Hypoxia and autophagic response of obese adult rat adipocytes which differ in nutritional state during childhood

Lailan Safina Nasution,1,2 Ahmad Aulia Jusuf,3 Sri Widia Jusman4,5 and Mohamad Sadikin4,5,*

1Doctoral Programme in Biomedical Sciences, 2Department of Histology, 3Department of Biochemistry and Molecular Biology and 4Center of Hypoxia and Oxidative Stress Studies, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Salemba Raya 4 Jakarta 10430, Indonesia
3Department of Nutrition, Faculty of Medicine, Universitas Muhammadiyah Jakarta, K.H. Ahmad Dahlan Jakarta 15419, Indonesia

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The prevalence of obesity in adults is increasing worldwide, which is problematic since obesity is associated with degenerative diseases. Nowadays Indonesia is facing an interesting phenomenon since there are adults who have been obese since childhood and others who conversely were undernourished while young. The biological differences of these two types of obesities are not well understood. This study aims to analyse the difference in the size and number of visceral adipocytes, HIF-1α, HIF-2α and MAP1LC3A/LC3 in obese adult rat groups that were undernourished at a young age compared to groups who were normal or even already fat from childhood. We analyzed Hif-1α, Hif-2α, Lc3 mRNA by RT-qPCR; HIF-1α, HIF-2α, MAP1LC3A/LC3 protein level by ELISA. The HIF-1α and HIF-2α protein level of visceral adipocytes derived from the group of rat which were undernourished while young increased significantly compared to the group which was overnourished. The visceral adipocytes of the group which was overnourished since childhood showed an increase in Hif-2α mRNA level. The LC3 mRNA of the rat group which were undernourished since young increased significantly compared to rat group which was obese since childhood.

Key Words: high-fat diet, low-caloric diet, adipocyte hypertrophy, HIF-α, LC3

The prevalence of overweight and obesity in adults is still increasing worldwide, including in Indonesia. This is a major concern since obesity is associated with comorbidities such as type 2 diabetes mellitus, cardiovascular diseases and cancer. Nowadays Indonesia is facing an interesting phenomenon, since there are adults who have been obese since childhood and others who conversely were undernourished while young.1(1) The biological differences of adipocytes derived from these two types of obesities are not well understood.

An improvement in socioeconomical status may cause people to shift to a more sedentary lifestyle and consume higher-energy food in greater amounts. This can cause the person to consume more calories than is used, leading the person to store excess calories as fat and eventually be obese. The excess calories are deposited in adipocytes in the form of triglycerides. The accumulation of triglycerides that happens chronically could cause excessive enlargement of adipocytes, which is called hypertrophy. Studies have shown that adipocyte hypertrophy, especially in the visceral fat compartment, may represent a critical marker and a driver of adipose tissue dysfunction and concomitant metabolic disease risk.2(2)

The adipose tissue expansion takes place in two phases. The first phase is hypertrophy wherein the adipocytes increase in size. Secondly, hyperplasia takes place where precursor cells known as preadipocytes are formed. Determination of adipocyte size is essential to determine endocrine functions and to detect the changes in adipose tissue morphology.

It is widely known that the size of fat cells will increase (hypertrophy) along with the obesity development, but the physiological characteristic of fat cells derived from person who were wellnourished or even overnourished at childhood compared to someone who suddenly experience an excess caloric intake in adulthood is not clearly understood. Along with the increase in cells size, the need for the oxygen will increase too. So, it is suggested that the adipocytes from individuals who were undernourished at childhood will undergo more severe hypoxia than individuals who were wellnourished or even overnourished since childhood.

Hypoxia is marked by an imbalance between the need and the supply of oxygen in cells. This could induce some reactions at the level of the cell, tissue or organism in order to reach the homeostatic situation and to minimize the detrimental effects. Metabolic reprogramming and changes in gene expressions are necessary for adaptation to this hypoxic condition. In mammals, the primary transcriptional response to hypoxic stress is mediated by the hypoxia-inducible factors (HIFs).3(3) HIFs revealed important roles as adaptive function in cellular stress response, both physiological and pathological processes. HIFs are heterodimers consisting of an α and β subunit and a stable β subunit. There are three isoforms of HIF-α: HIF-1α, HIF-2α and HIF-3α. HIF-1α is ubiquitously expressed at low, basal levels in all tissues in healthy individuals in normoxia. HIF-1α expression increases with transient, acute hypoxia exposure in most tissues and decreases to basal levels after reaching its maximum expression. HIF-2α and HIF-3α expressions are more tissue specific. HIF-2α expression increases with prolonged, chronic hypoxia exposure. HIF-1 and HIF-2 subtypes were suggested play different roles in cellular adaptation to acute and chronic hypoxia.4(4) There are multiple splice variants of HIF-3α, some of which inhibit HIF-1α and HIF-2α activity in a dominant-negative fashion.5(5)

