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Short Communication

Autophagy up-regulation by early weaning in the liver, spleen and skeletal muscle of piglets

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Abstract

Autophagy, a catabolic process responsible for the degradation of cytosolic components and the preservation of cellular homeostasis in virtually all eukaryotic organisms, is up-regulated when nutrient supplies are limited. However, whether early weaning induces autophagy in infants is not completely clear. In the present study, we used piglets as the early-weaning model to examine the autophagic activity in different tissues in response to nutrient status. Western blot analysis demonstrated that microtubule-associated protein 1 light chain 3-II, a promising marker protein for macroautophagy, was expressed at a notably higher level at 12 and 24 h weaning treatments than without weaning treatment ($P<0{\cdot}01$), and that the p62 (sequestome 1; SQSTM1) expression level was significantly attenuated after weaning treatments ($P<0{\cdot}01$) in the liver, spleen and skeletal muscle tissues. In addition, autophagic vacuoles detected by transmission electron microscopy were dramatically accumulated in these tissues ($P<0{\cdot}01$). Together, these results indicate that autophagy induced by early weaning may be helpful for the physiological system, which controls the balance of energy and nutrients for basic cell functions in the piglet model.

Key words: Autophagy: Microtubule-associated protein 1 light chain 3: p62: Early weaning: Piglets

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process by which portions of cytosol and organelles are sequestered into double-membrane vesicles and degraded upon fusion with lysosomal compartments[1]. Under physiological conditions, autophagy is active at a basal level and has a series of important roles, such as cellular development and differentiation, prevention of neurodegeneration, anti-ageing and tumour suppression[2–4]. On the other hand, autophagic activity can be quickly and markedly up-regulated by many stimuli, such as nutrient limitation, heat and oxidative stress[5]. The most well-known inducer of autophagy is nutrient starvation, both in cultured cells and in intact organisms, ranging from yeast to mammals[5]. A recent study has revealed that autophagy is vital for survival during neonatal starvation; this concept has been illustrated in an animal model of ATG5-deficient mice[6]. These mice, although nearly normal at birth, could not survive the early neonatal starvation period since they failed to induce autophagy[6].

Compared with the rodent model, the piglet model is more physiologically relevant for nutrition studies, as its digestive system is anatomically and functionally more similar to that of the infants[7]. To date, however, there have been no studies reporting on autophagic activity in piglets. Similar to the case of neonatal starvation in mice[6], at early weaning, the milk nutrient supply is suddenly interrupted, and the piglets (or infants) also face severe starvation until supply can be restored through feed (or food); however, whether early weaning can up-regulate autophagic activity in piglets is still unclear. In the present study, we have reported that the early-weaning piglets adapt to this adverse circumstance by inducing autophagy in the liver, spleen and skeletal muscle tissues. The results indicate that autophagy may contribute to the physiological system that controls the balance of energy and nutrients for basic cell functions during the early-weaning case in the piglet model.

Abbreviation: LC3, microtubule-associated protein 1 light chain 3.

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Experimental methods

Animals

For the early-weaning studies, fifteen Landrace × Yorkshire piglets (at 14 d of birth) were weaned for 0 h (five piglets as the control), 12 h (five piglets as the first time-course treatment) or 24 h (five piglets as the second time-course treatment) in the temperature-controlled (28 ± 2°C) metabolism cages, and had free access to drinking-water. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the Institutional Animal Care and Use Committee at Huazhong Agricultural University.

Tissue sampling

Liver, spleen and skeletal muscle tissue samples for Western blotting were aseptically excised, rinsed in cold PBS, snap-frozen in liquid N_2 and stored at −80°C until analysis. Tissue samples for transmission electron microscopy assessment were collected from similar areas on each organ and fixed in 0·1 M-sodium cacodylate-buffered (pH 7·4) 2·5 % glutaraldehyde solution.

