Enhanced Activity of Hormone-Sensitive Adenylate Cyclase during Dietary Restriction in the Rat

DEPENDENCE ON AGE AND RELATION TO CELL SIZE

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ABSTRACT Age-related decreases of hormone-sensitive adenylate cyclase activities of rat fat cell plasma membranes (ghosts) have been recently described. Glucagon-sensitive activity was completely lost between 1 and 6 mo, an interval in which fat cell size increases rapidly, while decreased activation by ACTH was gradual over the entire life span of the animal (24 mo), and epinephrine-sensitive enzyme diminished modestly and only during senescence. In the present studies an attempt was made by restricting food intake to assess the importance of changing cell size in the age-related alterations of hormone-sensitive enzyme activities. Enzyme activities were determined before restriction and at monthly intervals for 3 mo for the unstimulated enzyme (basal) and in the presence of maximally stimulating concentrations of glucagon, ACTH, epinephrine, and fluoride. Activities were calculated per milligram ghost protein or per cell. Restriction of food intake for 3 mo starting at 1 or 12 mo produced fat cells equal in size to those of 5-wk-old animals fed ad lib. In young animals restricted for 1 mo, hormone-stimulated activity expressed as fold increase (stimulated/basal) was not merely maintained as the cells were prevented from enlarging, but was enhanced two to three times over the initial values with all three hormones. With continued restriction epinephrine-sensitive activity remained two times increased. Glucagon and ACTH responses subsequently decreased, but even by 3 mo of restriction, responses to the latter hormones, although declining, were still 1.5–3 times greater than the unrestricted controls, regardless of whether activity was expressed as total activity per milligram ghost protein or per cell, or as fold-increase. In the young animals, basal and fluoride-sensitive activities after a 3-mo restriction were unchanged or had decreased only slightly, depending on the base line used. Dietary restriction of adult animals for 3 mo, in contrast to the results in the young, did not increase total hormone-stimulated activity but rather produced either 0% (per milligram protein) or 25% decrease (per cell) for epinephrine-sensitive enzyme, 25 or 50% decrease of ACTH response, and 40 or 60% decreases of basal- and fluoride-stimulated activities. Expression of activities of restricted adults as fold-increase (stimulate/basal) showed an "increase of responsiveness" for all three hormones, but this was a reflection of the marked decrease of basal activity. Nonetheless, the restricted adults showed significant restoration of a small amount of glucagon-sensitive activity (1.8-fold over basal). These results indicate that cell size, per se, is not a dominant factor affecting hormone-responsive adenylate cyclase under conditions of dietary restriction. The data do not support the postulation that increasing cell size is directly related to the enzyme's decreased activity during aging. Hormone-sensitive adenylate cyclase is, however, clearly under dietary control, while age determines the pattern of the observed response during dietary restriction.

INTRODUCTION

Rat fat cell adenylate cyclase is responsive to a variety of hormones, including epinephrine, ACTH, glucagon, luteinizing hormone, thyroid stimulating hormone, and secretin (1). The lipolytic response of the fat cell to these hormones is presumably mediated by

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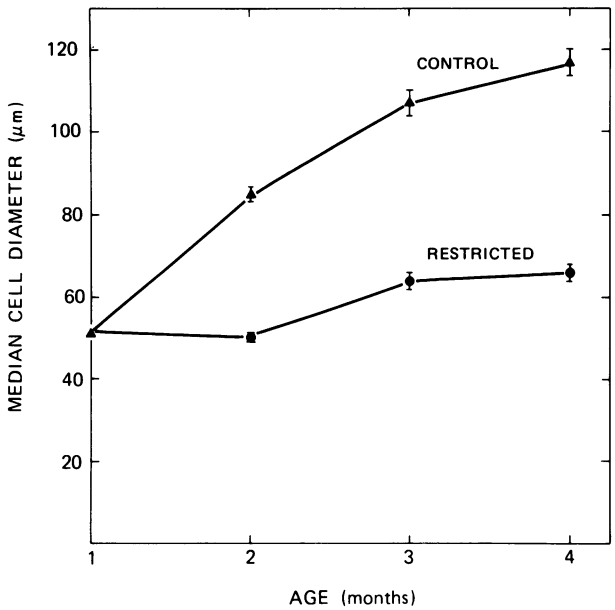

