The role of small proliferative adipocytes in the development of obesity: Comparison between Otsuka Long-Evans Tokushima Fatty (OLETF) rats and non-obese Long-Evans Tokushima Otsuka (LETO) rats

Takayuki Hanamoto, Kazuo Kajita, Ichiro Mori, Takahide Ikeda, Kei Fujioka, Masahiro Yamauchi, Hideyuki Okada, Taro Usui, Noriko Takahashi, Yoshihiko Kitada, Koichiro Taguchi, Toshiko Kajita, Yoshihiro Uno, Hiroyuki Morita and Tatsuo Ishizuka

Department of General Internal Medicine, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

Abstract. Obesity consists of hypertrophy and hyperplasia of adipocytes. Although the number of adipocytes is influenced by anatomical location, nutritional environment, hormone and genetic variation, it has been thought to be determined by the proliferation of precursor cells and subsequent differentiation. However, our recent research has identified the population of small adipocytes less than 20 µm in diameter, exhibiting tiny or no lipid droplets and expressing adipocyte marker proteins (small proliferative adipocytes: SPA) in isolated adipocytes. Notably, 5-bromo-2’-deoxyuridine (BrdU) incorporation and proliferating cell nuclear antigen (PCNA) expression were detected in these cells. In this study, we investigated the role of SPA in development of adipose tissue using genetically obese diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats and their non-obese and non-diabetic littermates, Long-Evans Tokushima Otsuka (LETO) rats. Proliferation of SPA was determined by measurement of PCNA at the protein level in isolated fractions of adipocytes with collagenase digestion. In general, expression levels of PCNA rose, reached a maximum, and declined in adipose tissues during aging. The expression levels of PCNA were maximum in epididymal fat at 32 w and 12 w of age in LETO and OLETF, respectively. They reached the maximum at 20 w of age both in LETO and OLETF in mesenteric fat. Although the PCNA expression level was higher in OLETF in the early period, it reversed later. Enlargement of adipocytes developed during aging, which was enhanced when the expression levels of PCNA declined. These results suggest that proliferation of SPA may prevent adipocyte hypertrophy and the resultant development of metabolic disorders.

Key words: Adipocyte growth, Obesity, Visceral adipocyte growth

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ferentiation of preadipocytes. However, this concept has not been clearly verified. On the other hand, some studies have raised the possibility that adipocytes may proliferate. Foster and Bartness [10] have shown that adipocytes in vivo incorporate 5-bromo-2′-deoxyuridine (BrdU). Sugihara et al. [11] have provided evidence that “ceiling” cultures of adipocytes exhibit morphologically typical of dividing cells. Our own recent research has revealed that adipocytes in vivo incorporate BrdU. Expression of proliferating cell nuclear antigen (PCNA) is also detected in adipocytes. Moreover, primary cultured adipocytes using ceiling culture showed an increase in cell number and incorporated BrdU. We have found that BrdU is incorporated more actively in small adipocytes (<20 µm in diameter) than large ones [12] in histological examination. However, it was hard to discriminate these cells from non-adipocytes. To confirm our results, a cytological study using adipocytes isolated by collagenase digestion, which facilitates evaluation of separated cells, was performed. We have identified a population of small cells (less than 20 µm in diameter) possessing tiny or no lipid droplets, which express adipocyte markers, such as adiponectin in fractions of isolated adipocytes. Since they actively incorporate BrdU, and express PCNA, we named them small proliferative adipocytes (SPA). In this study, we investigated the role of proliferation of SPA in proliferation of adipose tissue during aging.

Materials and Methods

Animals

Male Long-Evans Tokushima Otsula (LETO) rats and Otsuka Long-Evans Tokushima Fatty (OLETF) rats were fed using CE2 powder ad libitum. Food consumption was determined by subtracting the remaining food from that supplied every 2-3 days, and the averages of these values in one week were expressed as the weekly food consumption. They were housed in a specific pathogen-free facility with a 12-h light/12-h dark cycle. All procedures for animal care were carried out in accordance with protocols approved by the University of Gifu Institutional Animal Care Committee.