Western blotting

Piglet tissues were homogenised in nine volumes of ice-cold PBS supplemented with protease inhibitors. The homogenates were centrifuged at 500 ***g*** for 10 min at 4°C. Protein extracts (50 μg) were subjected to SDS-PAGE and immunoblotted using the anti-microtubule-associated protein 1 light chain 3 (LC3) antibody produced in rabbits (catalogue no. L7543; Sigma-Aldrich, St Louis, MO, USA) and anti-p62 antibody produced in rabbits (catalogue no. 5114; Cell Signaling Technology, Danvers, MA, USA).

Transmission electron microscopy

Tissue samples were fixed in 0·1 M-sodium cacodylate-buffered (pH 7·4) 2·5 % glutaraldehyde solution for 2 h and

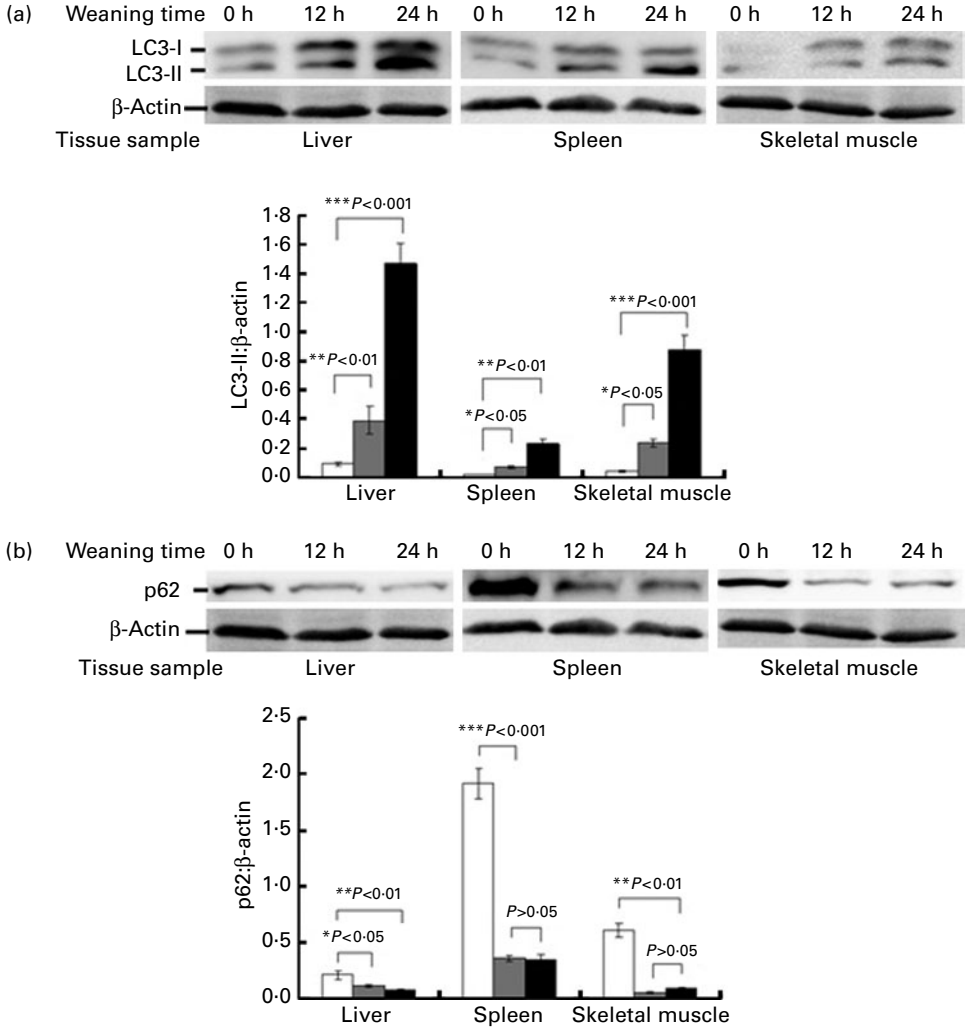

Fig. 1. Protein levels for (a) microtubule-associated protein 1 light chain 3 (LC3), (b) p62 and β-actin in the liver, spleen and skeletal muscle tissues of weanling piglets (top). The proteins were quantified by Western blotting. Levels for target proteins were normalised to those for β-actin (bottom). Values are means, with their standard errors represented by vertical bars. Mean values were significantly different: * $P<0·05$, ** $P<0·01$, *** $P<0·001$. □, 0 h; ▩, 12 h; ■, 24 h.

