Insensitivity of Adipocytes of Hypophysectomized Rats to Growth Hormone and Insulin[1] (35537)

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Kono (1) has recently found that treatment of adipose tissue from normal rats with trypsin, followed by digestion with various preparations of collagenase produced free adipocytes that were insensitive to insulin. In cells treated in this manner, insulin not only failed to increase glucose utilization, but it also failed to decrease the effects of epinephrine on lipolysis. Sensitivity to epinephrine, as judged by glycerol production, was virtually unaffected by this treatment with trypsin. The fact that the responsiveness to insulin was abolished while the response to epinephrine persisted suggested to Kono that the insulin "effector system" was selectively destroyed.

Since growth hormone, like insulin, increases glucose uptake in adipose tissue of hypophysectomized rats (2), and antagonizes the lipolytic effects of epinephrine (3), it is possible that both growth hormone and insulin act on the same receptor in the fat cell membrane. These studies were undertaken in the hope that trypsin treatment might be useful in determining whether insulin and growth hormone act via the same effector system in adipose tissue. Since animals with an intact pituitary are insensitive to the insulin-like effects of growth hormone (4), it was necessary to use epididymal adipose tissue from hypophysectomized animals. This paper describes the problems encountered in attempting to prepare fat cell suspensions sensitive to growth hormone and insulin.

Materials and Methods. Normal and hypophysectomized rats, weighing 100–200 g were obtained from the Charles River Breeding Laboratories. Hypophysectomized animals were fed a high carbohydrate, fat-free diet (5) *ad libitum* from the day of surgery until they were used 2–4 weeks later. Normal rats were fed the same diet for at least 1 week before use. In some experiments hypophysectomized rats were pretreated with growth hormone, L-triiodothyronine (Cytomel sodium, Smith, Kline and French Laboratories), or cortisone acetate (Merck, Sharp and Dohme). The injection schedules are given in the text. The animals were killed by cervical dislocation, and the epididymal fat pads were quickly excised and placed in physiological saline. When tissue fragments were studied, each pad was cut into 4–8 segments weighing 20–60 mg each. Only the thin distal portions were used. These were blotted and randomly divided among the control and experimental incubation vials. Three to 6 rats were used in each experiment, with one segment from each rat used for each experimental condition. In some experiments, the tissue segments were preincubated for 1 hr in Krebs–Ringer bicarbonate buffer (KRB) containing 20 mg/ml of bovine serum albumin (Armour Fraction V) and, where indicated, 1 mg/ml of D-glucose.

Suspensions of isolated fat cells were prepared by the method of Rodbell (6), with few modifications. Approximately 1 g of minced epididymal fat pooled from 4–8 rats was digested for 1 hr in a plastic vial containing 40 mg/ml of bovine serum albumin and 1800 U of crude collagenase EC 3.4.4.19 (Worthington Code C1S; lots 8BA, 9FC, and

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9LG) in 3.0 ml of KRB (47C, pH 7.4). Glucose was excluded from the digestion medium unless otherwise indicated. Following the digestion period, the cells were washed 3 times with 2% albumin in KRB. Pieces of undissociated tissue were removed with forceps and discarded. Aliquots of the suspension (0.2 ml, corresponding to 10–15 mg of triglyceride) were delivered to plastic incubation vials containing control and experimental media. The vials were capped with serum stoppers from which plastic cups were suspended for later collection of $^{14}CO_2$. Incubations were carried out, with shaking at 37°, for 1, 2, or 3 hr. The gas phase was 95% O_2:5% CO_2. In some experiments 0.2 mg/ml of soybean trypsin inhibitor (chromatographically purified, Worthington Code SI), 0.3 U/ml of phospholipase C inhibitor (*Clostridium perfringens* antitoxin contained in gas gangrene polyvalent antitoxin, Lederle), or D-glucose were added to the digestion medium. Each milliliter of the antitoxin contained 500 U of antitoxin to *Cl. perfringens* and to *Cl. septicum*, 150 U to *Cl. histolyticum*, and 75 U to *Cl. novyi* and to *Cl. oedematoides*. In other experiments, crude collagenase was replaced with 6 mg of chromatographically purified collagenase free from peptidase and trypsin-like activity (Worthington Code CLSPA) plus 0.1 mg/ml of trypsin (crystallized, Worthington Code TRL) or 1, 10 or 100 mg/ml of phospholipase C (partially purified, Worthington Code PHLC). When trypsin was included in the digestion medium, 2 mg/ml of soybean trypsin inhibitor was added to the buffer used to wash cells. The details of these experiments are given in the text.