The rats were then sacrificed and subcutaneous, epididymal and mesenteric fat were harvested. To obtain isolated adipocytes, collagenase digestion was performed. Adipose tissue was minced to about 0.5 mm diameter followed by treatment with type 1 collagenase (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) in KRB-HEPES buffer (pH 7.4) at 37°C for 30 min. After filtration through a nylon mesh, the filtrate was centrifuged at 8 × g for 1 min, and the floating layer was rinsed 4 times with KRB-HEPES and then used as isolated adipocytes. The remained layer was centrifuged at 210 × g for 3 min. Stromal vascular fraction (SVF) was obtained from the resulting pellet.

Immunocytochemical staining

Isolated adipocytes suspension were solidified using iPGell (Genostaff, Tokyo, Japan) according to the manufacturer’s instructions. Cell blocks were fixed with 10% formalin neutral buffer solution and embedded in paraffin using a standard method. Immunostaining was carried out using anti-adiponectin antibody and anti-BrdU antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) as first antibodies. Enzyme-labeled antibody method was performed using Vectastain ABC kit (Vector Laboratories Inc, Burlingame, CA).

Adipocyte size

Samples of subcutaneous, epididymal and mesenteric fat isolated from LETO and OLETF were fixed with formaldehyde and stained with hematoxylin/eosin. Adipocyte size in adipocyte areas was assessed by manual tracing of the long axis of 500 or more mature adipocytes per rat in each genotype, using a BZ-II analysis application (Keyence, Osaka, Japan).

Western blotting

Cell lysates were mixed with Laemmli sample buffer and boiled for 3 min. Equal amounts of cell lysate were subjected to SDS-PAGE, and transferred onto nitrocellulose paper. The paper was blocked with 5% skim milk tris-buffered saline (TBS), and incubated with anti-peroxisome proliferator-activated receptor γ (PPAR-γ) antibody or anti-actin antibody (Santa Cruz Biotech). Protein bands were visualized with an ECL system.

Statistical analysis

Statistical comparisons were performed with one-factor ANOVA (Dunnett test). All statistical tests were two-tailed. Data are given as mean ± SEM. Values of $P < 0.05$ were considered statistically significant.

Results

Growth of adipose tissue in LETO and OLETF rats

From 10 w of age, OLETF exceeded LETO in body weight, whereas weight gain terminated at 29 w of age.
in OLETF and at 40 w of age in LETO. Food consumption was increased more in OLETF than in LETO during the observation period (Table 1). In general, percentage of fat weight to body weight was greater in OLETF than in LETO. The percentage of subcutaneous and epididymal fat weight increased at 10 w of age (Fig. 1A, B), while the percentage of mesenteric fat weight increased at 14 w of age in both genotypes (Fig. 1C). Interestingly, fasting blood glucose level exceeded 150 mg/dL when the percentage of mesenteric fat expanded to 1.0% in both LETO and OLETF (Fig. 1D). Plasma insulin (IRI) level rose significantly at 16 w, and reached the maximum at 32 w in OLETF, whereas it rose more gradually in LETO (Fig. 1E). Next, we studied the alteration of adipocyte size associated with systemic growth in LETO and OLETF. No difference was observed in major cell diameter in epididymal fat in LETO (E-LETO), mesenteric fat in LETO (M-LETO) or mesenteric fat in OLETF (M-OLETF), while epididymal fat in OLETF (E-OLETF) displayed large cell size at 12 w of age.

Table 1 Changes in body weight and food consumption/day of LETO and OLETF (n = 4-32)

<table>
<thead>
<tr>
<th>wk of age</th>
<th>Body Weight (g)</th>
<th>Food Consumption (g/day)</th>
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<tbody>
<tr>
<td></td>
<td>LETO</td>
<td>OLETF</td>
</tr>
<tr>
<td>7</td>
<td>205 ± 4.4</td>
<td>232 ± 2.4</td>
</tr>
<tr>
<td>10</td>
<td>323 ± 6</td>
<td>385 ± 6</td>
</tr>
<tr>
<td>12</td>
<td>368 ± 4</td>
<td>432 ± 9</td>
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<tr>
<td>16</td>
<td>460 ± 3</td>
<td>556 ± 7</td>
</tr>
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<td>20</td>
<td>502 ± 3</td>
<td>599 ± 10</td>
</tr>
<tr>
<td>32</td>
<td>525 ± 4</td>
<td>680 ± 9</td>
</tr>
<tr>
<td>40</td>
<td>558 ± 4</td>
<td>715 ± 29</td>
</tr>
<tr>
<td>50</td>
<td>560 ± 3</td>
<td>703 ± 35</td>
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Fig. 1 The course of fat weight, levels of fasting plasma glucose and serum insulin (IRI)

