Regulation of new fat cell formation in rats: the role of dietary fats

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Abstract Factors that stimulate formation of new adipocytes during development of obesity are yet to be identified. We examined whether diet acts directly on preadipocytes to stimulate replication and differentiation or indirectly by interacting with adipocytes to release or modify local growth factors. Male Sprague-Dawley rats were fed chow or diets high in starch (HST), saturated (HFS) or polyunsaturated (HFP) fats until 5–7 months of age. We found that, compared to other diets, HFS induced acceleration of replication of preadipocytes in primary culture (doubling time of retroperitoneal-derived preadipocytes: HFS 17 ± 1 versus chow 32 ± 6 and HFP 29 ± 3 h, $P < 0.05$). HFS stimulated greater expansion of retroperitoneal fat than HFP even when caloric intake was equal and increased adipocyte number threefold. Preadipocyte pool size in inguinal and retroperitoneal fat pads changed relative to fat pad weight in rats fed all diets compared to chow, suggesting that the balance between the number of cells capable of replicating and those terminally differentiated was perturbed. Differentiation of preadipocytes and release of adipocyte growth factors in vitro were unaffected by diet. We concluded that dietary saturated fats induced expansion of adipose tissue mass more effectively than polyunsaturated fats and that this may, in part, be achieved by acceleration of preadipocyte replication.—**Shillabeer, G., and D. C. W. Lau.** Regulation of new fat cell formation in rats: the role of dietary fats. *J. Lipid Res.* 1994. **35:** 592–600.

Supplementary key words adipocytes • preadipocytes • differentiation • replication • adipose tissue • saturated fats

Although it is well established that an increase in the number of adipocytes occurs during the development of obesity in humans (1) and in rodents (2, 3), factors that stimulate the formation of new adipocytes are yet to be identified. New adipocytes are formed as a result of replication and differentiation of preadipocytes, which have been shown to be present throughout life (4). This process may be accelerated in obesity. Preadipocytes derived from massively obese adult humans have been shown to replicate faster in vitro than those derived from nonobese subjects (5) although those derived from genetically obese rats did not (6).

A role for paracrine factors in the control of adipose tissue growth is suggested by the observation of regional variations in growth in vivo (2) and in vitro (7, 8). We have shown that rapidly proliferating, human-derived preadipocytes released factors that promoted the replication of rat preadipocytes in culture (9). In addition, we recently reported that preadipocytes derived from Sprague-Dawley rats differentiated in primary culture in response to the presence of mature adipocytes (10). The adipogenic effect of the mature fat cells was not due to their release of triacylglycerols, fatty acids, or lactate but to an unidentified factor.

In vivo, such paracrine factors, released by constituent cells of adipose tissue, may be subject to modulation by caloric intake or specific dietary components and may play a role in the expansion of adipose tissue mass in the development of obesity. Lemmonier (11) and Herberg and colleagues (12) have previously shown that dietary fats induce obesity, independent of body weight gain. Later studies showed that this could be achieved without increasing caloric intake (13, 14). Dietary fats may act directly on preadipocytes to increase the rate of replication and/or differentiation. Alternatively, they may act by modulating the activity or release of paracrine factors, by modifying the hormonal milieu, or by affecting substrate delivery to adipose tissue. We have recently reported that dietary saturated fats fail to suppress hepatic de novo lipogenesis as effectively as polyunsaturated fats (15). Small changes in lipogenic enzyme activity induced by dietary fats of different composition may, over the long term, have significant impact on the total lipid accumulated in adipose tissue and, thus, on the development of obesity.

Abbreviations: HST, high starch diet; HFS, high saturated fat diet; HFP, high polyunsaturated fat diet; LPL, lipoprotein lipase; GPDH, glycerophosphate dehydrogenase; HSL, hormone-sensitive lipase; MEM, minimum essential medium; FBS, fetal bovine serum; HFS-R, high saturated fat-restricted diet; HFP-R, high polyunsaturated fat-restricted diet.

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In the study reported herein, we examined whether dietary factors act on preadipocytes to stimulate replication and differentiation during the development of diet-induced obesity. We also investigated the postulate that dietary components may interact with mature fat cells to release or modify local growth factors.

METHODS

Animals and diets

One-month-old male Sprague-Dawley rats (Charles River; initial weight 50–60 g) were housed individually in wire-bottomed cages and maintained on a 12 h:12 h light-dark cycle at 22°C. The rats were fed nutritionally adequate, semi-synthetic diets, differing in their major source of energy: beef tallow (Teklad Test Diets, Madison, WI) (high saturated fat, HFS), safflower oil (high polyunsaturated fat, HFP), or corn starch (HST) (ICN Canada, Montreal, PQ). The two high fat diets contained 45% of calories as fat and the starch diet contained 65% of calories as starch and 10% as sucrose. All diets contained 20% protein as casein (ICN) and adequate vitamins, minerals, and essential fatty acids. Sucrose and safflower oil were obtained from the local supermarket. To eliminate effects due to differences in intake of calories between the two high fat groups, two additional groups were included in which intake of the high fat diets was calorically pair-matched to the intake of the HST group (HFS-R and HFP-R). Rats of all groups were compared to rats fed standard chow (CHOW). Food intake was recorded daily and spillage was collected, weighed, and deducted from daily intake. After 15 (4–5 months of age) or 26 weeks (7 months of age) on the diets, the rats were killed in the fed state by exsanguination under light halothane anesthesia. Epididymal, perirenal-retroperitoneal, and inguinal fat pads from animals maintained on diets until 5 months old were used for cell counts, biochemical and enzyme analysis, while those from rats fed until 7 months old were used for cell culture studies. Blood was collected from rats of the older group for radioimmunoassay of plasma insulin by the double antibody method using rat insulin standards (16).

Biochemical and enzyme assays

To assess whether differences in lipid accumulation were due to diet-induced changes in lipid uptake and release, adipose tissue extracts were assayed for lipoprotein lipase (EC 3.1.1.34.; LPL), glycerophosphate dehydrogenase (EC 1.1.1.8.; GPDH), and hormone-sensitive lipase (EC 3.1.1.3.; HSL) activity.

