J. Dairy Sci. 101:1–11
https://doi.org/10.3168/jds.2017-13731

α-Lactalbumin-oleic acid complex kills tumor cells by inducing excess energy metabolism but inhibiting mRNA expression of the related enzymes

B. Fang,*[1] M. Zhang,† K. S. Ge,‡ H. Z. Xing,* and F. Z. Ren*‡
*Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, 100083, China
†School of Food Science and Chemical Engineering, Beijing Technology and Business University, Beijing, 100048, China
‡Key Laboratory of Functional Dairy, co-constructed by Ministry of Education and Beijing Government, and Beijing Laboratory of Food Quality and Safety, China Agricultural University, Beijing 100083, China

ABSTRACT

Previous studies have demonstrated that the anti-tumor α-lactalbumin-oleic acid complex (α-LA-OA) may target the glycolysis of tumor cells. However, few data are available regarding the effects of α-LA-OA on energy metabolism. In this study, we measured glycolysis and mitochondrial functions in HeLa cells in response to α-LA-OA using the XF flux analyzer (Seahorse Bioscience, North Billerica, MA). The gene expression of enzymes involved in glycolysis, tricarboxylic acid cycle, electron transfer chain, and ATP synthesis were also evaluated. Our results show that α-LA-OA significantly enhanced the basal glycolysis and glycolytic capacity. Mitochondrial oxidative phosphorylation, including the basal respiration, maximal respiration, spare respiratory capacity and ATP production were also improved in response to α-LA-OA. The enhanced mitochondrial functions maybe partly due to the increased capacity of utilizing fatty acids and glutamine as the substrate. However, the gene expressions of pyruvate kinase M2, lactate dehydrogenase A, aconitate hydratase, and isocitrate dehydrogenase 1 were inhibited, suggesting an insufficient ability for the glycolysis process and the tricarboxylic acid cycle. The increased expression of acetyl-coenzyme A acyltransferase 2, a central enzyme involved in the β-oxidation of fatty acids, would enhance the unbalance due to the decreased expression of electron transfer flavoprotein β subunit, which acts as the electron acceptor. These results indicated that α-LA-OA may induce oxidative stress due to conditions in which the ATP production is exceeding the energy demand. Our results may help clarify the mechanism of apoptosis induced by reactive oxygen species and mitochondrial destruction.

Received August 22, 2017.
Accepted November 18, 2017.
[1]Corresponding author: bingfang@cau.edu.cn or 49631263@qq.com

Key words: glycolysis, tricarboxylic acid cycle, mitochondrial oxidative phosphorylation, extracellular acidification rate, oxygen consumption rate

INTRODUCTION

The selective tumoricidal activity of α-lactalbumin-oleic acid complex (**α-LA-OA**) has been confirmed in murine models of glioblastoma xenograft and bladder cancer (Fischer et al., 2004; Mossberg et al., 2010) and in clinical studies of skin papillomas and bladder cancers (Gustafsson et al., 2004; Mossberg et al., 2007). To investigate the conserved survival mechanisms that are targeted by α-LA-OA, several screening techniques, such as metabolomic technology (Storm et al., 2011) and the isobaric tags for relative and absolute quantitation method (Fang et al., 2016), have been used to identify genes or proteins targeted by α-LA-OA.

In contrast to healthy cells, tumor cells must support continuous cell growth and proliferation and thus exhibit high rates of aerobic glycolysis, which converts pyruvate to lactate acid instead of acetyl-CoA, a necessary component of the tricarboxylic acid (**TCA**) cycle, even in the presence of oxygen (known as the Warburg effect; Hsu and Sabatini, 2008). A previous study showed that depletion of glucose or addition of 2-deoxyglucose (**2-DG**), a glycolysis inhibitor, increases the sensitivity of tumor cells to HAMLET, a similar complex of α-LA with OA (Storm et al., 2011), indicating that α-LA-OA may target the energy metabolism of tumor cells. The authors found that the sensitivity of tumor cells to α-LA-OA was dependent on c-Myc and glycolysis toward the pentose phosphate pathway by inhibiting hexokinase and pyruvate kinase isozymes M2 was increased by α-LA-OA (Storm et al., 2011). Furthermore, our previous proteomics study reported that α-LA-OA increased the expression of poly (ADP-ribose) polymerase 1 (PARP-1), which caused cell death by inhibition of hexokinase activity, leading to defects in glycolysis (Andrabi et al., 2014). Together

these studies indicate that α-LA-OA alters cancer cell metabolism and that it may disrupt glycolysis, which is altered in cancer cells as discussed above. However, in contrast with these results of decreased glycolysis by α-LA-OA, our proteomics results also demonstrated that α-LA-OA increased the expression of several proteins involved in carbohydrate and energy metabolism, such as fructose-bisphosphate aldolase A, pyruvate kinase isozymes M1/M2, succinate dehydrogenase, electron transfer flavoprotein, and ATP synthase (Fang et al., 2016), indicating an enhanced glycolytic capacity and energy metabolism.