Adipocyte hypertrophy especially in visceral adipose tissues which found in obesity could induce autophagy.6(6)–10(10) Autophagy is a lysosomal degradation pathway in which organelles and other components of the cell are compartmentalized within a double-membrane vesicle, the autophagosome, and undergoing degradation by lysosomal enzymes and recycled. It is essential for survival, differentiation, development, and cell homeostasis. Autophagy is activated as an adaptive catabolic process in response to different

To whom correspondence should be addressed.
E-mail: msadikin@fk.ui.ac.id, sadikinmohamad@gmail.com

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forms of metabolic stress, including nutrient deprivation, growth factor depletion, and hypoxia. Dysregulation of autophagy could cause various diseases including infections, cancer, neurodegeneration, aging, and heart diseases.\(^{(11,12)}\) Autophagy genes’ expression is upregulated in visceral fat in human obesity, which associated with obesity-related cardiometabolic risk.\(^{(7)}\)

Autophagy is tightly regulated by proteins encoded by autophagy-related genes (ATGs). Among these proteins, ATG8/microtubule associated protein 1 light-chain 3 (MAP1LC3), hereafter referred to as LC3, is essential for the autophagosome biogenesis and maturation. LC3 has been widely used to monitor the number of autophagosomes as well as autophagic activity. In mammalian cells, several homologs of yeast ATG8 such as MAP1LC3, GABARAP, and GABA type A receptor associated protein like (GABARAPL) 1 and 2 have been identified.\(^{(11)}\)

However, the difference of regulations between adipocytes which come from obese adult who were already fat from childhood compared to people who were normal or even undernourished at a young age is not fully understood. As mentioned by Spalding et al.,\(^{(13)}\) the amount of fat cells in an adult individual remains constant, and correlate with adipogenesis that happened during childhood and adolescence beforehand. Undernourished children will not undergo adequate adipogenesis, causing them to have fewer adipocytes as adults; contrasting with obese children who will grow up to have more adipocytes.

Therefore, in this study we want to further investigate the different responses that might occur to these obese adults having different nutritional statuses during childhood, especially the difference in the size and number of cells due to the different of nutritional status at childhood, the hypoxia-inducible factors HIF-1\(\alpha\), HIF-2\(\alpha\) and autophagy marker MAP1LC3/LC3 of visceral adipocytes between those derived from rats who were undernourished in childhood and became obese in adulthood and those derived from rats that were well-nourished or even obese since childhood.

**Materials and Methods**

**Animals.** Sprague-Dawley (SD) rats as the animal model in this study were obtained from the Laboratory Animal Management Unit, Faculty of Veterinary Medicine, Bogor Agricultural University. All procedures in this study were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Bogor Agricultural University (letter number 073/KEH/SKE/X/2017).

**Experimental design.** Thirty-five 4-week-old male SD rats weighing 65–110 g, after two weeks acclimatization, were randomly divided into 8-week and 28-week treatment groups. The 8-week groups consist of groups given a low-caloric diet (LCD8), a high-fat diet (HFD8), and a standard chow diet (SD8) as control. The 28-week groups consist of groups given LCD for 8 weeks followed by HFD for 20 weeks (LCD28), SD for 8 weeks + HFD for 20 weeks (SD28), HFD for 28 weeks (HFD28), and SD for 28 weeks as control. The body weight of each rat was monitored weekly. The animals were individually housed in stainless steel wire cages in an environmentally controlled clean air room with a 12-h light-dark cycle. At the end of the experiment, the rats fasted for 12 h and were terminated by ketamine-xylazin injection intramuscularly. To analyze the difference in the size and number of visceral adipocytes due to the different nutritional treatments at childhood, we terminated the LCD8, SD8 and HFD8 groups and analyzed the visceral adipose tissues through histopathological examination. Afterwards, after feeding the LCD28, SD28, and HFD28 groups 20 weeks of HFD, we analyzed the size and number of adipocytes (histopathological examination), the levels of Hif-1\(\alpha\), Hif-2\(\alpha\) and LC3 mRNA (RT-qPCR) and the levels of HIF-1\(\alpha\), HIF-2\(\alpha\) and MAP1LC3A/LC3 protein (ELISA) at the end of week 28.