Fat pads were rapidly excised from 5-month-old rats, taking care to conform to predetermined boundaries for each pad, and placed in tared vials containing phosphate-buffered saline (0.01 M K_2HPO_4, 0.01 M KH_2PO_4, 0.154 M NaCl, pH 7.4) at 37°C and weighed. One of each pair of pads was frozen in liquid nitrogen and stored at −70°C for later analysis while the other pad was processed immediately for cell counts.

The frozen fat pads were homogenized at 37°C with a tissue homogenizer (Kinematica, Lucerne, Switzerland) in 10 mM HEPES buffer containing 0.25 M sucrose, 1 mM EDTA, and 1 mM dithiothreitol. The homogenate was centrifuged at 100,000 *g* at 0–4°C for 20 min and the floating fat layer was discarded. Aliquots of the supernatant were assayed for GPDH, LPL, and HSL activity under optimal substrate and zero order kinetic conditions as previously described (15). GPDH activity was determined by the method of Kozak and Jensen (17). One unit of enzyme activity corresponded to the oxidation of 1 nmol of NADH/min. LPL activity was determined by the method of Ramirez et al. (18) and HSL activity was quantitated by the method of Khoo and Steinberg (19). For both LPL and HSL, one unit of enzyme activity was equivalent to 1 nmol oleate released/h. The specific activity of each enzyme was expressed as units/mg DNA. DNA was assayed using a fluorimetric method (20). Total protein content of fat pads was determined by the Coomassie blue method (21) and total lipids were determined gravimetrically using the chloroform–methanol 2:1 (vol/vol) method of Folch, Lees, and Sloane Stanley (22).

Mature fat cell counting and sizing

To isolate mature fat cells, fresh adipose tissue was minced and digested with collagenase (type II, Sigma, St. Louis, MO; 2 mg/ml in phosphate-buffered saline, 5 ml/g tissue) at 37°C with gentle shaking in a rotatory water bath for 10 min for epididymal and retroperitoneal pads, and 30 min for inguinal pads (8). The liberated adipocytes were fixed in 2% osmium tetroxide (Sigma) in 0.05 M collidine buffer according to the method of Hirsch and Gallian (23). Cell number and size were determined using an electronic particle counter and channelyzer (Coulter Electronics, Burlington, ON) with an aperture diameter of 400 μm and the window set to exclude cells of diameter smaller than 30 μm.

Cell culture

Fat pads were excised aseptically from the 7-month-old rats. Approximately 0.5 g of each pair of fat pads was finely chopped and kept as a source of mature fat cells in α-minimum essential medium (α-MEM, Gibco, Burlington, ON) and 5% fetal bovine serum (FBS, Hyclone, Logan, UT), containing penicillin (1.7×10^{-4} M) and streptomycin (9.2×10^{-5} M). Preadipocytes were isolated as previously described (8) from the remaining fat tissue of each pair for the determination of the rate of replication and of differentiation. Briefly, after digestion with collagenase (1 mg/ml; type IV, Sigma) in Hank's balanced salt solution (Gibco) and 5% bovine serum albumin

(Fraction V, Sigma) with shaking at 37°C for 45 min; the cell suspension was filtered through a 250-μm mesh Nitex filter (B and SH Thompson and Co. Ltd., Scarborough, ON) and centrifuged. The pellet was resuspended, filtered through a 25-μm mesh filter, and centrifuged again. The pellet obtained consisted mainly of preadipocytes that were cultured in α-MEM, supplemented as described above, and maintained at 37°C in a humidified atmosphere of 95% air–5% CO_2. Replication and differentiation assays were performed as previously described (6, 10) on preadipocytes isolated from each of the three regional fat pads from each rat individually.

Assay of preadipocyte differentiation

Preadipocytes were seeded at a density of 10^4/cm^2 in 35-mm-well plates and grown under three conditions: *i)* cells alone (SPONT) or *ii)* cells with approximately 100 mg finely chopped fat tissue from the same region of origin of the same rat (i.e., epididymal preadipocytes + epididymal fat; +FAT). Under condition *iii)*, cells were allowed to grow in α-MEM alone for 5 days, until confluent, at which time the medium was replaced with fresh α-MEM supplemented with 10^{-8} M insulin, 10^{-7} M corticosterone, and 0.5 mM 1-methyl-3-isobutylxanthine. After 48 h, this medium was replaced with fresh α-MEM containing, in addition to the supplements described above: 10^{-8} M insulin, 10^{-7} M corticosterone, and 0.5% (vol/vol) intralipid (Kabivitrum, Dorval, PQ) (24). The culture conditions described under *iii)* are referred to collectively below as differentiation mix (+MIX). Cells for each set of three conditions were derived from a single digest originating from one rat; each set was derived from a different rat.

After 5 days, cells under conditions *i)* and *ii)* were fixed in 10% formalin and stained with Oil-Red-O to show neutral cytoplasmic lipid droplets and nuclei were counterstained with 10% Giemsa. Cells grown under condition *iii)* were fixed and stained in the same manner after the additional 7 days of culture with MIX. Morphological assessment of differentiation was quantitated microscopically by counting cells containing visible fat droplets, and the results were expressed as a percentage of the total number of cells in eight randomly selected fields.

Assay of preadipocyte proliferation

Preadipocytes in primary culture were seeded at a density of 10^4/cm^2 in 24-well plates under two conditions: cells alone and cells with approximately 10 mg chopped fat tissue from the same region of origin. The rate of replication was measured on successive days in duplicate wells by enumeration of cells cultured under each condition, after trypsinization, using a Coulter counter. The mean of the duplicates was used to give one growth curve for each regional fat pad for each rat. Population doubling time of preadipocytes was calculated from the gradient of the logarithmic phase of each growth curve. The rate of replication of preadipocytes, derived from rats of each diet group was also measured in first subculture.