Incubations were carried out in 1 or 2 ml of KRB containing 1 mg/ml of D-glucose, (0.2 μCi/ml of glucose-U-^{14}C; Schwartz Bioresearch Inc., and New England Nuclear), 20 mg/ml of bovine serum albumin, and 0.1 mU/ml of insulin (regular, Lilly), or 1 μg/ml of bovine growth hormone (NIH GH B10)[4] where indicated.

Incubations were terminated by injecting 0.5 ml of 1 *N* H_2SO_4 through the serum cap into the medium. $^{14}CO_2$ was collected in either 0.2 ml of Soluene (Packard Instrument Company) or 0.5 ml of 2-phenethylamine (Eastman Organic Chemicals) injected into the cup suspended from the serum stopper. The vials were shaken for an additional hour, after which the cups containing $^{14}CO_2$ was dissolved in Soluene or 2-phenethylamine were transferred to counting vials containing 15 ml of scintillation fluid (4 g of BBOT; 2,5-bis(5-*tert*-butylbenzoxazolyl) thiopene; (Packard Instrument Company)/liter of toluene diluted 5:3 (v/v) with absolute ethanol.)

$^{14}CO_2$ production was used as an index of glucose utilization and was expressed as counts per minute per milligram of triglyceride per hour for cells, and counts per minute per milligram of wet weight per hour for tissues. Triglyceride was collected in the heptane phase after extraction by Dole's procedure (7), and was quantified gravimetrically in tared scintillation vials. Incorporation of ^{14}C into total lipid was determined after redissolving the lipid residue in 15 ml of scintillation fluid.

Results. In agreement with the earlier findings by Rodbell (6), insulin (0.1 mU/ml) produced a 2–3-fold increase in $^{14}CO_2$ production by adipocytes from normal rats (Table I). Higher concentrations of insulin failed to produce greater increases in $^{14}CO_2$ product. Data on incorporation of ^{14}C into lipid (not shown) paralleled those for CO_2 production. Since fat cells prepared with different lots of crude collagenase are known to exhibit varying sensitivity to hormones (1), three different lots of collagenase (8BA, 9FC, and 9LG) were tested. Sensitivity to insulin did not differ appreciably in cells prepared with any of these lots of collagenase. Growth hormone had little or no effect.

When epididymal fat from hypophysectomized rats was digested with the same lots of crude collagenase in a manner identical to that used for normal adipose tissue the resulting fat cells were insensitive to both growth hormone and insulin. In 13 experiments (Table I, Expts. 4–16) using a variety of preparative techniques, isolated fat cells from hypophysectomized rats uniform-

[4] The growth hormone was a gift of the Endocrine Study Section of the National Institutes of Health.

TABLE I. The Response of Isolated Fat Cells to Growth Hormone and Insulin.