**Diets.** Three kinds of diet were used in this study: standard, low-caloric and high-fat diet. The standard diet composition was made according to Indonesia National Agency of Drug and Food Control, containing 3,100 kcal/kg. The low-caloric diet contained 2,100 kcal/kg, reducing 30% from standard diet, and the high-fat diet contained 4,200 kcal/kg. All types of diet were given as pellets for ad libitum feeding. The diet compositions were analyzed using bomb calorimetry and the results are listed in Table 1.

**Quantification size and number of visceral adipocytes.** A part of visceral adipose tissue was fixed in 10% neutral buffered formalin for 6 h and dehydrated as standard before embedding in paraffin wax. Five \(\mu\)m thick slices were dyed by using hematoxylin and eosin (H&E) method. The images were captured at 40× optical magnification with microscope eyepiece camera. Using one field of view per slide image, manual measurements of adipocyte size and number were calculated using Image \(J\) program. The cell size is gathered through outlining each individual cell and using the size calculation from the application. This process is done for each cell in every relevant photograph taken. The final result is the number of cells and each cell’s size, stored conveniently in a spreadsheet.

**RNA extraction and quantitative polymerase chain reaction (qPCR) analysis.** The total RNA of visceral white adipose tissue was extracted using QIAamp \(^R\) RNA Blood Mini Kit Cat No. 52304 (Qiagen, Hilden, Germany) instructed by the manufacturer’s protocol. The purity of the total RNA was measured on a NanoDrop 2000 Spectrophotometer (ThermoScientific, Wilmington, DE). cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription Kit Cat No. 205311 (Qiagen) according to the manufacturer’s recommendations.

qPCR Relative Quantification was conducted using QuantiTect \(^R\) SYBR\(^\text{®}\) green PCR Kit (Qiagen). Each reaction contained 2 \(\mu\)l of cDNA template and 23 \(\mu\)l of the reaction mixture. The cycling condition is as follows: pre-denaturation, 95ºC for 15 min; denaturation, 94ºC for 15 s; annealing, 58ºC for 30 s; 40 cycles, extension, 72ºC for 30 s; and final extension 72ºC for 3 min. Then, a melting curve was generated by increasing the temperature from 72 to 95ºC with continuous collection of the SYBR Green fluorescence signal. 36B4 gene expression was used as an

| Table 1. Nutritional composition of the diet |
|-----------------|-----------------|-----------------|-----------------|
| Nutrient        | Standard diet   | Low-caloric diet| High-fat diet   |
| Gross energy (kcal/kg) | 3,121           | 2,188           | 4,280           |
| Dry content (%)  | 86.75           | 86.8            | 86.47           |
| Water content (%)| 13.25           | 13.2            | 13.53           |
| Ash (%)          | 9.84            | 14.34           | 5.02            |
| Gross/crude protein (%) | 20.15        | 15.4            | 20.1            |
| Gross/crude fiber (%) | 5.86            | 16.76           | 5.13            |
| Gross/crude fat (%) | 5.64            | 2.24            | 22.27           |
| Extract material without nitrogen | 43.76         | 36.56           | 32.45           |
| Ca (%)           | 0.9             | 0.9             | 0.9             |
| P (%)            | 0.6             | 0.6             | 0.6             |

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endogenous control. mRNA relative expression were calculated by the Livak-Schmittgen method (ΔΔCt method). All primer sequences are showed in Table 2.

**ELISA analysis.** Protein levels of HIF-1α, HIF-2α and LC3 in visceral adipocytes were determined by ELISA using commercially available kits (Rat HIF-1α ELISA Kit Cat No. MBS2886832, Rat HIF-2α ELISA Kit Cat No. MBS2601406 and Rat MAP1LC3A ELISA Kit Cat No. MBS076210 from MyBioSource.com). All procedures were conducted according to the manufacturer’s protocol. The protein content of the adipose tissue was analyzed using the Warburg-Christian method at λ 280 nm. 