post-fixed in 0·1 M-sodium cacodylate-buffered (pH 7·4) 1 % OsO_4 solution for 1 h. After dehydration in an ethanol gradient (70 % ethanol (20 min), 96 % ethanol (20 min) and 100 % ethanol (2 × 20 min)), samples were incubated with propylene oxide (2 × 10 min), impregnated with a mixture of propylenoid–LX-112 (1:1) and embedded in LX-112. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a FEI Tecnai G^2 20 TWIN transmission electron microscopy at 80 kV.

Autophagic vacuoles

All cells in the tissue samples were randomly assessed in a blind fashion for analysis of autophagic vacuoles by transmission electron microscopy, and then 100 electron micrograph images per sample were counted to quantify the number of autophagic vacuoles. Autophagic vacuoles were identified when they met two or more of the following criteria: double membranes (complete or at least partially visible); absence of ribosomes attached to the cytosolic side of the membrane; luminal density similar to cytosol; identifiable organelles or regions of organelles in their lumen.

Statistical analysis

Statistical significance was determined by a one-way ANOVA using Bonferroni's *post hoc* test ($P<0·05$, $P<0·01$ and $P<0·001$).

Results

Microtubule-associated protein 1 light chain 3-II and p62 protein levels

As shown in Fig. 1(a), little LC3-II was detected in the liver, spleen and skeletal muscle tissues of piglets under normal conditions, whereas the early weaning with 12 and 24 h treatments caused a significant accumulation of LC3-II. In addition, we also observed that the 12 and 24 h early-weaning treatments significantly attenuated p62 protein levels in the liver, spleen and skeletal muscle tissues of piglets (Fig. 1(b)).