FIGURE 1 Effect of dietary restriction on fat cell diameter in young rats. 1-mo rats were either continued on ad lib. diets (control) or maintained on restricted intake. Mean ±SEM; $n = 4$.

cyclic AMP (cAMP)[1] via activation of adenylate cyclase. Tissue from young rats has generally been used to demonstrate the hormone responsiveness of fat cell adenylate cyclase, but in our laboratory we have recently demonstrated an age-related loss of glucagon and ACTH-sensitive enzyme in rat fat cell membranes; epinephrine responsiveness decreased modestly and only during senescence (2). While the decrease of ACTH-activated enzyme occurred gradually over the life span of the rat (24 mo) the marked stimulation by glucagon of the enzyme in very young rats (1 mo) was already absent by 6 mo. Since cell size increases rapidly (surface area increases sixfold) between 1 and 6 mo, it was not clear whether this rapid loss of glucagon-activated enzyme represented a cell size effect or an age-related phenomenon. The basis for this concern is the reports which have suggested that cell size per se may play an important role in mediating hormone responsiveness in fat cells, especially those responses dependent on hormone binding to receptors on cell membranes (3, 4). Other investigators have shown that chronic dietary restriction can markedly affect rat fat cell size (5–7). With restricted diet we have now been able to prevent the normal cell enlargement associated with maturation in young rats and also to decrease cell size in older animals. By this manipulation we have been able to compare hormone-sensitive adenylate cyclase in rats of different ages having comparable fat cell size, thereby attempting to differentiate between age-related effects and those related to cell size, per se. An unexpected result of dietary restriction was an age-dependent enhancement of hormone-sensitive adenylate cyclase activity.

[1] *Abbreviation used in this paper*: cAMP, cyclic AMP.

MATERIALS AND METHODS

Animals and feeding. All studies used male Wistar rats from an outbred strain raised in the Gerontology Research Center. Rats of 6 mo are considered young adults and those of 12 mo mature adults. In this colony mortality is very low until 20 mo, but by 24 mo a 50% mortality is seen. All animals were maintained on a standard diet (National Institutes of Health) open formula containing approximately 24% protein, 5% fat, and 4.3 kcal/g) equivalent to Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.). Control animals were fed ad lib. Restricted animals were groups of either 1 or 12 mo with initial body weights comparable to controls which were maintained on approximately one-half of their normal dietary intake (10 g/day for 1 mo and 13 g/day for 12 mo). The restricted animals were given their food during the late afternoon and consumed their entire daily ration in a few hours. A small portion of the daily ration was given the restricted animals a few hours before sacrifice to assure that animals had not fasted for a prolonged period.

Preparation of fat cell ghosts and cell fixation. Isolated rat fat cells and plasma membranes ("ghosts") were prepared as described by the method of Birnbaumer et al. (8) but with addition of 1 mM dithiothreitol to the lysing medium. Ghosts were frozen in liquid nitrogen and stored up to 1 wk before assay without loss of activity. Fat cell size and number were determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.) after fixation with osmium tetroxide (2).

Assay of adenylate cyclase activity. Enzyme activity was measured by the method of Salomon et al. (9). The standard assay mixture contained 1.5×10^6 dpm [α^{32}P]ATP (New England Nuclear, Boston, Mass.), 10–30 Ci/mM; 1.6 mM ATP (Sigma Chemical Co., St. Louis, Mo.); 25 mM Tris-HCl (pH 7.4); 5 mM $MgCl_2$; 2 mM cAMP (Sigma Chemical Co.); 0.1% albumin (Pentex Biochemical, Kankakee, Ill., fraction V); 10 mM theophylline (Sigma Chemical Co.); and an ATP regenerating system consisting of 20 mM creatine phosphate and 1 mg/ml creatine kinase (Sigma Chemical Co.). Incubation mixtures contained a final vol of 50 μl. Reactions were initiated by addition of 20 μl of suspended ghosts (40–80 μg protein) and continued for 10 min at 30°C. All data were expressed as nmol cAMP/mg protein per 10 min or nmol cAMP/10^6 cells per 10 min. Determination of ghost protein was by the method of Lowry et al. (10). Hormones and activators included L-epinephrine bitartrate (Sigma Chemical Co.); porcine ACTH (Calbiochem, San Diego, Calif., grade B); glucagon (Sigma Chemical Co.); and sodium fluoride (Fisher Scientific Co., Pittsburgh Pa.). Ghosts were prepared from control and restricted rats of the same age on the same day. In a few cases epididymal fat pads from 1- or 2-mo rats were pooled because of the small amount of fat present in each pad. An n of 1 was assigned to each such pool. Statistical comparisons were made by Student's t test for unpaired samples.