**Statistical analysis.** Results are presented as mean ± SEM. One-way ANOVA was used to compare the differences between multiple groups, followed by LSD post-hoc test if the data has a normal distribution. Kruskall-Wallis was used to compare the differences between multiple groups, followed by Mann-Whitney post-hoc test if the data has abnormal distribution. Statistical analyses were performed using SPSS ver. 20 (IBM Corporation, Armonk, NY). The differences were considered significant when p values <0.05. The graphs were created using GraphPad Prism Software ver. 5.

**Results**

**The effects of the diet on the rat’s body weight.** Fig. 1 shows the effect of the diet on the rat body weight. At baseline, the rats used in the experiment were 6 weeks of age and had no significant body weight differences between all groups (p = 0.596) (Fig. 1A). Feeding rats with the low-caloric diet for 8 weeks caused a significantly lower body weight compared to control (p = 0.032), while feeding the high-fat diet caused a significantly higher body weight compared to control (p < 0.008), as shown in Fig. 1B. After this period, starting from when the rats were 14 weeks old, the rats were given a high-fat diet for 20 weeks, except the control group. This treatment caused the body weight of all the rats to increase and reach a significantly higher value compared to control, as shown in Fig. 1C. However, even though the highest body weight was found in the rats supplied a high-fat diet since childhood, the highest body weight increase actually happened to the rats given a low-caloric diet while young as shown in Fig. 2.

**The size and number of visceral adipocytes.** Analysis of the number and size of visceral adipocytes were conducted at the end of week 8 and 28. At the end of week 8, no difference was found between the visceral adipocyte counts of the treatment groups. 

### Table 2. Primer sequences

| Gen     | Accession number | Primer sequences                  | Amplicon size (bp) |
|---------|------------------|-----------------------------------|--------------------|
| Hif-1α  | NM_024359.1      | F 5’-CGT GAG CTC CCA TCT TGA TAA-3’ R 5’-CAA GAT CAC CAG CAG CAT GAA-3’ | 89                 |
| Hif-2α  | NM_923090.1      | F 5’-GTG ACC CAA GAC GGT GAT ATG-3’ R 5’-GAT GCT GTG TCC TGT TAG TTC T-3’ | 90                 |
| Lc3     | NM_199599.2      | F 5’-CCC ATC GCT GAC ATC TAT GAA C-3’ R 5’-GAA GGT TTC TTG GGA GGC ATA G-3’ | 78                 |
| 36B4    | NM_022402.2      | F 5’-AGC CAC ACT GCT GAA CAT-3’ R 5’-GTA GAT GCT GCC ATT GTC AAA C-3’ | 85                 |
groups and control, as shown in Fig. 3. The size of adipocytes of HFD8 group and SD8 group were bigger significantly compared with LCD8 group, as follows: HFD8 vs LCD8 \((p=0.000)\), SD8 vs LCD8 \((p=0.000)\), as shown in Fig. 4.

At the end of week 28, the LCD28 group had the least amount of adipocytes compared to the HFD28, SD28, and control group, as follows: LCD28 vs HFD28 \((p=0.001)\), LCD28 vs SD28 \((p=0.001)\), LCD28 vs CON \((p=0.000)\), as shown in Fig. 5. The biggest adipocyte size was found in the LCD28 group, and the LCD28 adipocytes were significantly different in size compared to SD28 \((p=0.001)\) and control \((p=0.000)\). There was an enlargement of the HFD28 cells, and they had a significant difference to the SD28 \((p=0.034)\) and control \((p=0.007)\), as shown in Fig. 6.

The visceral adipocytes hypoxia. There was no difference in the Hif-1α mRNA level between all the experimental groups compared to control \((p=0.466)\) as shown in Fig. 7. However, the HIF-1α protein level in the LCD28 group was higher significantly compared to HFD28 \((p=0.046)\) and control \((p=0.029)\), as shown in Fig. 8.

There was a significant increase in the level of Hif-2α mRNA in the HFD28 group compared to SD28 \((p=0.042)\), as shown in Fig. 9. The level of HIF-2α protein level in the LCD28 group was increased significantly compared to SD28 \((p=0.045)\) and HFD28 \((p=0.022)\), as shown in Fig. 10.
The autophagic activity of visceral adipocytes. There was a significant increase in the level of Map1lc3aA/Lc3 mRNA in the LCD28 group compared to HFD28 ($p=0.037$) and control ($p=0.047$), as shown in Fig. 11. However, there was no difference on the level of MAP1LC3A/LC3 protein between groups due to the nutritional treatments ($p=0.169$) as shown in Fig. 12.

