Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : December 18, 2023
Accepted : January 16, 2024
Published online : March 31, 2024
doi:10.24659/gsr.11.1_1

Original article

Rapid measurement of fumaric acid in culture medium as an evaluation of metabolic abnormality of adipocytes

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Abstract

It has been reported that adipocytes accumulate triglycerides and concurrently produce adiponectin, a beneficial adipocytokine, to assist insulin function, and conversely produce unbeneficial adipokines, which have negative effects on the organism. We previously reported that the production of fumaric acid increases under hyperglycemic conditions due to mitochondrial stress and that fumaric acid reacts with the thiol group of cysteine residue to form *S*-(2-succinyl)cysteine (2SC), which inhibits adiponectin secretion and modifies a variety of proteins, resulting in the dysfunction of adipocytes. Therefore, we considered that a simple measurement of fumaric acid produced by adipocytes would enable rapid evaluation of metabolic abnormalities in adipocytes, and investigated a measurement system for this purpose. After culturing adipocytes for 4 days, a decrease in pH was observed due to the production of organic acids. Hydroxylamine hydrochloride and iron (III) chloride, a reagent for carboxylic acid detection, reacted with fumaric acid, malic acid, and citric acid. Similarly, fumaric acid levels by the enzymatic method and organic acids were found to be elevated by the carboxylic acid qualitative reagent in the medium in which adipocytes were cultured. Therefore, it was confirmed that functional abnormalities of adipocytes can be easily evaluated by the carboxylic acid qualitative reagent.

***KEY WORDS*:** fumaric acid, adipocytes, *S*-(2-succinyl)cysteine (2SC), mitochondrial function

Introduction

Japan's aging population is increasing rapidly, and with it, advances in medical care for the elderly have made the country one of the longest-living countries in the world in terms of average life expectancy. However, according to a 2019 survey by the Ministry of Health, Labour and Welfare, there is a gap of 8.7 years between average life expectancy and healthy life expectancy for men and 12 years for women, which means that elderly people are forced to live with a deteriorated quality of life for approximately 10 years before their death [1)]. Diabetes mellitus is one of the most common lifestyle-related diseases that cause poor quality of life, and the number of people suffering from this disease, including those in the pre-diabetic group, has rapidly increased to 20.5 million in Japan. Since 95% of diabetic patients are type 2, which is mainly caused by lifestyle-related disorders, the disease can be treated with early intervention. However, due to poor glycemic control, various complications, mainly vascular disorders such as atherosclerosis, often develop within 5 to 10 years.

Previously, we analyzed non-enzymatic post-translational modifications in adipocytes, which play an important role in metabolic regulation. As a result, we found that fumaric acid, which increases due to abnormal mitochondrial function, reacts with the thiol group of cysteine, resulting in *S*-(2-discovered a phenomenon produced by succinyl)cysteine (2SC) [2)]. 2SC subsequently causes abnormalities in the function of adiponectin, a beneficial cytokine [3)], and GAPDH, which is essential for glucose metabolism [4)], leading to abnormal glucose metabolism. Furthermore, 2SC conversion of glutathione [5)] causes a decrease in antioxidant capacity, which in turn increases the production of AGEs, advanced glycation end products, through the production of carbonyl compounds [6)]. Recently, it has been reported that fumaric acid itself, a 2SC precursor, is involved in the development of inflammatory reactions [7)], for example, fumaric acid has been reported to promote DNA methylation in human monocytes and cause them to secrete inflammatory cytokines [8)]. In addition, α-ketoglutarate, succinate, fumarate, and malate are increased in macrophages under inflammatory conditions, and glutamate, pyruvate, and 2-oxoglutaric acid, succinate,

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fumarate, and malate are increased in obese adipose tissue. Therefore, chronic inflammation in adipose tissue contributes to increased organic acids in the TCA cycle[7, 9]. Therefore, suppressing the excessive production of fumaric acid associated with metabolic abnormalities may inhibit fumaric acid-mediated inflammatory reactions in living organisms. In this study, we investigated a simple method to evaluate functional abnormalities of adipocytes by measuring fumaric acid produced when adipocytes are cultured under a high glucose condition.

Materials and methods

Cell culture

The mouse preadipocyte cell line (3T3-L1 cells) was purchased from the JCRB cell bank. Cells were passaged in Dulbecco's Modified Eagle Medium (DMEM) (low glucose medium) containing phenol red with 100 µg/mL streptomycin, 100 µg/mL penicillin, and 10% calf serum (CS; Fujifilm Wako Ltd., Osaka, Japan) was used for passaging.

3T3-L1 was differentiated using DMEM medium containing 10% fetal bovine serum (FBS), 10 µg/mL insulin, 0.3 µM dexamethasone, and 0.5 µM 3-isobutyl-1-methylxanthine (IBMX)[2]. Subsequently, the medium was changed every 2 days using a maturation medium of DMEM supplemented with 10% FBS and 5 µg/mL of insulin. On the fourth day after differentiation, the medium was collected, treated with a 0.45 µm filter, and stored at -20 °C.

Measurement of pH

The measurements of pH were performed using a bench-top pH meter (F-51, Horiba, Kyoto, Japan) and a pH electrode (9615S). Measurements were calibrated prior to analysis with a neutral phosphate standard (pH 6.86), a phthalate standard (pH 4.01), and a borate standard (pH 9.18).