Assessment of preadipocyte pool size

To determine the total number of preadipocytes contained in each fat pad, known amounts of each pad, from rats both 5 and 7 months old, were thoroughly digested with collagenase and isolated by filtration as described above. Cells were enumerated using a Coulter counter and the total number in each pad was calculated.

Data analysis

Unpaired data were analyzed by one-way ANOVA and where significant intergroup differences were indicated, the data were further analyzed by Tukey's method for multiple comparisons. Intake-matched groups were compared by Student's *t*-test for paired data. The values are expressed as means ± SEM and all n values denote the number of rats compared.

RESULTS

Body weight gain and growth of abdominal fat tissues

Despite a lower total body weight ($P < 0.001$) (**Fig. 1**), the retroperitoneal fat pads of saturated fat-fed rats (HFS-R) were significantly larger than those of rats fed

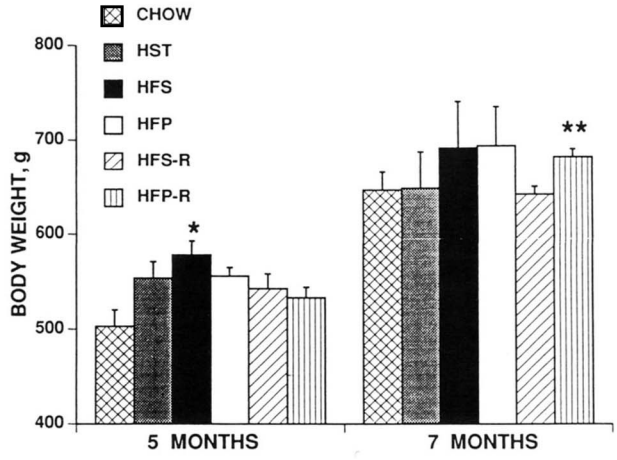

Fig. 1. Total body weights of 5- and 7-month-old male Sprague-Dawley rats fed one of the following diets (described in detail under Methods) for 15 or 26 weeks, respectively: standard rat chow (CHOW; n = 6); high starch (HST; n = 8); high saturated (HFS; n = 8) or polyunsaturated (HFP; n = 7) fats. These diets were fed ad libitum. Two additional diet groups in which intake of the high fat diets was pair-matched to the intake of the rats of the HST group are also shown: high saturated fat-restricted (HFS-R; n = 7); high polyunsaturated fat-restricted (HFP-R; n = 8); n is the number of rats in each group fed the diets for 5 months. The number of rats fed the diets for 7 months was: 4 in each of the CHOW, HST, HFS-R, and HFP-R groups and 3 in each of the HFS and HFP groups. Values shown are means ± SEM and are significantly different from CHOW when denoted by * ($P < 0.05$) and from HFS-R when denoted by ** ($P < 0.001$).

equicaloric amounts of polyunsaturated fat (HFP-R) diets at 7 months ($P < 0.05$) (**Fig. 2**). In addition, the retroperitoneal fat pads of the HFS-R group were larger than those of the pair-matched HST rats at 5 months (HST 18.9 ± 1.7 g, HFS-R 23.5 ± 2.3 g, $P < 0.05$).

Rats fed the saturated fat diet ad libitum were significantly heavier at 5 months than rats fed the CHOW diet (Fig. 1). Although this difference had disappeared at 7 months (Fig. 1), the epididymal, retroperitoneal, and inguinal fat pads of the saturated fat-fed rats were significantly larger than those of the chow-fed group (Fig. 2). Furthermore, the retroperitoneal pads of rats fed both high fat diets ad libitum were significantly larger than those from rats fed the starch diet. There was no difference in total caloric intake between the ad libitum-fed groups during the 15 weeks on the diets (HST 7845 ± 216; HFS 8321 ± 319; and HFP 7736 ± 266 kcal).

Plasma insulin

Plasma insulin concentrations of 7-month-old rats were (mU/l): CHOW 85 ± 31 (n = 3); HST 84 ± 20 (n = 3); HFS 65 ± 7 (n = 6); HFP 60 ± 13 (n = 7).

Enzymes

LPL, HSL, and GPDH activities were not markedly affected by the source of dietary energy (**Fig. 3**).

Growth characteristics of fat pads

The expansion of epididymal pads in the fat-fed rats relative to the chow-fed rats developed principally due to an increase in mean adipocyte size (**Fig. 4** and **Fig. 5**). In contrast, enlargement of the retroperitoneal fat pads was mainly due to an increase in fat cell number, which was particularly pronounced in the saturated fat-fed rats. Growth of the inguinal fat pads resulted from an increase in both adipocyte size and number in the fat-fed rats. These observations were consistent with changes in total protein, DNA, and lipid content of the three fat pads (**Table 1**).

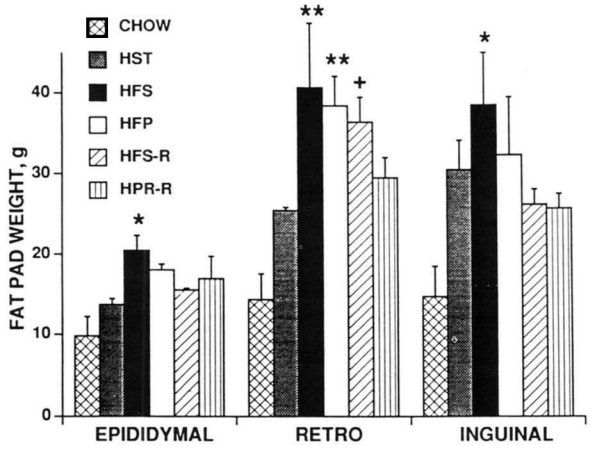

Fig. 2. Fat pad weights (mean ± SEM) of 7-month-old Sprague-Dawley rats. The procedure and diets were the same as in Fig. 1. The fat pads examined were the epididymal, retroperitoneal (RETRO), and inguinal. The number of rats in each group was: 4 in each of the CHOW, HST, HFS-R, and HFP-R groups and 3 in each of the HFS and HFP groups. *Values are significantly different: from CHOW; ** from CHOW and HST; • from HFP-R ($P < 0.05$).