Discussion

SD rats have been preferentially used as an animal model in diet-induced obesity since they mimic the pathophysiology of human obesity.\(^{(16)}\) Three kinds of diets were used in this experiment, which are the low-caloric diet, the high-fat diet and the standard chow diet. The high-fat diet comprised of 20% fat, in contrast to the standard chow diet which only contained 5% fat. The low-calorie diet contained 30% less calories than the standard chow diet. These kinds of diets were supplied to rats while they were between 6 to 14 weeks old, meaning the rats are still in their childhood growth period and not considered adults yet. Feeding rats with the low-caloric diet for 8 weeks caused a significantly lower body weight compared to control, while feeding the high-fat diet caused a significantly higher body weight compared to control. Even though LCD group had a lower body weight compared to control, they were still observed to be healthy and active. This is consistent with a research conducted by Keenan et al.\(^{(17)}\) which concluded that a 30% reduction of calorie intake compared to normal will result in a decrease in body weight, but not cause a nutrient deficiency in rat test subjects.

After this period, starting from the rats were 14 weeks old, the rats were given a high-fat diet for 20 weeks, except the control group. This treatment caused the body weight of all the rats to increase and reach a significantly higher value compared to control. However, even though the highest body weight was found in the rats supplied a high-fat diet since childhood (HFD28 group), the highest body weight increase actually happened to the rats given a low-caloric diet while young (the LCD28 group).

In this experiment, nutritional status classification is done by comparing the body weight of the treatment group rats and the body weight of control. This method is the most common method for classifying nutritional statuses, similar to most other studies regarding obesity. Some studies conversely use a method similar to calculating body mass index (BMI) in humans,\(^{(18)}\) and others use the Lee Obesity Index instead.\(^{(19)}\)
The number and size of the visceral adipocytes. Analysis of the number and size of visceral adipocytes were conducted at the end of weeks 8 and 28. The analysis was done through histopathological examination. The tissue preparations were placed under the microscope and viewed, and areas of the tissue which need further investigation were photographed and stored in a computer. Through the application ImageJ, cell size is gathered through outlining each individual cell and using the size calculation from the application. This process is done for each cell in every relevant photograph taken. The final result is the number of cells and each cell’s size, stored conveniently in a spreadsheet. The advantage of using this method is that it is simple, inexpensive, and we can easily discern the distribution of cell sizes. However, this method is tedious, time-consuming, and it relies on the tissue preparation having high quality since a bad histopathological preparation may caused artifacts and make an impact to the cells size. A number of methods to assess adipocyte size and number have been described but there has not been a single method that is deemed the gold standard.[2,6]

At the end of week 8, no difference was found between the visceral adipocyte counts of the treatment groups and control. At the end of week 28, the LCD28 group had the least amount of adipocytes at an average of 132 cells, compared with 284 in the HFD28 group; 277 in SD28 group, and 321 in control group. However, the adipocytes of the LCD28 group had the largest cells at an average of 9,626 μm² compared with 7,348 μm² in HFD28 group, 4,074 μm² in SD28 group and 2,900 μm² in control group. It is suspected that the lowered macronutrient intake during childhood caused the fat cells not to reach maturity and become functional. Consequently, excess caloric intake consumed during adulthood will be stored as fat in a limited number of adipocyte cells, causing these cells to hypertrophy. On the other hand, adequate adipogenesis causes someone to be protected in an excess caloric intake because it will be accumulated in more fat cells. The adequate adipogenesis during the childhood and adolescence period is critical to the function of adipocytes in adulthood. According to Spalding, the number of adipocytes in adulthood remains constant and is set during childhood and adolescence.[13]

It is known that adipose tissues with fewer but larger adipocytes are associated with a higher insulin resistance and a larger tendency for cardiovascular diseases and diabetes. On the other hand, adipose tissues with many adipocytes of a smaller size are associated with better metabolism.[21] Therefore, the LCD28 group, which had fewer but larger adipocytes, had an increased risk for degenerative diseases compared to the other groups and control.