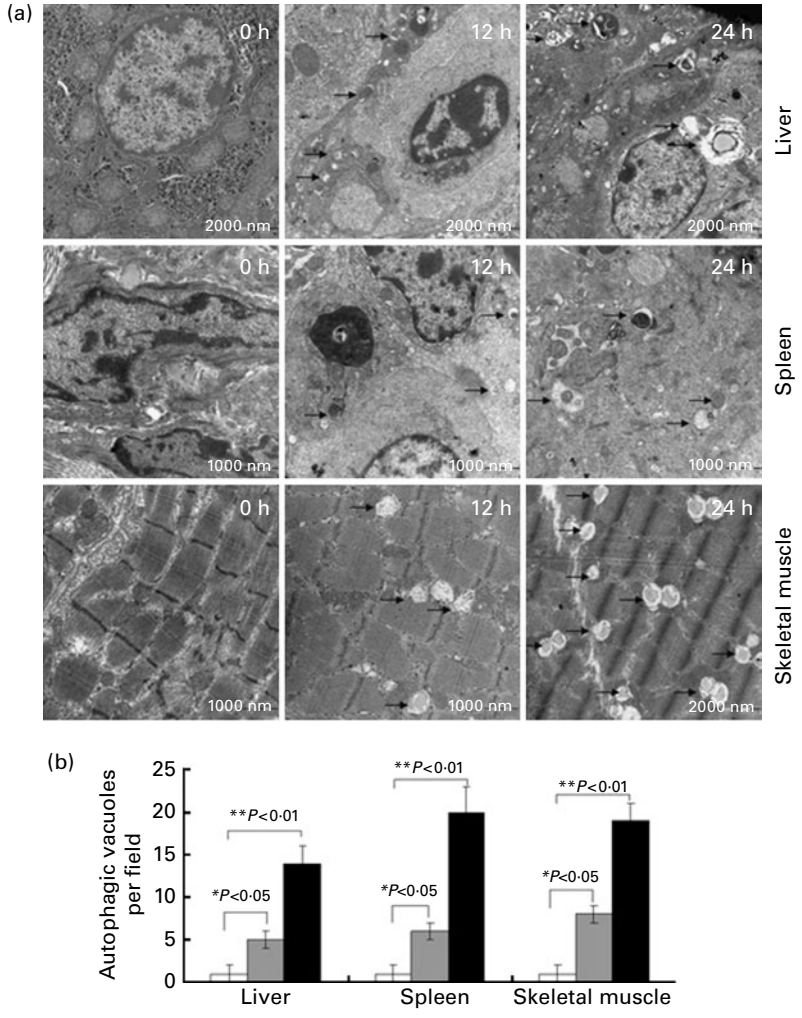

Fig. 2. (a) Weaning-induced autophagic activation in the liver, spleen and skeletal muscle tissues of piglets. Ultrastructural characterisation of the tissues from suckling piglets (0 h) and weaned piglets at 12 and 24 h post-weaning. (b) Accumulation of autophagic vacuoles (black arrows) per field was calculated and quantified. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different: * $P<0·05$, ** $P<0·01$. □, 0 h; ▩, 12 h; ■, 24 h.

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Autophagic vacuoles

Morphometric analysis of electron micrograph images revealed that double-membrane autophagic vacuoles (black arrows) were dramatically accumulated in the liver, spleen and skeletal muscle tissues of piglets under 12 and 24 h early-weaning treatments (Fig. 2(a)), and the average number of autophagic vacuoles per field in the liver, spleen and skeletal muscle tissues of piglets with early-weaning treatments was notably higher than that of the control group (Fig. 2(b)).

Discussion

In mammals, LC3, a mammalian homologue of yeast Atg8, has been widely used as a sole marker of autophagosomes[5,8]. During autophagy, the cytoplasmic form (LC3-I) is processed and recruited to the autophagosomes, where LC3-II is generated by site-specific proteolysis and lipidation near to the C-terminus. As LC3-II closely binds to autophagosome membranes, tracking the conversion of LC3-I to LC3-II is indicative of autophagic activity, and the amount of LC3-II closely correlates with the number of autophagosomes[5]. Besides LC3, p62 (also known as SQSTM1/sequestome 1) is selectively incorporated into autophagosomes through direct binding to LC3 and is efficiently degraded by autophagy[9]; thus, the total cellular expression levels of p62 inversely correlate with autophagic activity[5]. In the present study, LC3-II protein level in the liver, spleen and skeletal muscle tissues of piglets was significantly up-regulated at 12 and 24 h weaning treatments, and that the p62 expression level was significantly attenuated after weaning treatments. These results indicate that early weaning can induce autophagy in the liver, spleen and skeletal muscle tissues of piglets. We then performed ultrastructural studies to probe for double-membrane-bound autophagic vacuoles, a long-established analytical 'gold standard' for autophagy and further confirmed autophagy induction by early weaning in the piglet model.

It is accepted that autophagy can be transiently induced by stress, such as nutrient deprivation, as a survival response[10]. Under the food restriction or starvation condition, autophagy is activated to provide cells with the necessary nutrients through degradation of intracellular materials. Since early weaning is also an energy-limiting cellular stress, we aimed to obtain direct evidence concerning the tissue-specific and timing of autophagic response in the tissues of piglets during the early-weaning period. In animals, starvation induces the largest protein loss in the liver; mice and rats can lose approximately 25–40 % of their liver protein during the first 48 h of starvation[11]. The liver provides functions required to maintain homeostasis in the organism, and a great part of our current understanding of mammalian macroautophagy is derived from studies of the liver[12–14]. Muscle mass represents 40–50 % of the human body and, in mammals, is one of the most important sites for the control of metabolism[15]. Moreover, during catabolic conditions, muscle proteins are mobilised to sustain gluconeogenesis in the liver and to provide alternative energy substrates for organs. The spleen is an organ found in virtually all vertebrate animals with important roles with regard to erythrocytes and the immune system[16]. In addition to the relevance of autophagy as a physiological response to starvation, this pathway has also been suggested to play diverse important roles in innate and adaptive immunity[17], but whether early weaning can up-regulate autophagic activity in the spleen of piglets has not been fully examined. Thus, in the present study, we mainly focused on the liver, spleen and skeletal muscle tissues of piglets, and observed that autophagic activity in these tissues was all significantly up-regulated by early-weaning treatments.