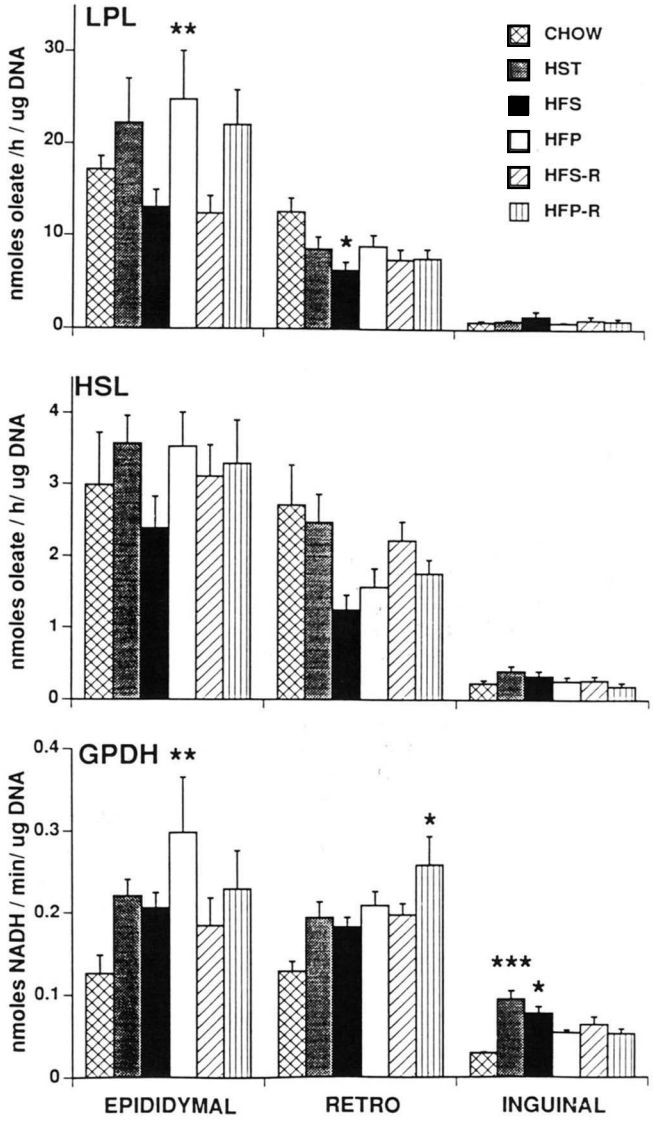

Fig. 3. Lipoprotein lipase (LPL), hormone sensitive lipase (HSL), and glycerophosphate dehydrogenase (GPDH) specific activity (mean ± SEM) in the epididymal, retroperitoneal (RETRO), and inguinal fat pads of 5-month-old rats. The diets were the same as in Fig. 1. The number of rats in each group was: CHOW n = 6, HST n = 8, HFS n = 8, HFP n = 7, HFS-R n = 7, HFP-R n = 8. *Values are significantly different from CHOW; **from HFS and HFS-R; *** from CHOW, HFP, and HFP-R ($P < 0.05$).

Preadipocyte differentiation

No regional differences in differentiation had been observed in our earlier study (10). Hence, the data from the three regional fat pads in the present study are shown

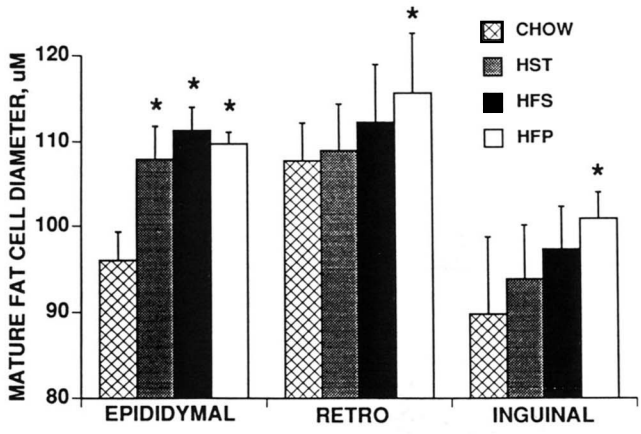

Fig. 4. The mean (± SEM) diameter (μm) of adipocytes isolated from epididymal, retroperitoneal (RETRO), and inguinal fat pads of 5-month-old rats. Diets were the same as in Fig. 1. The number of rats in each group was: CHOW n = 4, HST n = 8, HFS n = 6, HFP n = 7. *Values are significantly different from CHOW.

combined in **Fig. 6.** Preadipocytes, isolated from all three fat tissue regions of the diet-fed rats, that were cultured in the presence of mature fat tissue from the same source (+FAT) showed a 30- to 40-fold increase in the accumulation of lipid droplets compared to cells that were cultured in medium alone (SPONT; $P < 0.001$). An even greater accretion of lipid droplets was seen in response to differentiation mix (+MIX). However, there was no difference among diet groups in response to either treatment (Fig. 6). There was also no significant difference in response to mature fat tissue from diet-fed rats between preadipocytes derived from the same diet-fed rats (diet) and those from a pool of preadipocytes isolated from age-matched, chow-fed Sprague-Dawley rats (test): epididymal: HST 40 ± 16 and 37 ± 4%; HFS 49 ± 15 and 46 ± 13%; HFP 41 ± 12 and 66 ± 16%; retroperitoneal: HST 42 ± 11 and 55 ± 20%; HFS 61 ± 17 and 66 ± 18%; HFP 59 ± 8 and 59 ± 10%; inguinal: HST 48 ± 5 and 43 ± 12%; HFS 56 ± 12 and 63 ± 17%; HFP 46 ± 3 and 53 ± 11% for diet and test, respectively (n = 3–6).

Assay of preadipocyte replication

The population doubling times in primary culture of epididymal and retroperitoneal preadipocytes isolated from rats fed the high saturated fat diet were significantly decreased compared to those of preadipocytes derived from polyunsaturated fat-fed rats ($P < 0.05$) (**Fig. 7,** panel A). A similar trend was seen in cells from the inguinal pad. This difference in the rate of replication was not observed when the preadipocytes were tested in first subculture (data not shown).

