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Adipose Cell Hyperplasia and Enhanced Glucose Disposal in Transgenic Mice Overexpressing GLUT4 Selectively in Adipose Tissue*

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To gain insight into the molecular pathogenesis of obesity and specifically the role of nutrient partitioning in the development of obesity, we overexpressed the insulin-responsive glucose transporter (GLUT4) in transgenic mice under the control of the fat-specific aP2 fatty acid-binding protein promoter/enhancer. Two lines of transgenic mice were generated, which overexpressed GLUT4 6–9-fold in white fat and 3–5-fold in brown fat with no overexpression in other tissues. *In vivo* glucose tolerance was enhanced in transgenic mice. In isolated epididymal, parametrial, and subcutaneous adipose cells from transgenic mice, basal glucose transport was 20–34-fold greater than in nontransgenic littermates. Insulin-stimulated glucose transport was 2–4-fold greater in cells from transgenic mice. Total body lipid was increased 2–3-fold in transgenic mice overexpressing GLUT4 in fat. Surprisingly, fat cell size was unaltered and fat cell number was increased >2-fold. This is the first animal model in which increased fat mass results solely from adipocyte hyperplasia and it will be a valuable model for understanding the mechanisms responsible for fat cell replication and/or differentiation *in vivo*.

Obesity results from an imbalance between energy intake and energy expenditure that leads to excess storage of calories as triglyceride. All forms of obesity studied are associated with adipose cell hypertrophy, and more severe forms also show adipose cell hyperplasia (1, 2). Little is known about the mechanisms for this hyperplasia or for the regulation of fat cell number in general. A major hypothesis has been that alterations in nutrient partitioning can cause obesity; however, this has not been experimentally proven. The GLUT4 glucose transporter plays a pivotal role in insulin-stimulated glucose transport in fat and muscle (3). Since muscle is the major site of insulin-mediated glucose uptake (4) and adipocytes utilize comparatively little glucose (5, 6), the importance of adipocyte GLUT4 expression in *in vivo* insulin-responsiveness and in "the partitioning of nutrients" to fat as part of the etiology of obesity has not been clear. However, marked alteration of GLUT4 gene expression in adipocytes (3, 7–9) but not in muscle (3, 10, 11) in several models of obesity in humans and rodents suggests that regulation of GLUT4 expression in adipocytes may be important in the development of, or adaptation to, the obese state. We created transgenic mice that overexpress GLUT4 selectively in fat. These mice have enhanced glucose disposal *in vivo* and massively increased glucose transport into isolated adipocytes. Remarkably, these mice develop increased adiposity due to adipocyte hyperplasia. Thus, altering GLUT4 expression in fat without any other manipulation of the pathways for insulin signaling or cellular metabolism enhances whole body net glucose utilization. Furthermore, markedly increasing glucose transport into fat can result in increased adipose mass by promoting the replication of immature adipocytes and/or the differentiation of precursor cells into adipocytes.

MATERIALS AND METHODS

Transgenic Animals—The fat-specific GLUT4 transgene (Fig. 1) was constructed using the fat-specific promoter/enhancer (12) (gift of Dr. B. M. Spiegelman) from the fatty acid-binding protein gene, aP2. This 5.4-kb[1] DNA fragment, which contains a consensus transcription initiation site and has been shown to promote high levels of expression of the reporter gene chloramphenicol acetyltransferase selectively in fat (12), was ligated to a 6.3-kb *Bam*HI - *Pvu*II genomic DNA fragment corresponding to bases 2061–8396 of the human GLUT4 gene (13) (gift of Drs. J. B. Buse and G. I. Bell). The sequence begins 164 bases upstream of the ATG start codon and contains all 11 exons and 10 introns of the human GLUT4 gene and a consensus polyadenylation signal. The construct was injected into the pronucleus of fertilized zygotes from FVB mice and transferred to pseudopregnant females. Southern blotting of genomic DNA extracted from tail clippings was used to identify offspring carrying the transgene. Mice received standard Purina mouse chow *ad libitum*.

Glucose Tolerance Tests—After an overnight fast, D-glucose (1 mg/g body weight) was injected intraperitoneally in awake mice. Blood was sampled form the tail vein before injection (time zero) and 10, 20, 30, 60, 120, and 180 min after glucose injection.