RESULTS

Dietary restriction and cell size in young rats. Median fat cell diameter increased from 52 to 117

μm between 1 and 4 mo in the control rats (Fig. 1). In contrast, rats of comparable age on a restricted diet for 3 mo showed a much smaller increase of fat cell diameter from 52 to 66 μm. Both increases were significant ($P < 0.001$), as was the difference in cell size between the 4-mo controls and the restricted animals ($P < 0.001$). Mean cell size in the restricted group was equivalent to that of 5-wk rats maintained on an ad lib. diet (Fig. 1).

Activities of adenylate cyclase. Stimulated/basal in controls and after dietary restriction for 3 mo. In the 4-mo control animals adenylate cyclase was stimulated 4.8-fold relative to basal by epinephrine, 1.8-fold by ACTH, and 8.6-fold by fluoride; by this age glucagon no longer stimulated significantly (Fig. 2A). In the restricted animals hormone response was consistently enhanced 1.5–3 fold relative to the response in control animals depending on the hormone and the reference base (membrane protein or cell number). Expressed per milligram protein the differences were all highly significant ($P < 0.001$). When enzyme activity was expressed per cell (Fig. 2B), the restricted group showed a 35% decrease of basal activity and similar decrease of fluoride-simulated activity ($P < 0.05$). These results suggest the presence of less "total" enzyme per cell (diminished number of catalytic units) in the smaller cells of the restricted animals. Nonetheless, in the animals restricted for 3 mo, hormone-stimulated enzyme activity per cell was greater than in the controls on stimulation by epinephrine ($P < 0.05$), ACTH ($P < 0.025$), and glucagon ($P < 0.005$).

Comparison of hormone-sensitive responses during the period of dietary restriction. As shown in Fig. 3 the control group showed a rapid loss of glucagon responsiveness (stimulated/basal activity). From 4.2-fold at 1 mo, the response was 2.1-fold at 2 mo, and 1.3-fold at 3 mo; by 4 mo there was no significant activation of adenylate cyclase by glucagon. ACTH-sensitive enzyme activity decreased more gradually and was still twofold increased at 4 mo in the control animals.

In the restricted group, marked increases of epinephrine, ACTH, and glucagon responsiveness were noted. Epinephrine-responsive activity was increased twofold after only 1 mo of restriction and remained increased (Table 1). Fluoride response did not increase in the restricted animals at any time.