To the best of our knowledge, this is the first report to show the effect of early weaning on autophagic activity in mammals, especially in infants, as assessed by LC3-II, p62 and transmission electron microscopy using piglets as the early-weaning model. Overall, the results showed that autophagic activity was significantly up-regulated in the liver, spleen and skeletal muscle tissues of piglets upon early-weaning treatments, which may be helpful for maintaining cellular homeostasis and survival during the early-weaning period in the piglet model. It will be of high interest to determine whether such a phenomenon in infants is reproducible in order to define its role in nutritional regulation and develop adapted milk formula and therapeutics. Further investigation into the biochemical mechanisms will provide us more information of how human cells can work under early-weaning stress using cellular signalling pathways. More importantly, we should be able to identify the main feature of macroautophagy regulation by nutrients, in particular, amino acids as well as glucose and vitamins, and its mechanisms. Thus, these nutrients might be used nutritionally and therapeutically to infant health or disease caused by early weaning.

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References

1. Kundu M & Thompson CB (2008) Autophagy: basic principles and relevance to disease. *Annu Rev Pathol* **3**, 427–455.
2. Cecconi F & Levine B (2008) The role of autophagy in mammalian development: cell makeover rather than cell death. *Dev Cell* **15**, 344–357.
3. Mizushima N, Levine B, Cuervo AM, *et al.* (2008) Autophagy fights disease through cellular self-digestion. *Nature* **451**, 1069–1075.

4. Rubinsztein DC (2006) The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* **443**, 780–786.
5. Mizushima N, Yoshimori T & Levine B (2010) Methods in mammalian autophagy research. *Cell* **140**, 313–326.
6. Kuma A, Hatano M, Matsui M, *et al.* (2004) The role of autophagy during the early neonatal starvation period. *Nature* **432**, 1032–1036.
7. Herfel TM, Jacobi SK, Lin X, *et al.* (2009) Safety evaluation of polydextrose in infant formula using a suckling piglet model. *Food Chem Toxicol* **47**, 1530–1537.
8. Kabeya Y, Mizushima N, Ueno T, *et al.* (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* **19**, 5720–5728.
9. Bjørkøy G, Lamark T, Brech A, *et al.* (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* **171**, 603–614.
10. Levine B (2005) Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* **120**, 159–162.
11. Addis T, Poo LJ & Lew W (1936) The quantities of protein loss by the various organs and tissues of the body during a fast. *J Biol Chem* **115**, 111–118.
12. Seglen PO & Brinchmann MF (2010) Purification of autophagosomes from rat hepatocytes. *Autophagy* **6**, 542–547.
13. Strømhaug PE, Berg TO, Fengsrud M, *et al.* (1998) Purification and characterization of autophagosomes from rat hepatocytes. *Biochem J* **335**, 217–224.
14. Yin XM, Ding WX & Gao W (2008) Autophagy in the liver. *Hepatology* **47**, 1773–1785.
15. Sandri M (2010) Autophagy in skeletal muscle. *FEBS Lett* **584**, 1411–1416.
16. Mebius RE & Kraal G (2005) Structure and function of the spleen. *Nat Rev Immunol* **5**, 606–616.
17. Levine B & Deretic V (2007) Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol* **7**, 767–777.

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