Exp. no.	Collagenase used[a]	$^{14}CO_2$ production (cpm/mg of triglyceride/hr) Control	Growth hormone (1 μg/ml)	Insulin (0.1 mU/ml)
Normal rats				
1	9FC	425 ± 45 (3)[b]	—	1434 ± 46
2	9LG	335 ± 75 (4)	574 ± 67	1099 ± 127
3	8BA	395 ± 23 (4)	420 ± 50	1008 ± 112
Hypophysectomized rats				
4	9FC	265 ± 33 (3)	239 ± 34	282 ± 17
5[c]		66 ± 7 (3)	63 ± 3	98 ± 9
6	9LG	221 ± 3 (6)	239 ± 7	238 ± 16
7[d]		233 ± 6 (3)	241 ± 97	245 ± 28
8	8BA	196 ± 38 (3)	146 ± 4	192 ± 8
9[e]		163 ± 22 (3)	172 ± 37	207 ± 32
10		204 ± 29 (3)	252 ± 19	225 ± 47
11		214 ± 24 (3)	200 ± 18	196 ± 41
12		555 ± 37 (3)	64 ± 6	728 ± 111
13[e]		64 ± 6 (3)	64 ± 6	43 ± 7
14[e]		101 ± 8 (3)	112 ± 12	122 ± 4
15[f]		602 ± 48 (3)	452 ± 25	590 ± 84
16[f]	9FC	73 ± 3 (3)	83 ± 10	101 ± 7

[a] Digestions carried out for 1 hr in the absence of glucose except where otherwise indicated.

[b] Mean ± SEM; numbers in parentheses indicate numbers of replicate aliquots of cells studied in each experiment.

[c] Digestion carried out in the presence of: 3 mg of glucose/ml; [d] 1 mg of glucose/ml.

[e] Digestion carried out for 30 min instead of 60 min.

[f] Tissues preincubated for 1 hr prior to digestion in KRB containing 2% bovine serum albumin, 3 mg/ml of glucose, and 0.1 mU/ml of insulin. The tissues were then transferred to 3.0 ml of fresh medium containing 1800 units of collagenase.

ly failed to respond to either growth hormone or insulin. Incorporation of ^{14}C into lipid (data not shown) paralleled $^{14}CO_2$ production. Including glucose in the digestion medium (Table I, Expts. 5, 7, and 9), or shortening the digestion time (Expts. 13 and 14) did not improve the response to hormones. Preincubation of the tissues with 0.1 mU/ml of insulin (Expts. 15 and 16) for 1 hr before digestion with collagenase also failed to improve the response to hormones, although Czech and Fain (8) found that preincubation of normal parametrial white fat cells for 1 min with 0.25 U/ml of insulin protects the insulin effector system from destruction by high doses (0.25 mg/ml) of trypsin.

Segments of tissue from the same hypophysectomized animals were also studied. The data in Table II (Expts. 2–6) indicate that growth hormone (1 μg/ml) and insulin (0.1 mU/ml) both caused significant increases in the production of $^{14}CO_2$. Tissues from normal rats responded to insulin, but not to growth hormone (Table II, Expt. 1). In one experiment (Table II, Expt. 6), tissue segments were incubated in the presence of hormones and free cells prepared from hypophysectomized rats. The hormones in the medium were still able to increase glucose uptake 2–3-fold by the tissue segments, indicating that substances leaking from cell suspensions or residual peptidase activity from the collagenase treatment (9) did not destroy the hormones or inhibit their action.

Crude collagenase preparations are contaminated not only with Clostridiopeptidase B (EC 3.4.4.20) (1), an enzyme with a substrate affinity similar but not identical to

trypsin (10), but also with enough phospholipase C [*Clostridium perfringens* alpha toxin (11)] to hydrolyze a significant amount of cellular phosphatidyl choline (12). Trypsin (1) and phospholipase C (13) in high concentrations destroy the ability of normal fat cells to respond to insulin. In an effort to protect them from the contaminating enzymes, fat cells were prepared with crude collagenase (lot 8BA) in the presence of 0.2 mg/ml of soybean trypsin inhibitor, 0.3 U/ml of phospholipase C inhibitor [*Cl. perfringens* antitoxin (14)], or a combination of 0.3 mg/ml of trypsin inhibitor and 0.3 U/ml of phospholipase C inhibitor. Including these inhibitors in the digestion medium in these concentrations failed to improve the response of cells from hypophysectomized rats to growth hormone and insulin, as judged by $^{14}CO_2$ production.