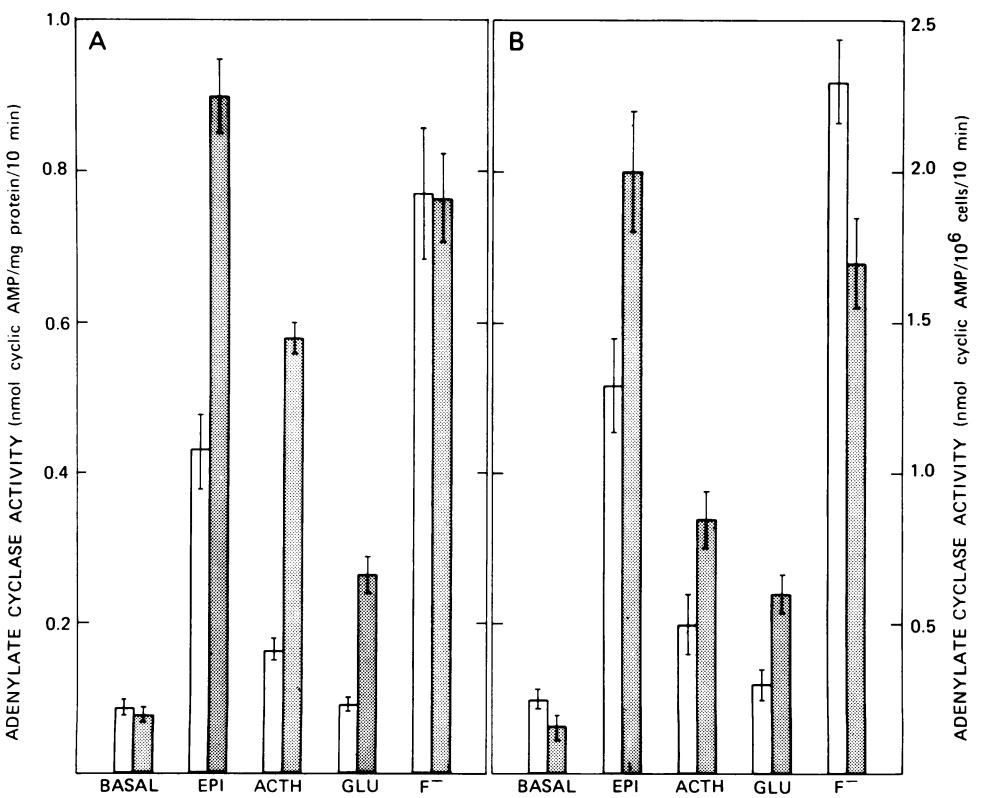

FIGURE 2 Effects of dietary restriction on fat cell adenylate cyclase activity per milligram ghost protein (A) and per cell (B) in 4-mo rats. Open bars, animals fed ad lib. (control); stippled bars, rats maintained on restricted intake for 3 mo. Additions were 1 mM epinephrine (EPI); 0.1 mM ACTH; 10 μM glucagon (GLU); and 10 mM fluoride (F^-). Mean±SEM; $n = 4$.

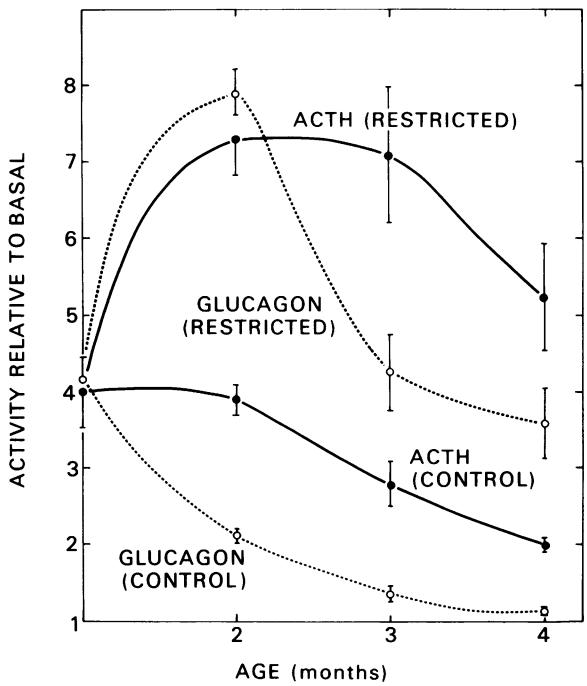

FIGURE 3 Effects of dietary restriction on the age-related loss of glucagon and ACTH-sensitive adenylate cyclase. 1-mo rats were fed ad lib. (control) or were maintained on restricted intake. Concentrations of hormones were 0.1 mM ACTH and 10 μM glucagon. Mean±SEM; n = 4. When activity is expressed as the ratio of hormone-stimulated activity relative to basal activity, the value is the same whether ghost protein or cell number is used.

After 1 mo of restriction, adenylate cyclase responsiveness was 7.9-fold with glucagon and 7.3-fold with ACTH (Fig. 3). Despite continued restriction, loss of hormone responsiveness occurred with both hormones; glucagon decreased to 3.6-fold ($P < 0.001$) and ACTH to 5.3-fold ($P < 0.05$) after 3 mo of restriction. Epinephrine and fluoride responses, in contrast, showed no age-related loss in either the control or restricted groups (Table 1).

TABLE I
Effect of Dietary Restriction on Epinephrine and Fluoride-Stimulated Adenylate Cyclase

Addition	Group	Age of animals 1	2	3	4
				mo	
			Activity relative to basal*		
Epinephrine, 1 mM	Control	5.7	6.5±0.4	6.7±0.3	5.3±0.8
	Restricted	—	11.6±0.8	11.0±0.8	12.6±2.2
Fluoride, 10 mM	Control	9.8	9.3±0.6	12.7±1.3	9.4±0.5
	Restricted	—	9.3±0.6	10.7±0.6	10.7±1.7

Control values are means of duplicate determinations for three animals. All other values are mean±SEM; n = 4.
* When activity is expressed as the ratio of hormone-stimulated activity relative to basal activity, the value is the same whether ghost protein or cell number is used.