Blood Glucose and Plasma Insulin Determinations—Blood glucose was measured with a One Touch II glucose meter (Lifescan, Inc.). Plasma insulin was measured with a radioimmunoassay kit (Linco Research, Inc., St. Louis, MO) using rat insulin standards.

Western Blotting—Post-nuclear membranes from brown fat, white fat (epididymal), hindlimb skeletal muscle, heart, brain, liver, and kidney were prepared, separated by SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose and immunoblotted with an antiserum specific for the COOH-terminal of GLUT4 (gift of Dr. M. Mueckler), all as previously described (7, 11).

Body Composition—Carcasses were digested by ethanolic KOH hydrolysis. Body lipid content was determined by enzymatic measurement of glycerol (14), and body protein was determined by biuret assay of the digest (14).

Fat Cell Glucose Transport—Adipocytes were isolated from epididymal, parametrial, or subcutaneous fat pads by collagenase (1 mg/ml) digestion (15, 16). Cells were incubated at 37 °C with constant shaking in an 8% suspension by volume, in Krebs-Ringer-Hepes (30 mM) buffer (pH 7.4) with 2.5% bovine serum albumin (fraction V), 200 nM adeno-

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[1] The abbreviation used is: kb, kilobase pair(s).

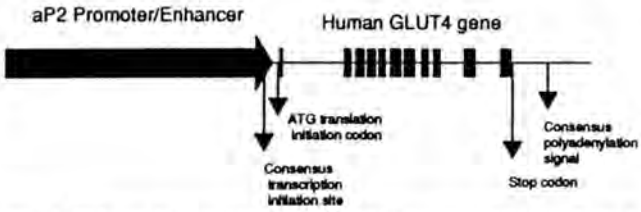

FIG. 1. **Schematic representation of the transgene construct.** 5.4 kb of mouse genomic DNA containing the mouse aP2 fatty acid-binding protein promoter and enhancer elements was fused to 6.3 kb of human genomic DNA containing all 11 exons of the human GLUT4 gene as described under "Materials and Methods."

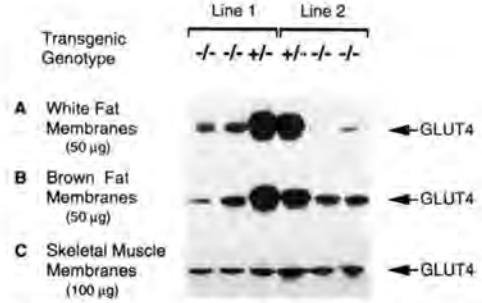

FIG. 2. **Fat-specific overexpression of GLUT4 protein in two independent lines (1 and 2) of transgenic FVB mice.** Fat and muscle membranes from mice heterozygous for the transgene (+/−) or nontransgenic littermates (−/−) were prepared, and SDS-polyacrylamide gel electrophoresis and immunoblotting for GLUT4 were performed as described under "Materials and Methods." Micrograms of protein loaded in each lane are indicated on the figure.

sine, and without (basal) or with (insulin-stimulated) 80 nM crystalline porcine insulin. Following the initial 30-min incubation with or without insulin, [U-^{14}C]glucose (3 μM) was added for 60 min and the reaction was terminated by separating cells from media by spinning the suspension through dinonyl phthalate oil (17).

Fat Cell Size and Number—Isolated adipocytes were fixed with osmic acid and counted in a Coulter counter (18, 19), and cell size (μg of lipid/cell) was calculated as previously described. Briefly, total lipid was extracted from an aliquot of cells by the method of Dole (20) and the total lipid per aliquot was divided by the number of cells in an aliquot of equal volume yielding lipid content per cell (18). Fat cell number per fat depot was calculated by dividing the milligrams of lipid per fat depot by the micrograms of lipid per cell (18).

Statistical Analysis—Statistical analyses were carried by analysis of variance using the Beth Israel Hospital analyzer system. Differences were accepted as significant at $p < 0.05$.