Since these inhibitors may not be effective against the contaminating enzymes, attempts were made to prepare a suspension of cells with pure collagenase, which contains little or no peptidase or trypsin-like activity (15), and little phospholipase C activity (12). However, as found by Kono (1), tissues incubated with pure collagenase alone did not dissociate. Inclusion of trypsin (0.1 mg/ml) or phospholipase C (1, 10, or 100 μg/ml) in the digestion medium along with pure collagenase caused the tissues to dissociate, but the cells were unresponsive to growth hormone or insulin.

Attempts were made to prepare hormone-sensitive cells from hypophysectomized animals by shortening the exposure time of the tissues to crude collagenase and trypsin. As already noted, cells from tissues digested for 0.5 hr with crude collagenase 8BA did not respond to hormones (Table I, Expts. 13 and 14). In one experiment (Table III), tissue from normal and hypophysectomized animals was incubated for 15 min in KRB containing 4% albumin and 0.1 mg/ml of trypsin. The tissues were then washed with buffer containing trypsin inhibitor (2 mg/ml) and transferred to buffer containing pure collagenase (2 mg/ml) for an additional hour. In normal cells prepared in this manner, insulin caused a 3-fold increase in $^{14}CO_2$ production and a 2-fold increase in the incorporation of $^{14}CO_2$ into lipids. Cells from hypophysectomized animals, however, did not respond to growth hormone or insulin.

TABLE II. The Response of Segments of Epididymal Fat From Normal and Hypophysectomized Rats to Growth Hormone and Insulin.

Expt. no.	$^{14}CO_2$ production (cpm/mg of wet wt/hr) Controls	Growth hormone (1 μg/ml)	Insulin (0.1 mU/ml)
Normal rats			
1	44 ± 4 (6)[a]	58 ± 8	84 ± 10
Hypophysectomized rats			
2	41 ± 8 (3)	91 ± 18	159 ± 21
3[b]	48 ± 4 (4)	73 ± 6	67 ± 4
4[c]	11 ± 1 (6)	36 ± 4	28 ± 4
5	23 ± 6 (6)	54 ± 7	57 ± 9
6[d]	11 ± 2 (5)	30 ± 3	37 ± 7

[a] Mean ± SEM; numbers in parentheses indicate numbers of animals studied in each experiment.

[b] Tissues preincubated for 1 hr in KRB containing: 2% bovine serum albumin and 1 mg/ml of glucose; [c] 2% bovine serum albumin, but no glucose.

[d] Tissue segments incubated along with isolated cells (20–30 mg of triglyceride). Values given are calculated $^{14}CO_2$ due only to tissue segments, *i.e.*, $^{14}CO_2$ produced by cells subtracted.

The failure of free adipocytes from hypophysectomized rats to respond to hormones presumably stems from their hormonal deficiency. In an effort to replace hormones, but leave the animals sensitive to growth hormone, hypophysectomized rats were pretreated with cortisone (2 mg/rat/day, ×4) or triiodothyronine (5 μg/rat/day, ×4). Cortisone severely reduced (80% inhibition of the rate of glucose utilization seen in uninjected controls) and T3 markedly enhanced (6-fold increase over uninjected controls) glucose utilization, but neither restored the responsiveness to growth hormone or insulin. Even giving 200 μg of growth hormone in two doses 24 and 48 hr before use, alone or in a combination with cortisone and T3 (cortisone, 2 mg/rat/day; triiodothyronine, 5 μg/rat/day; growth hormone, 100 μg/rat/

TABLE III. The Response of Fat Cells Isolated by Incubation with Trypsin and Collagenase to Growth Hormone and Insulin.

