** 562 (1976).
4. Nutting, D. F., Isaksson, O., Kostyo, J. L., and Reagan, C. R., Fed. Proc. **36,** 323 (1977).
5. Hjalmarson, Å., and Ahrén, K., Acta Endocrinol. **56,** 347 (1967).
6. Hjalmarson, Å., Acta Endocrinol. **57,** Suppl. 126, 19 (1968).
7. Ramaley, J. A., Steroids **21,** 433 (1973).
8. Greengard, O., *in* "Biochemical Actions of Hormones" (G. Litwack, ed.), Vol. I, p. 53. Academic Press, New York (1970).

9. Kon, S. K., and Cowie, A. T. (eds.), "Milk: The Mammary Gland and Its Secretions," Vol. II. Academic Press, New York (1961).
10. Goldberg, A. L., Martel, S. B., and Kushmerick, M. J., *in* "Methods in Enzymology" (J. G. Hardman and B. W. O'Malley, eds.), Vol. 39, Part D, p. 82. Academic Press, New York (1975).
11. Rillema, J. A., Kostyo, J. L., and Gimpel, L. P., Biochim. Biophys. Acta **297,** 527 (1973).
12. Winer, J., "Statistical Principles in Experimental Design," 2nd ed. McGraw-Hill, New York (1971).
13. Herzfeld, A., and Greengard, O., J. Biol. Chem. **244,** 4894 (1969).
14. Harding, H. R., Rosen, F., and Nichol, C. A., Amer. J. Physiol. **201,** 271 (1961).
15. Fulks, R. M., Li, J. B., and Goldberg, A. L., J. Biol. Chem. **250,** 290 (1975).
16. Kostyo, J. L., and Schmidt, J. E., Endocrinology **70,** 381 (1962).
17. Li, C. H., *in* "Handbook of Physiology" (E. Knobil and W. H. Sawyer, eds.), Vol. 4, Part 2, p. 103. American Physiological Society, Washington, D. C. (1974).
18. Fellows, R. E., Jr., Rogol, A. D., and Mudge, A., *in* "Growth and Growth Hormone" (A. Pecile and E. E. Müller, eds.), p. 42. Excerpta Medica Foundation, Int. Congr. Ser. No. 244, Amsterdam (1972).
19. Nicoll, C. S., *in* "Handbook of Physiology" (E. Knobil and W. H. Sawyer, eds.), Vol. 4, Part 2, p. 253. American Physiological Society, Washington, D. C. (1974).
20. Whitworth, N. S., and Grosvenor, C. E., Fed. Proc. **35,** 220 (1976).
21. Goldberg, A. L., J. Biol. Chem. **244,** 3223 (1969).
22. Nutting, D. F., Endocrinology **99,** 1423 (1976).
23. Kostyo, J. L., Endocrinology **76,** 604 (1965).
24. Kostyo, J. L., and Redmond, A. F., Endocrinology **79,** 531 (1966).
25. Wool, I. G., Amer. J. Physiol. **199,** 715 (1960).
26. Blecher, M., Amer. J. Physiol. **205,** 446 (1961).
27. Eichhorn, J., Feinstein, M., Halkerston, I. D. K., and Hechter, O., Proc. Soc. Exp. Biol. Med. **106,** 781 (1961).

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