The presence of mature fat tissue did not affect the rate of replication of preadipocytes derived from rats of any of the diet groups compared to that of cells grown alone over the 6-day period tested (inset in Fig. 7).

Preadipocyte pool size

At both 5 and 7 months, the size of the preadipocyte pool in fat pads of rats fed palatable diets was not significantly different from that of chow-fed rats despite the fact that the fat pads of the former were 2- to 4-fold larger. To further examine this surprising finding, the relationship between the weight of a fat pad and the number of preadipocytes contained by that pad was investigated. To do this, preadipocytes from an additional group of male, chow-fed Sprague-Dawley rats (body weight range: 452–766 g) were isolated and counted. These data, together with values for the 5- and 7-month-old chow-fed rats, were plotted as scatter plots of preadipocyte number versus fat pad weight (**Fig. 8**). Correlation between preadipocyte number and fat pad weight was highly significant for each of the three regional fat pads examined. At 5 months, the retroperitoneal and inguinal fat pads from rats of all diet groups contained a significantly reduced pool of preadipocytes relative to predicted values (Fig. 8). At 7 months, the pool size in these pads remained significantly reduced compared to predicted values in both the fat-fed groups. In contrast, the epididymal fat pads of the diet-fed rats mainly conformed to the expected relationship.

DISCUSSION

In order to identify factors that initiate or promote the formation of new adipocytes, we have examined the effect of dietary factors on preadipocyte replication and differentiation during the development of diet-induced obesity. We have found that the rate of replication of preadipocytes derived from rats fed saturated fats was accelerated in primary culture compared to those from other diet

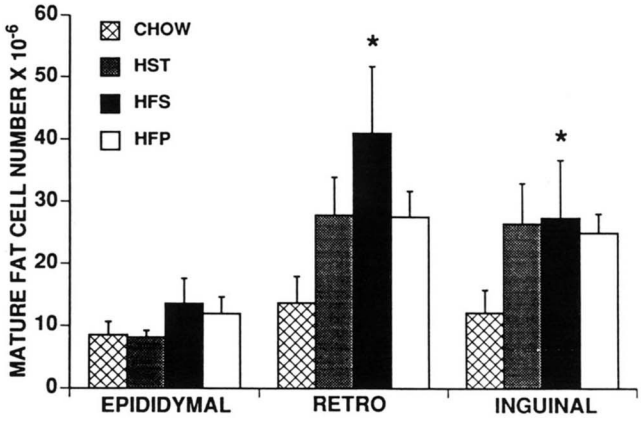

Fig. 5. The mean (± SEM) number of adipocytes in epididymal, retroperitoneal (RETRO), and inguinal fat pads of 5-month-old rats. Diets were the same as in Fig. 1. CHOW n = 4, HST n = 7, HFS n = 7, HFP n = 7. *Values significantly different from CHOW ($P < 0.05$).

TABLE 1. Total protein, DNA, and triglyceride (TG) content of fat pads of 5-month-old, diet-fed rats

Fat Pad	Diet					
	Chow	HST	HFS	HFP	HFS-R	HFP-R
Epididymal						
Protein, mg	28 ± 1^{a}	48 ± 4^{a}	71 ± 6^{b}	44 ± 4^{a}	55 ± 8^{a,b}	40 ± 5^{a}
DNA, μg	175 ± 15^{a}	263 ± 21^{a}	417 ± 41^{b}	242 ± 30^{a}	295 ± 32^{a,b}	265 ± 34^{a}
TG, g	1.7 ± 0.6^{a}	5.6 ± 0.7^{b}	11.8 ± 0.5^{c}	7.8 ± 0.7^{b}	7.9 ± 1.4^{b}	8.5 ± 1.2^{b,c}
Retroperitoneal						
Protein, mg	69 ± 9^{a}	115 ± 17^{a,b}	154 ± 20^{b}	128 ± 14^{a,b}	140 ± 15^{a,b}	129 ± 16^{a,b}
DNA, μg	443 ± 83^{a}	677 ± 102^{a,b}	947 ± 84^{b}	799 ± 93^{a,b}	786 ± 67^{a,b}	744 ± 75^{a,b}
TG, g	5.5 ± 0.8^{a}	14.9 ± 1.3^{b}	23.8 ± 2.2^{c}	18.3 ± 0.9^{b,c}	20.1 ± 2.0^{b,c}	19.0 ± 2.0^{b,c}
Inguinal						
Protein, mg	150 ± 13	161 ± 24	240 ± 59	208 ± 25	218 ± 28	233 ± 15
DNA, μg	1285 ± 120	1486 ± 234	1799 ± 333	1951 ± 198	1929 ± 180	2332 ± 119^{a}
TG, g	2.9 ± 0.3^{a}	11.3 ± 1.4^{b}	12.3 ± 1.6^{b}	11.0 ± 1.1^{b}	10.7 ± 0.6^{b}	13.0 ± 1.9^{b}

Values that have different superscripts within a line are significantly different, $P < 0.05$.

groups (Fig. 7). Commensurate with this, the three regional fat pads of the saturated fat-fed rats showed the greatest gain in mass (Fig. 2), and in the retroperitoneal and inguinal pads, significant increases in the number of adipocytes (Fig. 5).

The present findings confirmed earlier studies demonstrating that dietary fats induce adipose tissue growth independent of body weight gain (11, 12) and caloric intake (13, 14). In addition, we have demonstrated that dietary saturated fats were more effective than polyunsaturated fats in causing retroperitoneal pad growth when caloric intake was equal (Fig. 2). The differences in fat accumulation between the two fat diets could not be explained by changes in lipogenic or lipolytic enzyme activities (Fig. 3). HSL activity, and hence lipolysis, has been shown to be reduced in saturated compared to polyunsaturated fat-fed rats (25). While HSL activity tended to be reduced in the saturated compared to the polyunsaturated fat-fed rats in the present study, LPL activity also tended to be lower.