Dietary restriction, cell size, and hormone-sensitive adenylate cyclase in mature rats. The effect of dietary restriction on the fat cell diameter of 12-mo rats is shown in Fig. 4. By 6 wk of restriction, mean fat cell diameter had decreased from 124 to 77 μm and was almost at the plateau level of 70 μm reached after 3 mo of restriction. At this point cells were comparable to 5–6-wk rats maintained on an ad lib. diet (cf. Fig. 1). This decrease of fat cell size was associated with a 40% loss of body weight. The median cell diameters of the control animals did not change between 12 and 15 mo.

The effects of dietary restriction on fat cell adenylate cyclase are shown in Fig. 5. After 10–12 wk of restriction basal and fluoride-stimulated activity, expressed per milligram protein, decreased by 40% of control values for rats of the same age ($P < 0.005$). There was no demonstrable difference in epinephrine, glucagon, or ACTH-sensitive enzyme between the control and restricted groups. When enzyme activity was expressed per cell (Fig. 5B), basal and fluoride-activated

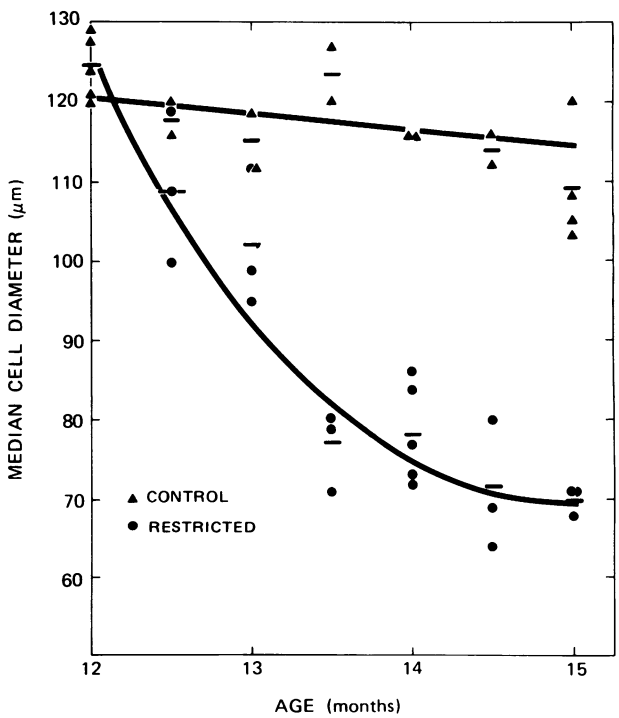

FIGURE 4 Effect of 3 mo of dietary restriction on median fat cell diameter of 1-yr-old rats. Animals were either continued on ad lib. diets (control) or maintained on restricted intake.