	$^{14}CO_2$ production (cpm/mg of triglyceride/hr)		
	Control	Growth hormone (1 μg/ml)	Insulin (0.1 mU/ml)
Normal rats	266 ± 71[a]	298 ± 48	846 ± 115
Hypophysectomized rats	128 ± 21	131 ± 23	101 ± 31

[a] Mean ± SEM; each member represents the mean of 3 replicate aliquots of cells. Minced adipose tissue was incubated for 15 min in KRB containing 4% bovine serum albumin and 0.1 μg/ml of trypsin. The chunks of tissue were then washed with KRB (2% albumin) containing 2 μg/ml of trypsin inhibitor, and were transferred to KRB containing 4% albumin and 2 mg/ml of purified collagenase (Worthington Code CLSPA).

day for 4 days) failed to restore the response to insulin and growth hormone.

Discussion. The present data clearly indicate that fat cells isolated from the epididymal adipose tissue of hypophysectomized rats are insensitive to the insulin-like effects of growth hormone and to insulin itself, while cells prepared from normal adipose tissue by the same procedures exhibited normal responsiveness to insulin. Since tissue segments from the same animals responded in the normal manner to these hormones, it is likely that hypophysectomy increased the susceptibility to some aspect of the digestion procedure which destroyed the responsiveness of the isolated cells. While the reasons for the lack of sensitivity to insulin and growth hormone are unknown, several explanations are possible: (a) Since low concentrations of many proteolytic enzymes (16–20) and phospholipases (13, 21, 22) are known to have insulin-like effects in adipose tissue, and since proteases (1, 23, 24) and phospholipase C (12) are known to contaminate crude collagenase, it is possible that glucose uptake by the fat cells of hypophysectomized rats was already maximally stimulated by these or other insulin-like enzymes during the digestion procedure. This, however, seems unlikely. Basal rates of glucose oxidation were comparable in cells from normal and hypophysectomized rats, yet normal cells doubled or tripled their output of $^{14}CO_2$ in response to insulin. Furthermore, the basal rate of glucose oxidation was markedly increased in cells from hypophysectomized rats pretreated with triiodothyronine, indicating that hypophysectomized rats are capable of utilizing glucose at higher rates. (b) Some anterior pituitary hormone(s) or the secretions of their target glands may be needed for the production of some material on the surface of the adipocytes or in the associated connective tissue. Loss of such material after hypophysectomy might render hormone receptor sites on the cell membranes more sensitive to attack by the proteases and phospholipase C found in crude collagenase preparations. (c) Fat cells from hypophysectomized rats, unlike those of normal rats, may be unable to repair membrane components damaged during the digestion procedure.

Pretreating hypophysectomized rats with cortisone, triiodothyronine, and growth hormone failed to restore hormone sensitivity. However, other pituitary hormones or a longer pretreatment period may be required for the tissue to reconstitute hypothesized protective elements, or for the cells to regain the capacity to repair membrane components. Attempts to inhibit enzymes in the collagenase preparation that might attack the cell membrane also failed, but as noted earlier, these inhibitors may not have been specific for the contaminating enzymes, or may have been added in amounts inadequate to inhibit enzymes present. On the other hand, some other enzymatic component of collagenase, or collagenase itself may be destroying those surface elements necessary for the response to growth hormone and insulin.

Finally, it is also possible that fat cells isolated from hypophysectomized rats may

produce an inhibitor or leak enzymes that might interfere with or destroy insulin and growth hormone. This seems unlikely, because in one experiment (Table II, Expt. 6) tissue segments incubated for 1 hr in the presence of cells responded to both growth hormone and insulin in the normal manner.

Despite their loss of response to the effects of insulin and growth hormone on glucose metabolism, fat cells from hypophysectomized rats may retain sensitivity to other hormones. Although it was beyond the scope of this study to investigate other hormonal effects, in one preliminary experiment, fat cells prepared from hypophysectomized rats remained sensitive to the lipolytic effects of epinephrine as judged by glycerol production. In these cells insulin failed to decrease epinephrine-stimulated lipolysis. This is consistent with the experiments of Kono (1), and Fain and Loken (25), who found that trypsin treatment of normal cells destroyed the antilipolytic effect of insulin but left the cells sensitive to epinephrine. As a general rule, it appears that loss of antilipolytic effects accompanies loss of stimulatory effects on glucose metabolism.