RESULTS AND DISCUSSION

Two independent lines of transgenic mice overexpressing GLUT4 selectively in fat were obtained and were designated lines 1 and 2. GLUT4 was overexpressed 6–9-fold in white fat and 3–5-fold in brown fat in both lines (Fig. 2, *A* and *B*). GLUT4 protein levels were unaltered in hindlimb muscle of transgenic mice compared to the endogenous level of GLUT4 in nontransgenic mice (Fig. 2*C*). Results in heart (not shown) were similar to those in skeletal muscle, and there was no overexpression of GLUT4 in liver, brain, or kidney in transgenic mice (not shown). Since the transgenic human GLUT4 protein can not be distinguished from the endogenous mouse GLUT4 by Western blotting, we distinguished the expression of the transgene from the endogenous gene on Northern blot where the mouse mRNA transcript is 2.7 kb (21) and the human is 3.5 kb (22). Northern blotting revealed high levels of human GLUT4 expression selectively in white and brown fat (not shown).

The effects of adipose-specific GLUT4 overexpression on *in vivo* glucose homeostasis were assessed by measuring ambient glucose and insulin levels and by glucose tolerance tests. Fasting blood glucose was slightly decreased in line 1 male and female transgenic mice compared to nontransgenic littermates (Fig. 3, *A* and *B*) and unchanged from nontransgenic levels in line 2 mice (Fig. 3, *C* and *D*). Table I shows lower fasting blood glucose values in a large number of line 1 mice. Strikingly, in *both* lines of transgenic mice the normal rise in blood glucose was blunted at 20, 30, 60, 120, and 180 min after glucose injection in both males and females, and glucose levels fell below base-line levels at 120 and 180 min (Fig. 3). These results indicate enhanced net glucose disposal in transgenic mice. Fasting insulin levels (Table I) were low in both nontransgenic and transgenic mice and no differences were detected between groups. In contrast, fed insulin levels were significantly lower in transgenic male mice. Fed insulin levels were not determined in female mice.

To assess the effects of overexpression of GLUT4 on glucose transport in fat cells, we measured basal and maximally insulin-stimulated glucose transport into isolated adipose cells from three different fat depots in transgenic mice and nontransgenic littermates (Fig. 4). In both lines of nontransgenic mice, insulin increased glucose transport in epididymal adipose cells 15-fold over basal levels (absence of insulin) (Fig. 4*A*). In epididymal adipocytes from GLUT4-overexpressing mice, basal glucose transport was 20-fold greater than in those from nontransgenic mice and ~1.5-fold greater than maximally stimulated transport in nontransgenic mice. Insulin stimulated glucose transport an additional 2.5-fold in cells from transgenic mice, to a maximal level that was ~4-fold greater than in nontransgenic littermates in both lines 1 and 2. In parametrial fat cells from nontransgenic mice, insulin increased glucose transport 29-fold