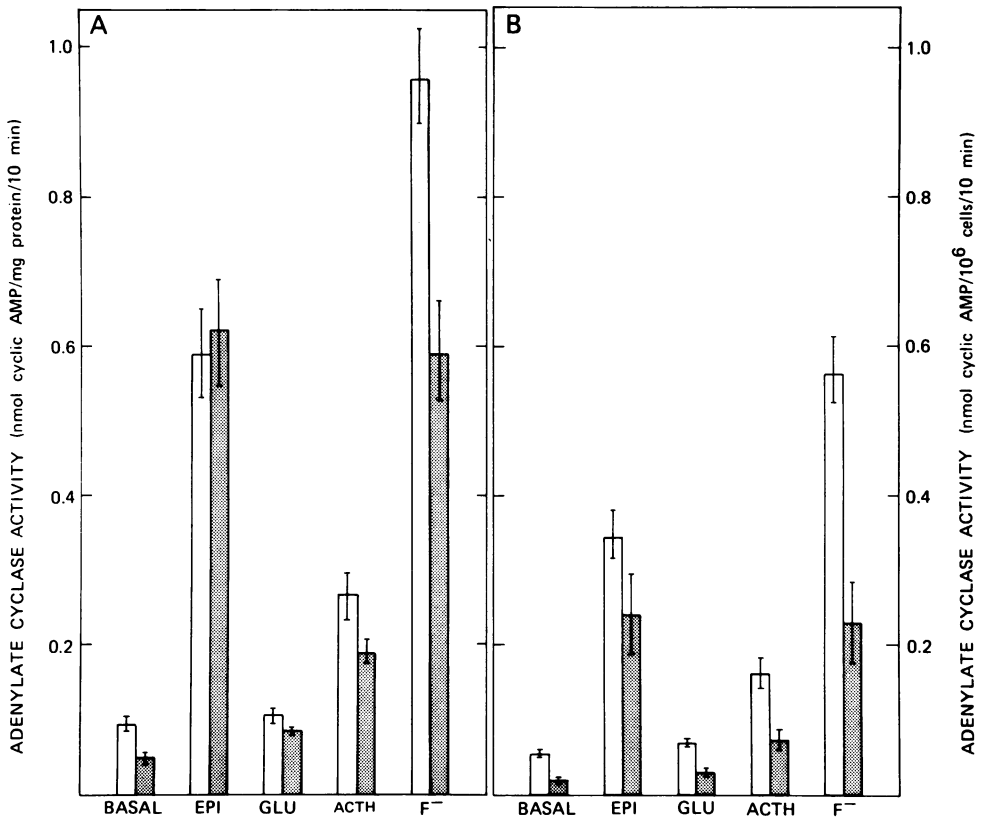

FIGURE 5 Effects of dietary restriction on fat cell adenylate cyclase activity per milligram ghost protein (A) and per cell (B) of 1-yr-old rats restricted for 3 mo and tested at 15 mo. Open bars, animals fed ad lib. (control); stippled bars, rats maintained on restricted intake. Additions as in Fig. 2. Mean±SEM; $n = 6$.

cyclases decreased by 60% of control values in the restricted animals ($P < 0.001$). Glucagon and ACTH-sensitive enzymes were also 50% less when expressed per cell in the restricted group ($P < 0.01$), although no difference in epinephrine-sensitive enzyme was seen. The decreased activities of basal and fluoride-sensitive enzymes suggest the presence of less total enzyme in the smaller cells of the diet-restricted 15-mo animals as compared to control animals of the same age.

Although absolute (total) values for hormone-sensitive adenylate cyclases showed no increases during restriction of the adult animals, hormone-sensitive activities expressed as ratios of stimulated/basal were enhanced by dietary restriction (Fig. 6). Epinephrine increased activity 12.4-fold compared to 6.1-fold stimulation in the control group ($P < 0.001$). ACTH stimulation was 3.8-fold in the restricted group and 2.8-fold in the control group; this difference was not statistically significant. While glucagon did not stimulate the enzyme of the control 15-mo rats, significant activation did occur (1.8-fold) with the restricted animals of the same age ($P < 0.025$).

DISCUSSION

Restriction by 50% of the food intake of rats markedly affected fat cell size in the young (1 mo) and adult (12 mo) rats in our studies. After 3 mo, median cell diameters in both groups of restricted animals approximated those of 5-wk animals fed ad lib. Other investigators have noted similar effects on cell size, as well as effects on cell number, in rats exposed to chronic semistarvation (6, 7).

The loss of glucagon-sensitive adenylate cyclase occurred over an age interval which was associated with a rapid increase of cell size. However, this decrease in responsiveness is not likely to be related solely to altered cell size. Cells from 15-mo restricted rats were comparable in size to cells of 5-wk animals, yet *absolute* levels of glucagon-sensitive adenylate cyclase activity were much decreased in the older cells.[2] Nonetheless, when expressed as a ratio of

[2] The term *hormone-sensitive adenylate cyclase* is time-honored and is used here simply to denote adenylate cyclase whose activity increases in the presence of hormone.