The excess growth in the retroperitoneal fat pads of saturated compared to high starch- or polyunsaturated fat-fed rats was mainly due to the surplus formation of approximately 13 × 10^{6} new fat cells (Fig. 5). As formation

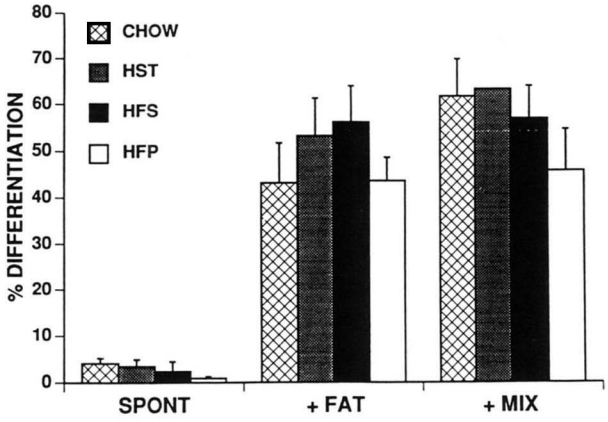

Fig. 6. Induction of preadipocyte differentiation by mature fat. The number of cells containing visible lipid droplets by Oil-Red-O staining is shown as a percentage of the total cells counted (mean ± SEM; n values for SPONT, +FAT, and +MIX, respectively, were: CHOW n = 16, 16, and 8; HST n = 5, 5, and 1; HFS n = 12, 11, and 13; HFP n = 16, 16, and 10). Preadipocytes derived from fat pads of 7-month-old, diet-fed rats were grown in medium alone (SPONT) or with the addition of finely chopped mature fat tissue (+FAT) or with differentiation mix (+MIX). The conditions are described in Methods and the diets were the same as in Fig. 1. The data from the three regional fat pads were combined.

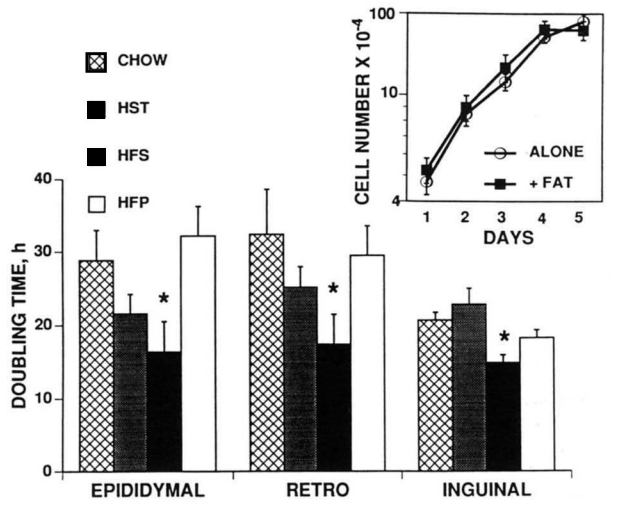

Fig. 7. The time (h) taken for the number of preadipocytes to double in primary culture (mean ± SEM). Preadipocytes were isolated from the epididymal, retroperitoneal (RETRO), and inguinal fat pads of rats fed chow (CHOW; n = 4); high starch (HST; n = 5 or 6); high saturated (HFS; n = 6) or polyunsaturated (HFP; n = 4) fats until 7 months of age. The method and conditions are described in the text. *Values significantly different were: epididymal, HFS vs. HFP; retroperitoneal, HFS vs. CHOW and HFP; inguinal, HFS vs. HST ($P < 0.05$). Inset: The rate of retroperitoneal preadipocyte replication in primary culture in the presence (+FAT) or absence (ALONE) of finely chopped mature fat tissue.