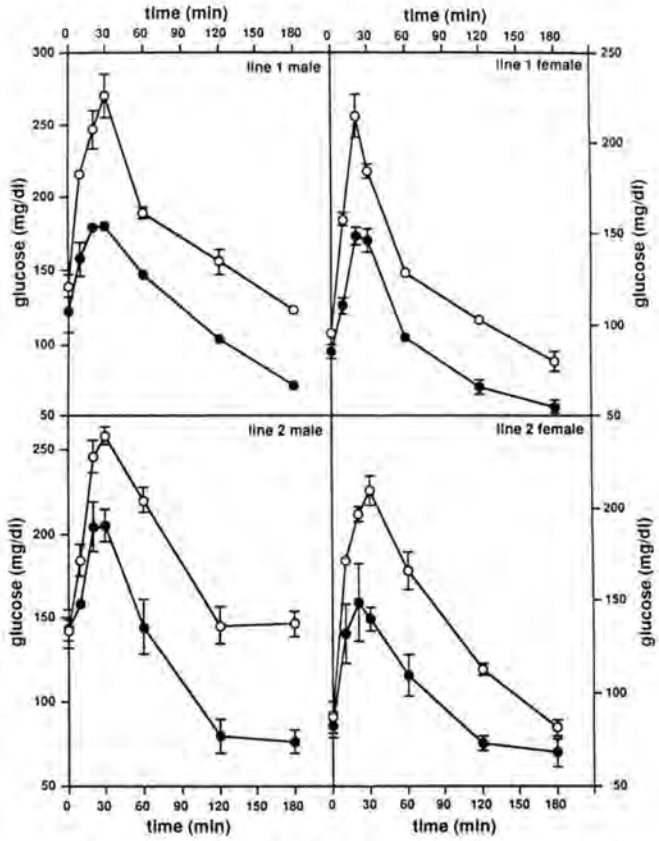

FIG. 3. **Glucose tolerance tests in mice heterozygous for the transgene (●) and in nontransgenic littermates (○).** After an overnight fast, D-glucose (1 mg/g body weight) was injected intraperitoneally in awake mice and blood glucose was determined on samples from the tail vein. Data shown in each panel represent 2–3 mice/group and are representative of results in a total of 17 nontransgenic and 20 transgenic mice, comprising seven sets of littermates ranging in age from 7 to 11 weeks.

TABLE I

Blood glucose and plasma insulin levels in nontransgenic and transgenic littermates

Values are means ± S.E. Number of mice is indicated in parentheses. Fasting values were obtained after an overnight fast in mice that were 8–18 weeks of age. Fed values were obtained between 8:30 and 10:00 a.m. in mice that were 18–25 weeks of age.

	Fasting glucose		Fasting insulin		Fed insulin	
	Nontransgenic	Transgenic	Nontransgenic	Transgenic	Nontransgenic	Transgenic
	mg/dl	*mg/dl*	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*
Male	107 ± 4 (14)	89 ± 6 (14)[a]	0.63 ± 0.31 (9)	0.82 ± 0.11 (7)	7.20 ± 1.19 (6)	4.37 ± 0.89 (6)[a]
Female	102 ± 6 (14)	76 ± 3 (17)[a]	0.21 ± 0.07 (4)	0.49 ± 0.04 (5)		

[a] $p \leq 0.025$.

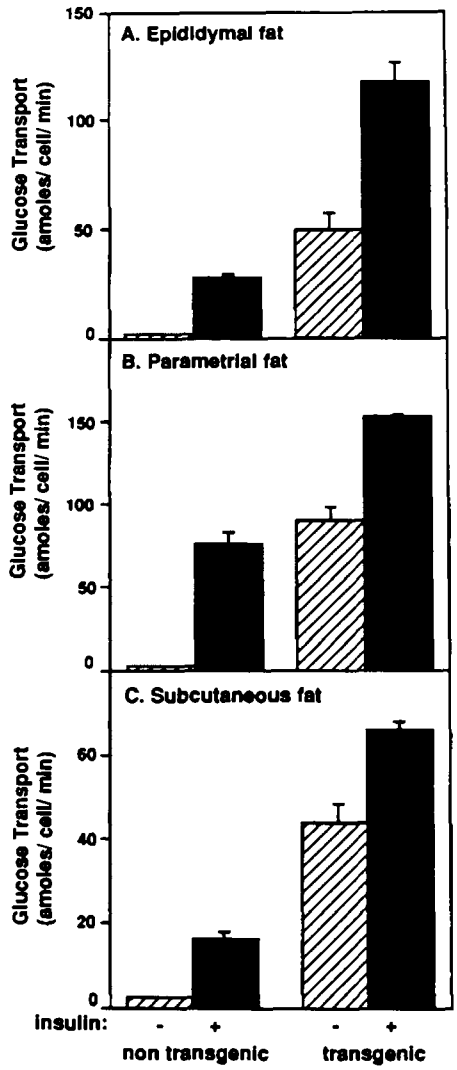

FIG. 4. **Glucose transport in isolated adipocytes from mice heterozygous for the transgene and nontransgenic littermates.** *A*, epididymal adipose cells from 19.5-week-old line 1 male mice. *B*, parametrial adipose cells from 10-week-old line 2 female mice. *C*, subcutaneous adipose cells from 12-week-old line 2 male mice. Each experiment was carried out on pooled cells from 2–3 littermates/group. Results are means ± S.E. and are representative of six separate glucose transport experiments.