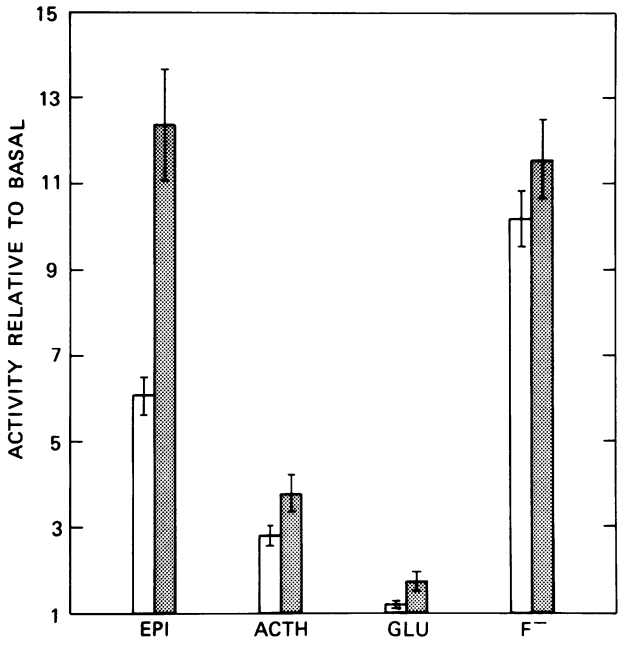

FIGURE 6 Effects of dietary restriction on ratio of hormone and fluoride-activated adenylate cyclase/basal in 1-yr-old rats restricted for 3 mo. Open bars, fed ad lib. (control); stippled bars, maintained on restricted intake. Additions as in Fig. 2 and activities as in Fig. 3. Mean±SEM; $n = 6$. The apparent enhancement of hormone sensitivity (EPI) is due to decreased basal activity rather than increased absolute stimulation (cf. Fig. 5.). A significant glucagon response (GLU) is seen in the restricted adult animals but not in the control group.

stimulated/basal activity, glucagon activated 1.8-fold in the adult restricted animals while stimulation was not significant in the controls.

If young animals had been studied at only one point in time, e.g. after 3 mo of restriction, the data would have suggested that the age-related loss of hormone responsiveness had been prevented, perhaps by preventing the normal age-related increase of cell size between 1 and 4 mo. However, our serial measurements of glucagon and ACTH-activated enzyme between 1 and 4 mo (Fig. 3) clearly demonstrated that dietary restriction initially enhanced hormone responsiveness to glucagon and ACTH, while an age-related loss of response to these hormones was still occurring. It is probably fortuitous that the activity ratios for the animals restricted for 3 mo had returned almost to those of 1-mo rats before restriction.

In contrast to the age-related decrease of absolute and relative glucagon and ACTH-sensitive adenylate cyclase activities, epinephrine activity was enhanced by dietary restriction in absolute terms only in the young animals (Fig. 2). In the adult animals, restriction caused no increase of absolute level of epinephrine-sensitive activity (Fig. 5). However, activity in the adults expressed as a ratio of stimulated/basal was increased as a result of restriction (Fig. 6); this increase resulted entirely (except for the case of glucagon) from the decrease of basal in the restricted animals. These age-dependent differences suggest an alteration of the coupling mechanism between the hormone receptors and their catalytic units. We have previously reported that no age-related decrease of epinephrine activation (stimulated/basal) is seen in animals fed ad lib. Absolute activity with epinephrine decreased between 2 and 6 mo but thereafter only slightly until after 12 mo; at 24 mo a modest decrease of epinephrine- and fluoride-stimulated activity was apparent, suggesting loss of adenylate cyclase catalytic units by that time (2). In the present studies both basal and fluoride-responsive enzyme (per milligram protein and per cell) were decreased in 15-mo restricted rats compared to control animals of the same age, suggesting that adenylate cyclase catalytic units might also be lost during dietary restriction in these animals. Decreased basal and fluoride-responsive adenylate cyclase (per cell) was also seen in young restricted rats. The increase of hormone-sensitive enzyme in young restricted animals may reflect an adaptive response in the fat cell which allows enhanced responses to hormones despite the presence of less total enzyme. Viewed in this light, the adult animals show considerably less ability to adapt than do the young. Although stimulation by fluoride may not reflect total adenylate cyclase activity, fluoride stimulation and that with GMP-P (NH)P (plus high Mg^{2+}) were closely parallel in our earlier report (2). The latter stimulus produces the highest adenylate cyclase activities seen to date.

Only a few previous studies have demonstrated effects of dietary manipulation on hormone-sensitive rat fat cell adenylate cyclase. Gorman et al. (12) studied 150-g rats (our estimated age, 6 wk). Short-term starvation (48 h) produced a modest (50%) increase of epinephrine-sensitive activity but did not affect glucagon responsiveness. Animals fed a high fat diet for 3 days had markedly reduced epinephrine-sensitive fat cell adenylate cyclase and total loss of