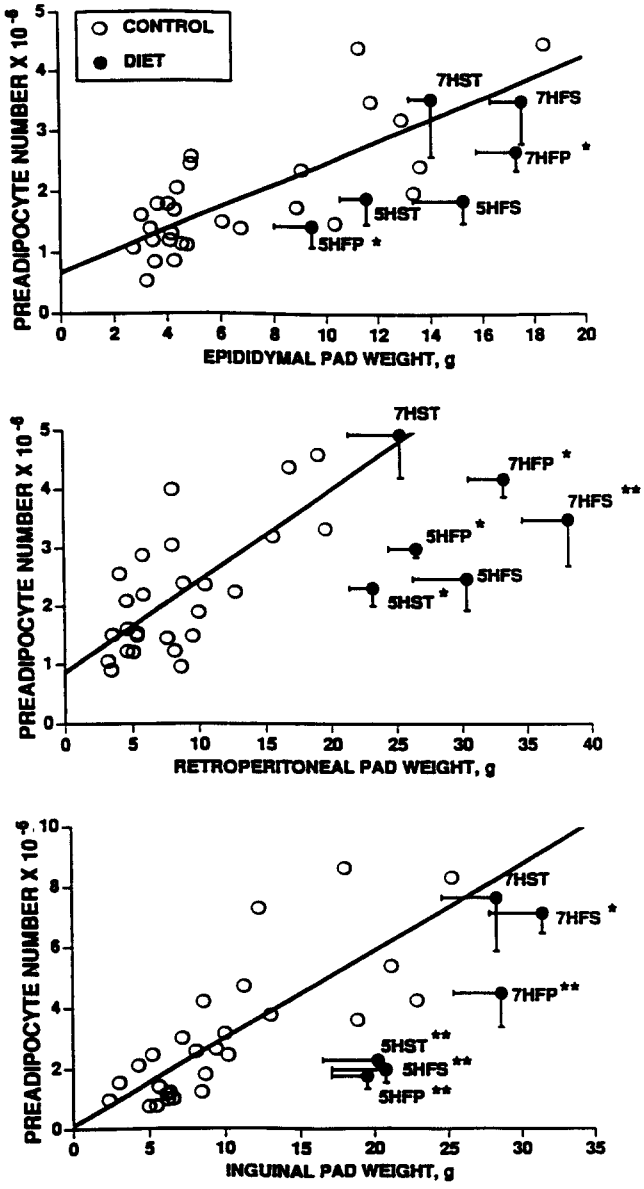

Fig. 8. Diet-induced deviation from the predicted ratio of total preadipocytes:fat pad weight. Scatter plots with estimated regression lines of preadipocyte number vs. fat pad weight were plotted for (A) epididymal, (B) retroperitoneal, and (C) inguinal fat pads of 26 chow-fed, male Sprague-Dawley rats (including the CHOW-fed experimental group of the present study and control rats from other studies). Correlation coefficients were: (A) $r = 0.76$ ($P < 0.001$); (B) $r = 0.71$ ($P < 0.0001$); (C) $r = 0.79$ ($P < 0.0001$). Mean (± SEM) values for the diet-fed rats at 5 (5HST, 5HFS, and 5HFP; diets were the same as in Fig. 7) and 7 (7HST, 7HFS, and 7HFP) months are shown on the graphs. Experimental values significantly different by paired *t*-test from values predicted by the regression line, $^{*}P < 0.05$; $^{**}P < 0.001$.

of new fat cells is a result of the replication and differentiation of preadipocytes, we expected to find that both processes would be increased in the saturated fat-fed rats. The doubling time of preadipocytes derived from saturated fat-fed rats was reduced compared to those of other diet groups (Fig. 6, panel A) indicating that preadipocytes from the saturated fat-fed rats had an accelerated rate of growth in primary culture. This increase in mitogenic activity may have been a direct effect of dietary saturated fat on the preadipocytes. However, none of the obesity-inducing diets significantly changed the rate of differentiation compared to the chow diet, although the retroperitoneal pads of the saturated fat-fed rats contained 3-fold more mature fat cells than those from the chow-fed rats. It can be argued that a constant rate of differentiation of preadipocytes from a pool enlarged by an increased rate of replication, as in the saturated fat-fed rats, would result in a greater number of mature fat cells. It should be stressed that these in vitro data represent a potential for increased replication and do not necessarily represent the in vivo rates of replication or differentiation.

Apart from the specific effects of dietary fats, it is evident that all caloric overloading leads to a perturbation of adipose tissue growth. A balance between cell proliferation and differentiation is a fundamental property of normal tissues. This relationship was clearly demonstrated by the correlation between preadipocyte number and fat pad weight for all three fat pads examined (Fig. 8). But an increased rate of preadipocyte replication with no apparent change in the rate of differentiation in vitro suggested the possibility that this balance may be perturbed in the saturated fat-fed rats. Indeed, we found that this relationship did not hold in the retroperitoneal and inguinal pads of all the diet-fed rats (Fig. 8). These observations suggested that a constant ratio between cells capable of replicating and those terminally differentiated was not maintained during adipose tissue expansion. On the other hand, the fact that the epididymal fat pads of the diet-fed rats conformed to the predicted relationship is consistent with our observation that growth in this pad principally resulted from an increase in adipocyte size rather than by a gain in adipocyte number.

Since we have used fat pad weight rather than the total number of mature fat cells to illustrate the replicating cell:terminally differentiated cell ratio (because no adipocyte count was made in 7-month-old rats), an increase in adipocyte size will result in ratios less than predicted. Indeed, the experimental values for the epididymal pads of the diet-fed rats tend to be below the regression line. However, as the differences in cell size in the retroperitoneal and inguinal pads between chow and diet-fed rats were less pronounced than in the epididymal pads (Fig. 4), increased cell size only explains a small part of the shift in the ratio. Moreover, in the 7-month-old rats when cell size was unlikely to have decreased, the ratios tended towards the predicted values (Fig. 8). Thus, the downward shift in the ratios must, primarily, be due to a reduced preadipocyte pool relative to the total number of mature fat cells. The 2- and 3-fold increases observed in retroperitoneal and inguinal preadipocytes, respectively, between the 5- and 7-month-old rats suggest that replication

of preadipocytes in vivo may not be a continual process. An alternative explanation might be due to the fact that we only studied the rate of differentiation of preadipocytes derived from the 7-month-old rats. In these rats, the preadipocyte pool had expanded, which is consonant with a slow rate of differentiation and a fast rate of replication. If we had investigated these processes in cells from 5-month-old rats, we might have observed an elevated rate of differentiation. However, it is evident from the time-related changes in the ratio of preadipocyte number:fat pad weight in rats fed obesity-inducing diets that replication and differentiation were not tightly coupled during the expansion of adipose tissue mass.

Diet-induced changes on the release of putative paracrine factors by mature fat cells were not apparent in vitro. Mature fat cells derived from rats of all diet groups stimulated adipose conversion of preadipocytes derived from diet-fed or test rats (see Results) to the same extent (Fig. 6), implying that the diets did not amplify the capacity of preadipocytes to differentiate in culture. Nor did they influence the release of the differentiation-inducing factor from adipocytes. Similarly, preadipocyte growth rate was unaffected by the presence of finely chopped fat tissue derived from the rats fed the obesity-inducing diets, suggesting that these diets did not act indirectly by stimulating the release of mitogenic factors within adipose tissue (Fig. 7, inset). Nevertheless, the differences between diet groups in the number of mature adipocytes gained imply that diet played a role in the formation of new cells, in addition to the acceleration of preadipocyte proliferation seen in the saturated fat-fed rats.

Our cell culture system did not assess the part played by the quantity of substrate delivered to adipose tissue in the formation of new adipocytes. We have shown earlier that a saturated fat diet was less effective than a polyunsaturated fat diet in the suppression of hepatic de novo lipogenesis (15). This may result in delivery of more substrate to adipose tissue in saturated than in polyunsaturated fat-fed rats. Indeed, both the epididymal and retroperitoneal pads of the saturated fat-fed rats contained a significantly greater amount of triglycerides (Table 1). The question is, how does substrate supply influence new fat cell formation? It could be hypothesized that differentiation and/or growth factors are released from mature fat cells when a critical size is reached.