To clarify the energy metabolism changes induced by α-LA-OA, here we used the XF flux analyzer to evaluate glycolysis and mitochondrial function in HeLa cells. This study may help clarify the effects of α-LA-OA on the energy metabolism of tumor cells and may help identify some potential targets for tumor therapy.

MATERIALS AND METHODS

Materials

Bovine α-LA (≥85% purity), oleic acid (**OA**, C18: 1:9 *cis*, ≥99.0% purity, cell culture tested), glucose, L-glutamine, and pyruvate were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). HyClone Dulbecco's modified Eagle's medium (**DMEM**) containing a high level of glucose was purchased from GE Healthcare (Chicago, IL). Fetal bovine serum was purchased from Gibco (Thermo Fisher Scientific, Carlsbad, CA). All other chemicals used were of analytical grade.

α-LA-OA Preparation

The α-LA-OA was prepared by heat treatment according to previously published methods (Fang et al., 2014), with modifications. We dissolved α-LA in phosphate buffer solution (Na_2HPO_4 and NaH_2PO_4 10 m*M*, pH 8.0) to a final concentration of 1 m*M*. Next, OA was directly added to the solution at 30 molar equivalents (OA:protein). The mixture was vortexed for 10 s and then incubated at 45°C in a water bath for 10 min. The sample was centrifuged at 4°C, 12,000 × *g* for 15 min to remove the unbound OA. The α-LA solution treated as above but without addition of OA was used as the control α-LA. Phosphate buffer added with same dose of OA with α-LA-OA and treated as above was used as control buffer.

Cell Culture and Cell Viability Assay

Human cervical carcinoma HeLa cells (Cell Bank of Chinese Academy of Science, Shanghai, China) were cultured in DMEM supplemented with 10% FBS in an incubator with 5% CO_2 at 37°C.

For the viability assays, HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well and grown for 24 h. The medium was removed and then replaced with fresh medium containing α-LA-OA, the control α-LA at final concentrations of 20, 40, 60, 80, or 100 μ*M*. The same volume of control buffer corresponded to 20, 40, 60, 80, or 100 μ*M* of α-LA-OA was added into medium and taken as the control treatments. After 24 h incubation at 37°C, cell viability was detected by the cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Next, 10 μL of CCK-8 solution was added to each well, and the 96-well plate was incubated at 37°C for 1 h. The optical density (OD) value for each well was read at 450 nm on a microplate reader (Infinite 200 PRO, Tecan, Zurich, Switzerland) to determine the cell viability. Wells did not contain cells and those treated as above were taken as blank wells. The assay was repeated 6 times. Cell viability was calculated using the following formula:

$$\text{cell viability (\%)} = \frac{\text{OD (experiment)} - \text{OD (blank)}}{\text{OD (control buffer)} - \text{OD (blank)}} \times 100.$$

Glucose Uptake

Cells were plated in 96-well plates at 1×10^4 cells per well and cultured for 24 h. Next, α-LA-OA or control α-LA was added at various concentrations and cells were incubated for another 24 h. Extracellular glucose levels were determined by the glucose oxidase method using a glucose determination kit (Applygen Technologies Inc., Beijing, China; Jiang et al., 2016). The glucose uptake of cells was subtracted the extracellular glucose levels from the glucose levels in the media.

Measurement of Glycolysis as Extracellular Acidification Rate

Cells were plated in Seahorse XF24 well plates (Seahorse Bioscience, North Billerica, MA) at 2×10^4 cells/well and incubated for 12 h until confluent. After 24-h treatment of α-LA-OA or control α-LA, the culture medium was replaced with glucose-free XF24 Seahorse medium containing 1 m*M* L-glutamine. Glycolytic flux (basal glycolysis, glycolytic capacity, and glycolytic reserve) in HeLa cells as assessed by extracellular acidification rate (**ECAR**) was measured by the sequential addition of glucose (10 m*M*), oligomycin (1 μ*M*), and 2-DG (50 m*M*) in an XF24 Extracellular Flux Analyzer according to the manual of XF glycolysis stress test kit (no. 103020–100, Seahorse Bioscience). The ECAR