Time course of changes in fat weight/body weight, levels of fasting plasma glucose and serum IRI were measured in LETO and OLETF. A: Subcutaneous fat weight/body weight (%), B: Epididymal fat weight/body weight (%), C: Mesenteric fat weight/body weight (%), D: Fasting plasma glucose level, E: Serum insulin level, LETO: dashed line-open circle, OLETF: solid line-closed square, (n = 4). *: p <0.05, LETO vs OLETF
Cell size increased equally in E-LETO, E-OLETF and M-OLETF, whereas it remained unchanged in M-LETO at 20 w of age. Cell sizes of E-LETO and M-LETO increased equally, while those of E-OLETF and M-OLETF increased more markedly at 32 w and 50 w of age (Fig. 2). These results indicate that adipocyte hypertrophy develops earlier in epididymal fat than in mesenteric fat, and that adipocyte hypertrophy is facilitated in obese animals. Therefore, in the presence of excess energy intake, first, the adipocyte number may increase, followed by an increase in adipocyte size.

**Proliferation of Adipocytes**

Our recent research demonstrate that adipocytes, especially small-sized adipocytes, incorporate 5-bromo-2′-deoxyuridine (BrdU), express PCNA protein, and increase cell number in culture medium [12]. As shown in Fig. 3A, PCNA-labeled nuclei were detected in adipocytes (typical large adipocyte: dashed arrow, small adipocyte: solid arrow). To assess proliferation of adipocytes, it is essential to exclude the possibility of mistaking non-adipocytes which co-exist with adipocytes in the tissue for adipocytes. Adipocytes were separated from non-adipocytes (stromal vascular fraction: SVF) as floating cells (isolated fraction of adipocytes) by collagenase digestion. Floating adipocytes observed with phase contrast microscopy exhibited a vast diversity in cell size ranging from 10-300 µm (Fig. 3B). Fig. 3C shows that numerous small-sized adipocytes less than 20 µm in diameter were observed among medium-sized adipocytes. Although the number of these small-sized adipocytes is great, it is difficult to discriminate them from non-adipocytes by cell size. To overcome this difficulty, we observed individual adipocytes using ceiling culture and smeared specimens in a previous study [12]. At present, use of iPell, which quickly gels around floating cells, facilitates morphological and immunocytochemical studies of isolated adipocytes.
Proliferation of adipocytes in OLETF

In addition, PCNA-labeled cells (solid arrow) were detected among these small cells (Fig. 4D). Double immunostaining for adiponectin (purple) and PCNA (brown) detected PCNA-labeled nuclei in only a few medium-sized (20-50 µm in diameter) adipocytes (dashed arrow, Fig. 4E), while numerous small cells were stained for PCNA and adiponectin (solid arrow, Fig. 4E). The 4-7% of PCNA-labeled adipocytes were medium-sized adipocytes in these specimens. Taken together, these small cells, characterized by expression of mature adipocyte marker proteins and lacking large unilocular fat droplets, display proliferative activity.

As shown in Fig. 3E, small mononuclear round cells were detected among large typical adipocytes in isolated adipocytes rinsed once. Unexpectedly, 3 additional rinses did not influence the amount of these small cells (Fig. 3F). It remains to be determined whether these cells are small adipocytes or contamination of non-adipocytes. Although some of these cells showed a tiny lipid droplet-like structure (solid arrow, Fig. 3G), most cells were indistinguishable from cells in SVF (Fig. 3H). Immunocytochemical examination revealed that almost all of these small cells expressed adiponectin (brown) (Fig. 4A), leptin (Fig. 4B), and Glut4 (Fig. 4C). In addition, PCNA-labeled cells (solid arrow) were detected among these small cells (Fig. 4D). Double immunostaining for adiponectin (purple) and PCNA (brown) detected PCNA-labeled nuclei in only a few medium-sized (20-50 µm in diameter) adipocytes (dashed arrow, Fig. 4E), while numerous small cells were stained for PCNA and adiponectin (solid arrow, Fig. 4E). The 4-7% of PCNA-labeled adipocytes were medium-sized adipocytes in these specimens. Taken together, these small cells, characterized by expression of mature adipocyte marker proteins and lacking large unilocular fat droplets, display proliferative activity.
expression of PCNA was observed in SVF cells isolated from epididymal fat and mesenteric fat in LETO at 12 w of age (Fig. 5A).