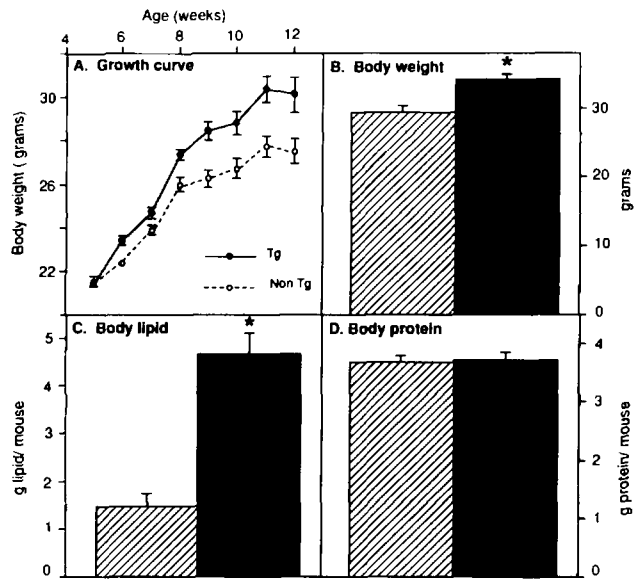

FIG. 5. **Adiposity characteristics in mice heterozygous for the transgene (■) and nontransgenic (▨) littermates.** *A*, representative growth curves in line 1 male mice 5–12 weeks of age. Weights are the the same in transgenic and nontransgenic littermates up to 5 weeks of age. *B*, body weight in male mice 18–21 weeks of age. *C*, total body lipid in male mice 18–21 weeks of age. *D*, total body protein in male mice 18–21 weeks of age. Results shown in *panels B–D* are for combined line 1 and line 2 male mice (n = 6–8 in each group). Similar results were obtained for female mice from both lines 1 and 2, ranging in age from 9 to 26 weeks. Results are mean ± S.E. *, $p < 0.02$.

over basal levels (Fig. 4*B*). In parametrial adipose cells from GLUT4 overexpressing mice, basal glucose transport was increased 34-fold over nontransgenic levels and insulin stimulated glucose transport to a level 2-fold greater than nontransgenic. Subcutaneous adipose cells were somewhat less responsive to insulin; in nontransgenic male mice, insulin stimulated glucose transport 7.4-fold (Fig. 4*C*). The effects of GLUT4 overexpression were also marked in subcutaneous adipose cells from transgenic mice; basal glucose transport was increased 20-fold over nontransgenic levels, and insulin stimulated glucose transport to a level 4-fold greater than in nontrangenic mice.

Thus, in all three fat depots, overexpression of GLUT4 resulted in markedly increased basal glucose transport with substantial increases in insulin-stimulated glucose transport as well. The magnitude of the increase in basal glucose transport (Fig. 4) appears greater than the magnitude of the increase in GLUT4 levels in total adipose tissue membranes (Fig. 2). Normally most of the GLUT4 glucose transporters are sequestered in an intracellular pool in the absence of insulin (3). Future studies will address whether an increased proportion of the overexpressed GLUT4 transporters are in the plasma membranes in cells from transgenic mice. This could account for the increase in basal transport, which is greater than the increase in the number of GLUT4 in total membranes.

To determine whether the massively increased glucose transport into fat in transgenic mice was affecting fat mass and adiposity, we measured body weight and lipid content. A typical growth curve (Fig. 5*A*) shows that transgenic mice weighed the same as nontransgenic littermates up to ~5 weeks of age, when transgenic mice began to gain weight more rapidly. In male transgenic mice, weight at 18–21 weeks was 3–4 g greater than nontransgenic mice (Fig. 5*B*). Body lipid in nontransgenic mice was ~1.5 g/mouse (5% of body weight) in males at 18–21 weeks

of age, while in transgenic littermates body lipid was increased to ~4.7 g/mouse (~14% of body weight) (Fig. 5*C*). Total body protein per mouse was the same in transgenic and nontransgenic mice (Fig. 5*D*), indicating that lean body mass was not altered by overexpression of GLUT4 in fat. A 2.3–4-fold increase in body lipid (grams/mouse) with no change in body protein was seen in both male and female transgenic mice from both lines 1 and 2. Thus, the increase in body weight was totally accounted for by the increase in fat mass. Furthermore, the total mass of fat per mouse exceeded the total mass of protein in transgenic mice (Fig. 5, *C* and *D*).