The term *hormone-responsive* is used synonymously. *Absolute* or *total* activity refers to activity in the presence of stimulator, i.e. hormone or fluoride without regard to and uncorrected for basal activity. Activity has also been expressed as the ratio of stimulated/basal or *relative* activity. This expression is convenient, although its use has been questioned because of the difficulty of interpreting the significance of basal activity (11). In contrast, altered *hormone sensitivity* is a phrase which implies an altered hormone response based on a change of the dose-response relationship. We have avoided the term hormone sensitivity, since our results, based only on the use of maximal levels of agonists, do not examine this issue.

glucagon-activated enzyme. In a subsequent paper these authors found that the lost epinephrine responsiveness on fat feeding was rapidly restored when rats were subsequently refed a high protein diet for 3 days (13); surprisingly, no such restoration was noted with the glucagon-sensitive enzyme. Since the recovery of epinephrine responsiveness occurred without a reduction in fat cell volume, the loss of responsiveness on fat feeding was evidently related to metabolic factors and was clearly independent of cell size. With a different dietary manipulation (intermittent feeding, i.e., "gorging") Braun and Fabry (14) referred to unpublished observations which noted that fat cell adenylate cyclase from fasted to refed rats had increased "sensitivity" to epinephrine (higher specific activity). Interestingly, epinephrine responsive lipolysis is enhanced under these conditions, as well.

Our results with the effects of dietary restriction on hormone-sensitive adenylate cyclase prompt comparison with some available information on the effects of chronic dietary restriction on catecholamine-stimulated lipolysis. Reardon et al. (5) maintained 4-wk rats on restricted caloric intake (7 g chow/100 g body wt per day) for 6, 8, 15, and 23 wk and compared in vitro basal and epinephrine-stimulated lipolysis (per cell) to groups of rats fed ad lib. Significant differences from control rates of lipolysis occurred after 15 wk, by which time the restricted animals had lower basal levels; the stimulation of lipolysis by epinephrine (relative to basal) was unchanged in the two groups. In contrast, Hubbard and Matthew (7) found that norepinephrine-stimulated lipolysis (expressed per microgram DNA) in older rats of unspecified age was significantly less than 10-wk-old younger animals, while dietary restriction for 10 wk prevented loss of norepinephrine-stimulated lipolysis. Knittle and Ginsberg-Fellner (15) noted decreased basal and epinephrine-stimulated lipolysis per cell in human adipose tissue fragments after a 6–12-mo period of caloric restriction (600 calorie/day) in markedly obese subjects; relative stimulation of lipolysis by epinephrine was unchanged. These observations clearly suggest that factors in addition to catecholamine-sensitive adenylate cyclase may be important determinants of lipolytic response during dietary restriction.

While decreased lipolytic responsiveness to glucagon has been found in fat cells of large rats (16–18), no published studies deal with the effects of dietary restriction on this phenomenon. However, in unpublished observations Hansen et al. (18) noted that dietary restriction appeared to prevent the decrease of glucagon-stimulated lipolysis found as cells enlarge. Our own data on glucagon-sensitive adenylate cyclase would suggest that dietary restriction might delay but would not prevent such an age-related loss of hormone responsiveness.

Future studies of hormone-sensitive lipolysis will require correlations of lipolytic responses with adenylate cyclase activities, hormone receptor numbers, intracellular cAMP levels, and phosphodiesterase activities. Several studies of size effects on lipolysis already indicate that decreased numbers of glucagon receptors and increased levels of phosphodiesterase may both contribute to altered lipolytic responsiveness (17, 19). The number of glucagon receptors decreases by about 50% over the time when glucagon responsive adenylate cyclase disappears almost entirely (17). Loss of glucagon receptors alone does not appear, therefore, to account for the loss of hormone-sensitive enzyme responsiveness during aging (maturation). In the future, consideration will have to be given to the now obviously important variables of age, dietary composition, and total caloric intake.

Our data do not provide information on the mechanism by which dietary restriction and (or) age affect hormone-sensitive adenylate cyclase activity. However, some recent observations by others suggest an approach to additional investigation of these phenomena. Lipids are clearly involved in the coupling of hormone receptors to adenylate cyclase catalytic units (20). The lipid composition of the plasma membrane of liver appears to be age-dependent and is associated with altered activity of membrane bound ATPases (21) and adenylate cyclase.[3] Furthermore, experimentally induced deficiency of essential fatty acids affects activity of liver plasma membrane ATPases and adenylate cyclase (22). The possibility seems worth exploring that age and diet-related alterations of hormone-sensitive adenylate cyclase activity may be dependent upon perturbations of the lipid composition and structure of the fat cell membranes.

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