In this study, we have endeavored to elucidate both direct and indirect effects of dietary components on the formation of new adipocytes during the expansion of adipose tissue mass. While we have demonstrated that dietary saturated fats may act directly on preadipocytes to accelerate replication, an indirect action on growth by promoting the release of the putative paracrine differentiation factor from adipocytes or by increasing preadipocyte responsiveness to this factor was not apparent in vitro. Other factors must be active within adipose tissue to account for the regional and diet-induced differences observed in vivo. It is possible that the cells in culture are liberated from inhibitory influences that operate in vivo.

In conclusion, we have demonstrated that diets that promote adipose tissue growth disturb the balance between the number of cells capable of proliferating and those terminally differentiated. Also, we have shown that this occurs in a region-dependent manner. Adipose tissue mass expanded to a greater extent in response to dietary saturated than polyunsaturated fats and this may, at least in part, be achieved by the acceleration of preadipocyte proliferation.

This work was supported by grants from the Medical Research Council of Canada (MA-9178) and the Canadian Diabetes Association. Dr. D. C. W. Lau was a Scholar of the Alberta Foundation for Medical Research and the Medical Research Council of Canada during the tenure of this study.

Manuscript received 25 March 1993 and in revised form 9 September 1993.

REFERENCES

1. Hirsch, J., and B. Batchelor. 1976. Adipose tissue cellularity in human obesity. *Clin. Endocrinol. Metab.* **5:** 299–311.
2. Faust, I. M., P. R. Johnson, J. S. Stern, and J. Hirsch. 1978. Diet-induced adipocyte number increase in adult rats: a new model of obesity. *Am. J. Physiol.* **235:** E279–E286.
3. Faust, I. M., W. H. Miller, Jr., A. Sclafani, P. F. Aravich, J. Triscari, and A. C. Sullivan. 1984. Diet-dependent hyperplastic growth of adipose tissue in hypothalamic obese rats. *Am. J. Physiol.* **247:** R1038–R1046.
4. Roncari, D. A. K. 1984. Pre-adipose cell replication and differentiation. *TIBS.* **9:** 486–489.
5. Roncari, D. A. K., D. C. W. Lau, and S. Kindler. 1981. Exaggerated replication in culture of adipocyte precursors from massively obese persons. *Metabolism.* **30:** 425–427.
6. Shillabeer, G., J. M. Forden, J. C. Russell, and D. C. W. Lau. 1990. Paradoxically slow preadipocyte replication and differentiation in obese JCR:LA-corpulent rats. *Am. J. Physiol.* **258:** E368–E376.
7. Djian, P., D. A. K. Roncari, and C. H. Hollenberg. 1983. Influence of anatomical site and age on the replication and differentiation of rat adipocyte precursors in culture. *J. Clin. Invest.* **72:** 1200–1208.
8. Lau, D. C. W., K. L. Wong, and S. C. Tough. 1987. Regional differences in the replication rate of cultured rat microvascular endothelium from retroperitoneal and epididymal fat pads. *Metabolism.* **36:** 631–636.
9. Lau, D. C. W., C. H. Hollenberg, and D. A. K. Roncari. 1987. Release of mitogenic factors by cultured preadipocytes from massively obese subjects. *J. Clin. Invest.* **79:** 632–636.
10. Shillabeer, G., J. M. Forden, and D. C. W. Lau. 1989. Induction of preadipocyte differentiation by mature fat cells in the rat. *J. Clin. Invest.* **84:** 381–387.
11. Lemmonier, D. 1972. Effect of age, sex and site on the cellularity of the adipose tissue in mice and rats rendered obese by a high-fat diet. *J. Clin. Invest.* **51:** 2907–2915.

12. Herberg, L., W. Döppen, E. Major, and F. A. Gries. 1974. Dietary-induced hypertropic-hyperplastic obesity in mice. *J. Lipid Res.* **15:** 580–585.
13. Kanarek, R. B., and N. Orthen-Gambill. 1982. Differential effects of sucrose, fructose and glucose on carbohydrate-induced obesity in rats. *J. Nutr.* **112:** 1546–1554.
14. Oscai, L. B., M. M. Brown, and W. C. Miller. 1984. Effect of dietary fat on food intake, growth and body composition in rats. *Growth.* **48:** 415–424.
15. Shillabeer, G., J. Hornford, J. M. Forden, N. C. W. Wong, and D. C. W. Lau. 1990. Hepatic and adipose tissue lipogenic enzyme mRNA levels are suppressed by high fat diets in the rat. *J. Lipid Res.* **31:** 623–631.
16. Morgan, C. R., and A. Lazarov. 1963. Immunoassay of insulin: two antibody system. *Diabetes.* **12:** 115–126.
17. Kozak, L. P., and J. T. Jensen. 1974. Genetic and developmental control of multiple forms of L-glycerol-3-phosphate dehydrogenase. *J. Biol. Chem.* **249:** 7775–7781.
18. Ramirez, I., A. J. Kryski, O. Ben-Zeev, M. C. Schotz, and D. L. Severson. 1985. Characterization of triacylglycerol hydrolase activities in isolated myocardial cells from rat heart. *Biochem. J.* **232:** 229–236.
19. Khoo, J. C., and D. Steinberg. 1974. Reversible protein kinase activation of hormone-sensitive lipase from chicken adipose tissue. *J. Lipid Res.* **15:** 602–610.
20. Labarca, C., and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102:** 344–352.
21. Spector, T. 1978. Refinement of the Coomassie Blue method of protein quantitation. *Anal. Biochem.* **86:** 142–146.
22. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226:** 497–509.
23. Hirsch, J., and E. Gallian. 1968. Methods for the determination of adipose cell size in man and animals. *J. Lipid Res.* **9:** 110–119.
24. Wiederer, O., and G. Löffler. 1987. Hormonal regulation of the differentiation of rat adipocyte precursor cells in primary culture. *J. Lipid Res.* **28:** 649–658.
25. Awad, A. B., and J. P. Chattopadhyay. 1986. Effect of saturated fatty acids on hormone-sensitive lipolysis in rat adipocytes. *J. Nutr.* **116:** 1088–1094.