Expression levels of PCNA increased in parallel with age in the early period, reached a maximum, and declined subsequently in isolated adipocytes from epididymal (Fig. 5B) and mesenteric fat (Fig. 5C) in both species. Expression levels of PCNA were maximal at 12 w of age in epididymal fat isolated from OLETF, and at 32 w of age in LETO. Expression levels of PCNA were more pronounced in mesenteric fat isolated from OLETF than those from LETO until 20 w of age. They reached an equivalent maximum level at 20 w of age followed by a gradual decline, in which PCNA level was higher in LETO at 32 w of age. These

Therefore, they form a distinct population of adipocytes (small proliferative adipocytes: SPA). Although the gene expression profile and biological activity of SPA are unknown, we speculate that SPA may represent the middle stage of differentiation as shown in Fig. 4F.

**Proliferation of adipocytes and non-adipocytes in adipose tissue**

Next, we assessed the role of proliferation of SPA in adipose tissue. The expression levels of PCNA in isolated fractions of adipocytes were regarded as quantified proliferative activity of SPA in adipose tissue. Marked expression of PCNA was detected in isolated fractions of adipocytes from epididymal and mesenteric fat, but not subcutaneous fat. Prominent expression of PCNA was observed in SVF cells isolated from epididymal fat and mesenteric fat in LETO at 12 w of age (Fig. 5A).

Fig. 4  Immunocytochemical evaluation of SPA in isolated adipocytes
A: Solidified isolated fraction of adipocytes immunocytostained for adiponectin (brown). Arrows show typically stained cells (Counter stained with hematoxylin). B: Solidified isolated fraction of adipocytes immunocytostained for leptin (brown). Arrows show typically stained cells (Counter stained with hematoxylin.). C: Solidified isolated fraction of adipocytes immunocytostained for Glut4 (brown). Arrows show typically stained cells (Counter stained with hematoxylin.). D: Solidified isolated fraction of adipocytes immunocytostained for PCNA (brown). An arrow shows typically stained cell (Counter stained with hematoxylin.). E: Double immunocytostaining for adiponectin (purple) and PCNA (brown) in typical mature adipocyte (dashed arrow) and SPA (solid arrow). F: Schematic representation of the concept of SPA. SPA concomitantly expresses adipocyte marker proteins (yellow) and proliferative activity. Scale bars represent 50 μm.
results indicate that the age-dependent increase of the expression levels of PCNA is accelerated in obese animals during early age, while decreasing faster than in control animals after reaching a maximum level. Therefore, it is suggested that the ability to proliferate in adipocytes might be limited.

Combining these results with mean adipocyte major diameter, it is suggested that, although the cell size of adipocytes increased with age, it was accelerated when expression of PCNA started to decline in epididymal (Fig. 6A) and mesenteric fat (Fig. 6B).

To assess proliferative activity of total cells in SVF, the expression levels of PCNA were measured. After increasing until 12 w of age, it decreased gradually in both LETO and OLETF, with the expression levels of PCNA higher in LETO than in OLETF. No difference was observed between epididymal (Fig. 6C) and mesenteric fat (Fig. 6D). We could not estimate the amount of newborn adipocytes via differentiation from preadipocytes. Since SVF consist of numerous cell types including vascular cells, connective tissue cells, immune cells, mesenchymal stem cells and preadipo-
cytes, our result does not demonstrate proliferative activity of preadipocytes. However, considering the fact that the expression levels of PCNA decreased after 12 w of age, it may be difficult to explain the subsequent adipocyte hyperplasia with new cell production in SVF. Taken together, our results support the contention that proliferation of SPA does contribute to cell growth in adipose tissue.