Importantly, in GLUT4-overexpressing mice, fat cell size was not altered in epididymal fat pads of transgenic mice (Fig. 6*A*). Similarly, in parametrial and subcutaneous fat depots, fat cell size was not significantly altered in transgenic mice compared to nontransgenic littermates (parametrial 0.239 ± 0.014 *versus* 0.243 ± 0.016 μg of lipid/cell; subcutaneous 0.116 ± 0.008 *versus* 0.096 ± 0.003 μg of lipid/cell; mean ± S.E.). The increase in body lipid without a corresponding increase in fat cell size indicates that the increased adiposity is primarily due to increased fat cell number. Calculation of fat cell number in epididymal fat pads revealed a 2-fold increase in transgenic mice at 18–21 weeks of age (Fig. 6*B*).

The high level of expression of GLUT4 in adipocytes of transgenic mice results in glucose transport in the basal state, which exceeds maximally insulin-stimulated transport in adipocytes from nontransgenic mice. Constant flooding of the otherwise normal fat cell with glucose results in no increase in cell size and a doubling of cell number. The fat cell hyperplasia is unexpected and distinctly unusual, inasmuch as the many models of rodent and human obesity that have been studied universally show an increase in fat cell size (1, 2). In the more severe forms of obesity, cell number is also increased (1); however, no obese states have been identified in which cell number is increased without a concomitant increase in cell size. In our transgenic model, fat cell size may not be increased because ambient (fed) insulin levels tend to be lower than in nontransgenic littermates. This would favor increased lipolysis and decreased lipogenesis. Furthermore, at physiological glucose concentrations, transport is not rate-limiting for glucose metabolism (23) and the actual increase in lipogenesis may not be as great as the increase in transport.

Little is known about the mechanisms for regulating adipocyte number in rodents or humans, and these transgenic mice represent a unique model for use in investigating the role of *in vivo* factors in the regulation of adipocyte number. Our results suggest two distinct possibilities. Since the aP2 promoter used in our transgene expresses in a differentiation dependent manner in adipocytes (24), the overexpression of GLUT4 in our transgenic mice is virtually entirely in cells that have embarked on differentiation into adipocytes. Our results indicate that, at the early stage of differentiation when aP2 is first expressed (25), the immature fat cell retains the ability to replicate. Furthermore, the constitutive and massive influx of glucose may promote replication of these immature adipocytes. Alternatively, the adipocyte hyperplasia may be due to a paracrine or systemic effect by which increased glucose metabolism in existing adipocytes promotes differentiation of other precursor cells into adipocytes. In at least one model of obesity that has characteristics similar to human obesity, the Zucker fa/fa rat, GLUT4 levels are increased in adipocytes from young obese rats but decreased in older obese rats compared to lean littermates (7). Our data in transgenic mice now suggest that the initial increase in adipocyte GLUT4 gene expression in some forms of obesity may play a role in the development of obesity and the subsequent decrease in expression may be an adaptation aimed at limiting the progression of obesity.

These observations in these transgenic mice demonstrate that the number of GLUT4 glucose transporters is rate-limiting for glucose transport by adipose tissue and that increasing glucose transport into fat can enhance whole body glucose tolerance. Furthermore, these data directly demonstrated that increased expression of the GLUT4 gene in fat may alter nutrient partitioning so as to increase adipose mass, and that increased glucose transport into fat may set into motion events that foster the replication of immature adipocytes and/or the differentiation of precursor cells into adipocytes.

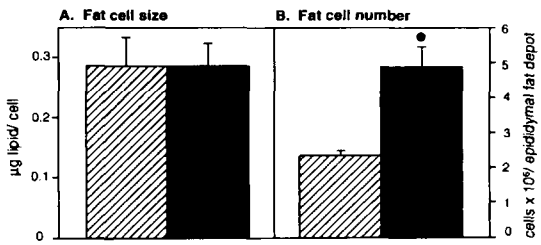

FIG. 6. **Adipocyte size and number for mice heterozygous for the transgene (■) and nontransgenic (▨) littermates.** Adipocyte size and number were calculated as described under "Materials and Methods." Results are means ± S.E. for 6–10 mice/group. *A*, epididymal fat cell size. *B*, number of fat cells per epididymal fat depot. *, $p < 0.005$.

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