The visceral adipocytes hypoxia. The increase of HIF-1α and HIF-2α indicates that a cell underwent hypoxia. In our observation, LCD28 adipocytes have increased HIF-1α and HIF-2α proteins, whereas the Hif-1α and Hif-2α mRNA levels were similar to control. This condition suggests that the LCD28 adipocytes underwent hypoxia, which needs to be overcome by inhibiting the degradation of HIF-α. The increased HIFs level and the normal Hifs mRNA levels show that the LCD28 adipocytes were able to overcome the hypoxia without increasing de novo synthesis.

On the other hand, the HFD28 adipocytes did not experience hypoxia, as indicated by normal HIF-1α and HIF-2α levels. However, these adipocytes seem to be preparing for a potential hypoxic condition, as shown by the increase of the Hif-2α mRNA level. Hif-2α mRNA can be translated into HIF-2α protein immediately, when needed, in a process of protein synthesis which is known to consume a lot of ATP. Matsunaga et al.[22] had observed that cells in a hypoxic condition have reduced ATP production. This reduced ATP production forces the cell to be very selective in energy utilization. They synthesize only the proteins which are necessary for survival.[22] The LCD28 adipocytes were much larger than HFD28’s, proven by their average of 9,626 μm² in size compared to the average size of HFD28 adipocytes, 7,348 μm². Previous studies have shown that hypoxia occurs in adipocytes with a diameter of more than 100 μm (or 7,857 μm²).[2,3] This confirms that the LCD28 adipocytes did experience hypoxia, but not the HFD28 adipocytes. We also found that adipose tissues derived from the LCD28 group had the least amount of adipocytes. The hypoxic condition could reduce the number of adipocytes in line with Matsunaga et al.’s[8] findings that hypoxia reduces cell proliferation.

The visceral adipocytes autophagy. Cells in hypoxia experience a decrease of ATP production[22] whereas the need of energy is still the same. It is known that HIFs would bind to p53, a tumor suppressor protein, resulting in p53 stabilization and inducing cell apoptosis.[4,6] This is one of the adaptation processes of hypoxic cells in order to use energy efficiently.

As another way to overcome this condition, hypoxic cells, under the HIFs control, could consume their macromolecules as an energy source in a process called autophagy. LC3, a biogenesis substance of autophagosome, is an essential part of autophagy.[4,11] In this study, we found that Lc3 mRNA within the adipocytes of the LCD28 group was higher compared to the other groups. This indicates a tendency for the LCD28 group adipocytes to undergo autophagy. Interestingly, Matsunaga et al.[22] found that in a stress condition, including hypoxia, there would be an upregulation of Nuclear protein 1 (NUPR1), a newly found cell cycle suppression protein. They found that the upregulation of NUPR1 could induce this autophagic activity to help the cell to survive. We did not analyze the NUPR1 in our study, so we cannot say that NUPR1 was involved in this phenomenon. However, Matsunaga et al.’s study[22] as well as our own showed that a hypoxic condition induced the autophagy.

Our findings showed an increase of Lc3 mRNA in the hypoxic cells of the LCD28 group. It should be interesting to analyze whether this autophagic gene is under the control of HIFs.

The previous studies had shown that an increased transcription of autophagy-related genes and proteins is associated with a dysfunction of adipose tissues and a higher risk of the development of cardiometabolic diseases.[15,17] If this is the case, the LCD28 group, which demonstrated autophagic activities, had a higher risk for cardiometabolic diseases compared to the other groups and control.

In conclusion, the result of our study indicates that there are differences in the visceral adipocyte characteristics of obese adult rats which differ in nutritional state at a young age. The obese adult rat adipocytes that were undernourished at childhood were hypertrophic, hypoxic and had increased autophagic gene expression. The adipocytes of rat that were obese since young were hypertrophic and had increased hypoxic gene expression.

Nowadays, Indonesia, and likely similar developing countries, is facing a double burden of malnutrition at the population level in that there is a high prevalence of both undernutrition and overnutrition. An individual who grew up undernourished but went into overnutrition as an adult, can be said to experience “the individual double burden of malnutrition”. These individuals have a larger tendency to develop degenerative diseases, as compared to obese adults who received either normal levels of nutrition or overnutrition while young.

Acknowledgments

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Abbreviations

ATGs autophagy-related genes
ELISA enzyme-linked immunosorbent assay
GABARAPL GABA type A receptor associated protein like
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Conflict of Interest

No potential conflicts of interest were disclosed.