Discussion

Various methods to estimate cell size and number of adipocytes have been developed. Recently, cell size has been measured precisely using sophisticated apparatuses such as Coulter Multisizer, and then cell number has been determined according to a mathematical model [8] or calculation based on the tissue weight, mean adipocyte weight and adipocyte density [13]. However, few studies have clarified the property of growth of subcutaneous and visceral adipose tissue during aging. DiGirolamo et al. reported that distinct differences in growth patterns take place among inguinal, epididymal, mesenteric and retroperitoneal fats, with hypertrophy dominant in mesenteric and epididymal fats, in contrast to hyperplasia in retroperitoneal and inguinal fats [2].

In most of these investigations, the increased number of adipocytes has been regarded as reflecting the mobilization of adipocyte precursor cells and subsequent differentiation into mature adipocytes. However, no significant data concerning the inability of adipocytes to proliferate have been published. Conversely,
the possibility of the proliferation of adipocytes has been proposed by several investigators [10, 11]. A recently developed adipose tissue-organotypic culture system demonstrated proliferation of adipocytes [14]. In a previous study, we have shown the existence of a population of adipocytes (SPA) characterized by small size, having tiny or no lipid droplets, expressing adipocyte markers, and exhibiting evidence of proliferation [12]. More recently, we have found that iP Gel facilitates morphological observation of separated adipocytes including SPA. As shown in Fig. 3C, there are a considerable number of small adipocytes in the fraction of floating cells. On the other hand, numerous small mononuclear cells were observed in the fraction of isolated adipocytes solidified by iP Gel. A major problem, however, is determining whether these cells represent contamination of non-adipocytes, or small adipocytes. Although morphological discrimination between small adipocytes and non-adipocytes as shown in Fig. 3G and H is difficult, immunocytochemical study revealed that they were small adipocytes (Fig. 4A-C). The amount of these small cells was not influenced by repeated rinses as shown in Figures 3E and F, suggesting that these cells may float as is typical of mature adipocytes. We speculate that the large number of small adipocytes observed with phase contrast microscopy as shown in Fig. 3C may be recognized as cells exhibiting no or only a few tiny lipid droplets in these specimens. It is possible that the amount of lipid in these small adipocytes is too low to be detected as lipid droplets. Since both PCNA- and adiponectin-labeled cells were detected (Fig. 4E), they were considered to be SPA. Although their physiological significance in the differentiation process is uncertain, it is reasonable to surmise that SPA represent the middle stage of cell differentiation, and will progress into lipid-laden large adipocytes. The next issue is whether medium-sized (20-50 µm in diameter) or large adipocytes (more than 50 µm in diameter) actually proliferate. The number of medium-sized adipocytes expressing PCNA as shown in Fig. 4E was small (4-7%) out of the total PCNA-labeled adipocytes. Since the amount of lipid content in SPA varies among SPA as shown Fig. 3G, it is possible that medium-sized PCNA-labeled adipocytes may represent the variance of SPA. In addition, our previous study revealed that only small adipocytes showed increased cell numbers in ceiling culture [12], therefore, proliferative ability is considered to be limited to SPA. On the other hand, we have no information as to whether large adipocytes convert to SPA. Our concept of SPA is shown in Fig. 4F.