Although these studies failed to shed light on the question whether the insulin-like effect of growth hormone is exerted at the same cellular receptors as those affected by insulin, they nevertheless served a useful purpose by calling attention to a potential hazard inherent in studies on isolated cells. Since preparation of cells necessitates exposure to variable amounts of enzymes which can act on and presumably destroy portions of the cell surface, some aspects of the behavior of free fat cells may be artifactual and may lead to erroneous conclusions. Had our earlier studies on the early effects of growth hormone been made with cells rather than tissue segments, the insulin-like effect of growth hormone on adipose tissue would have been missed. Other subtle differences between free cells and segments of tissue, particularly with regard to sensitivity to fatty acids (Schimmel and Goodman, unpublished study), have also been noted. It would seem prudent to suggest therefore, that observations made with isolated cells be compared with data derived from segments of fat or preferably *in vivo* observations before firm conclusions regarding the physiology of adipose tissue are made.

Summary. Free fat cells prepared from epididymal fat of hypophysectomized rats by digestion with collagenase failed to exhibit increased glucose oxidation when exposed to insulin or growth hormone. Segments of epididymal fat from the same hypophysectomized rats responded normally to these hormones. When obtained from normal rats, both segments of epididymal fat and free adipocytes also exhibited normal sensitivity to insulin. Hormonal responsiveness of adipose cells of hypophysectomized animals was not restored by shortening the digestion period, addition of trypsin or phospholipase inhibitors or pretreatment of the donor animals with cortisone, triiodothyronine, or growth hormone.

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1. Kono, T., J. Biol. Chem. **244**, 1772 (1969).
2. Goodman, H. M., Endocrinology **76**, 1134 (1965).
3. Goodman, H. M., Metabolism **19**, 849 (1970).
4. Goodman, H. M., Endocrinology **76**, 216 (1965).
5. Goodman, H. M., Endocrinology **75**, 140 (1964).
6. Rodbell, M., J. Biol. Chem. **239**, 375 (1964).
7. Dole, V. P., J. Clin. Invest. **35**, 150 (1956).
8. Czech, M. P., and Fain, J. N., Endocrinology **87**, 191 (1970).
9. Schreibman, P. H., Wilson, D. E., and Arky, R. A., Life Sci. **7**, 1295 (1968).
10. Ogle, J. D., and Tytell, A. A., Arch. Biochem. Biophys. **42**, 327 (1953).
11. Zamecnik, P. C., Brewster, L. E., and Lipmann, F., J. Exp. Med. **85**, 381 (1947).
12. Elsbach, P., and Rizack, M. A., Biochim. Biophys. Acta **198**, 82 (1970).
13. Rodbell, M., J. Biol. Chem. **241**, 130 (1966).
14. Zamechnik, P. C., and Lipmann, F., J. Exp. Med. **85**, 395 (1947).
15. Keller, S., and Mandl, I., Arch. Biochem. Biophys. **101**, 81 (1963).
16. Kuo, J. F., Holmlund, C. E., and Dill, I. K., Life Sci. **5**, 2257 (1966).
17. Kuo, J. F., Holmlund, C. E., and Dill, I. K., Arch. Biochem. Biophys. **117**, 269 (1966).
18. Rieser, P., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **25**, 441 (1966).

19. Kuo, J. F., J. Biol. Chem. **243**, 211 (1968).

20. Kuo, J. F., Dill, I. K., and Holmlund, C. E., J. Biol. Chem. **242**, 3659 (1967).

21. Rodbell, M., J. Biol. Chem. **241**, 140 (1966).

22. Blecher, M., Biochem. Biophys. Res. Comm. **23**, 68 (1966).

23. Kono, T., Biochemistry **7**, 1106 (1968).

24. Mandl, I., MacLennan, J. D., and Howes, E. L., J. Clin. Invest. **32**, 1323 (1953).

25. Fain, J. N., and Loken, S. C., J. Biol. Chem. **244**, 3500 (1969).

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