In the present study, we investigated the role of proliferation of SPA in adipose tissue growth in lean and obese rats. OLETF, a CCK1 receptor deficient rat, is characterized by the phenotype of obesity, hyperphagia, late onset hyperglycemia (after 18 w of age), hyperplastic islets in the early stage, and subsequent islet destruction [15, 16]. Our results that OLETF exhibits hyperphagia and elevated plasma glucose level after 20 w of age are consistent with these data. Although Schroeder et al. reported the development of adipocyte size and adipocyte number in LETO and OLETF, they did not measure them in mesenteric fat [17]. Mesenteric fat volume has been considered to be closely related to various metabolic disorders [18]. Our result that fasting plasma glucose level exceeded 150 mg/dL, when mesenteric fat weight/body weight (%) increased to more than 1%, and that distinct insulin resistance was associated with enlargement of mesenteric adipocytes, may support this notion. Moreover, the result that growth of subcutaneous and epididymal fat preceded growth of mesenteric fat agreed with the concept that subcutaneous fat is sometimes ‘protective’ in avoiding pathogenic visceral fat obesity [6]. Although epididymal fat is regarded as a visceral fat, mesenteric fat is considered to be related more closely to metabolic complications, as mesenteric fat secretes free fatty acids and other substances directly into the portal vein [19]. Therefore, enlargement of epididymal fat may protect against mesenteric fat obesity. In an early study, adipocyte hypertrophy was stated to precede hyperplasia. Although the critical fat cell size hypothesis, the notion that specific fat cell size triggers a subsequent increase in adipocyte number was advocated in the 1980s, a modification was proposed in 2001 to explain conflicting evidence [4]. Lately, since the fact that enlarged adipocytes impair systemic insulin sensitivity has been demonstrated [20], adipocyte hypertrophy has been regarded as a decompensated state, in which hyperplasia is unable to compensate for excess nutrition [21]. Insulin-induced glucose uptake is attenuated in large adipocytes [22], although the mechanism by which adipocyte hypertrophy is implicated in the pathogenesis of systemic insulin resistance is controversial. Recent research has revealed that enlarged adipocytes secrete FFA and cytokines to induce adipose tissue inflammation, leading to insulin resistance and subsequent metabolic disorders [23, 24]. Our results regarding cell size demonstrate that enlargement of adipocyte
size was accelerated in OLETF. Interestingly, hypertrophy of mesenteric adipocytes followed hypertrophy of epididymal adipocytes (Fig. 2, Fig. 6A, B). Taken together, these results also support the idea that hypertrophy of mesenteric adipocytes, which is most harmful, is protected by the enlargement of other fat tissues.

On the other hand, insulin is a potent lipogenic hormone, and is essential in adipogenesis, although the effect of hyperinsulinemia on adipocyte size and number in vivo is not fully understood. Adipocytes in adipose tissue are heterogeneous in size and intrinsic insulin sensitivity, with smaller adipocytes responding to insulin, whereas larger adipocytes are insulin resistant [25]. As the expression level of sterol regulatory element-binding protein 1 is regulated by phosphatidylinositol 3-kinase in adipocytes, lipogenesis is suppressed in the state of insulin resistance [26]. Clinical data suggest that adipogenesis is impaired in insulin resistance [27]. Insulin has been suggested to increase proliferation of adipocytes and preadipocytes in organotypic culture system [14], but this has not been confirmed. Our result that proliferation of adipocytes was facilitated in the early stage, followed by an early decline in obese rat, was considered to be derived from excess energy intake. However, it is possible that the associated hyperinsulinemia might modify the proliferation rate.

In this study, we assessed the time course of proliferation of SPA by measuring the PCNA at the protein level in epididymal and mesenteric adipocytes isolated from LETO and OLETF. As reported previously [12], PCNA was detected more abundantly in visceral fat, including epididymal and mesenteric fat, than in subcutaneous fat. Since the expression level of PCNA was very low in subcutaneous fat, we estimated it in epididymal and mesenteric fat. We found that the expression levels of PCNA were elevated in OLETF, and that it increased in early age. Interestingly, while it typically reached a maximum level followed by gradual decline, the age of each maximum point was different depending on the region of fat tissue and species. It reached a maximum at 12 w and 32 w in epididymal fat isolated from OLETF and LETO, and at 20 w in mesenteric fat isolated from both LETO and OLETF, respectively. The expression levels of PCNA were greater in LETO than in OLETF after 20 w. These results suggested that the proliferative ability of SPA may be limited.

As shown in Fig. 6A, the decline in the expression levels of PCNA and the increase in adipocyte size showed opposite tendencies. This result suggests that impaired proliferation of SPA may lead to adipocyte hypertrophy to compensate for excess nutrition. In other words, proliferation of SPA may be a mechanism to prevent adipocyte hypertrophy. Our previous research demonstrated that pioglitazone treatment increases proliferation of SPA in subcutaneous fat [12]. Therefore, up-regulation of proliferation of SPA might be a new strategy to treat adipocyte dysfunction. To confirm this, further study will be necessary.

Acknowledgment

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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