Qualitative analysis for carboxylic acid

All organic acids in the TCA cycle have a carboxy group (R-COOH). Determination of organic acids was performed by a modification of the method of Murata *et al.*[10]. Specifically, to 50 µL of each sample, 50 µL of a saturated ethanol solution of hydroxylamine hydrochloride (Fujifilm Wako Ltd., Osaka, Japan) and 50 µL of a 1% ethanol solution of dicyclohexylcarbodiimide (DCC; Fujifilm Wako Ltd., Osaka, Japan) were added, mixed, and left for 2 minutes. The iron (III) chloride reagent was prepared by dissolving 10 mg of iron (III) chloride hexahydrate (Nacalai Tesque Co., Ltd., Kyoto, Japan) in 1 mL of ethanol, adding 10 µL of hydrochloric acid, and 50 µL was added to the above reaction solution. Then, 200 µL was added to each 96-well plate, and the absorbance was measured at 550 nm with a microplate reader (Infinite 200 PRO, Tecan). The color tone of the iron chelate complex is not changed by organic acids. Therefore, with a carboxylic acid qualitative reagent, it is not possible to detect each organic acid individually, and only the total amount is detected. Present experiment was conducted to measure the total amount of organic acids in order to confirm the correlation between the increase in fumaric acid and the increase in organic acids. Therefore, in order to specifically measure fumarate with this method, it is necessary to establish a measurement system using HPLC.

Measurement of fumaric acid

Fumaric acid was determined using the Fumarate assay kit (MAK060, Sigma-Aldrich, St. Louis, MO, USA). Ten µL of the assay sample was added to a 96-well plate. Master reaction mix (100 µl) was added to each well and pipetted. The absorbance at 450 nm was then measured in a microplate reader. Fumaric acid standard was added to each 0 (blank), 2, 4, 6, 8, and 10 nmol/well to generate a calibration curve for quantifying fumaric acid and calculate the concentration of fumaric acid in the samples.

Statistical analysis

All data were expressed as mean ± standard deviation ± standard deviation (SD). One-way analysis of variance and Bonferroni's post test were used to examine statistical significance. Statistical analysis was performed using the EZR software package[11].

Results

Color changes in adipocyte culture media

To confirm the variation in organic acid production by 3T3-L1 as a function of glucose concentration, we evaluated the color changes in the culture medium of 3T3-L1 adipocytes cultured in DMEM medium for 4 days at different glucose concentrations. No change in color tone was observed in the medium alone, while the supernatant cultured in low glucose became slightly lighter in color and changed to yellow in high glucose (***Fig. 1-a***). Since phenol red may have changed the color tone, the pH of the culture medium was next measured. The results showed that when 3T3-L1 adipocytes were cultured in high glucose, the pH was significantly lower than in other conditions (***Fig. 1-b***).

Determination of organic acids by qualitative reagents

The color reaction of carboxylic acids was used to measure organic acids in the culture medium. In this study, DCC was used as a condensing agent for carboxylic acids, which were converted to hydroxamic acid by condensing with hydroxylamine via DCC, and then reacted with trivalent iron chloride to form a colorful iron chelate complex that can be detected (***Fig. 2-a***). As a result of the measurement of carboxylic acids contained in organic acids using this property, a concentration-dependent increase in the amount of carboxylic acid was confirmed in the standards of fumaric acid, citric acid, and malic acid, which are representative organic acids (***Fig. 2-b***).

Comparison of accuracy of fumaric acid determination

To confirm the accuracy of the detection of carboxylic acid content by qualitative reagents, a comparison was made using the Fumarate assay kit. Concentration increased

approximately 4-fold under high glucose conditions (*Fig. 3-a*). Similarly, carboxylic acid levels were significantly increased in the supernatant of 3T3-L1 cultures grown under high glucose conditions (*Fig. 3-b*).

A strong correlation of fumarate levels was obtained between the enzymatic assay and the colorimetric method (*Fig. 3-c*).

Discussion

Previously, we measured 2SC by GC-MS, but due to the difficulty of pretreatment, we developed a system in which 2SC was measured by LC-MS/MS without pretreatment. As a result, 2SC accumulated in the brain of mice with aging, but no age-related changes were observed in the liver [12], indicating that 2SC may also be involved in age-related changes in brain function. In addition, blood 2SC increases with decreasing renal function, and a longitudinal study before and after renal transplantation showed that blood 2SC significantly decreased with transplantation, indicating that some fumaric acid is involved in the decrease in renal function [6].

Although the interaction between macrophages and adipocytes via free fatty acids is generally known in normal chronic inflammation [13], the involvement of fumaric acid with respect to inflammation has been reported in recent years. For example, it is known that inflammatory stimuli with lipopolysaccharide induce mitochondrial remodeling and increased fumaric acid production in macrophages [14] and that the fumaric acid produced induces interferon-β (IFN-β) production by macrophages [7]. Therefore, fumaric acid has been reported to be closely related to inflammatory reactions, but since fumaric acid itself is metabolized, it is not a marker of fumaric acid-derived inflammatory reactions and it is difficult to explain the relationship with inflammatory reactions. The measurement of 2SC, a derivative measurement of fumarate, is expected to become an important marker to explain fumaric acid-derived biological phenomena *in vivo*.

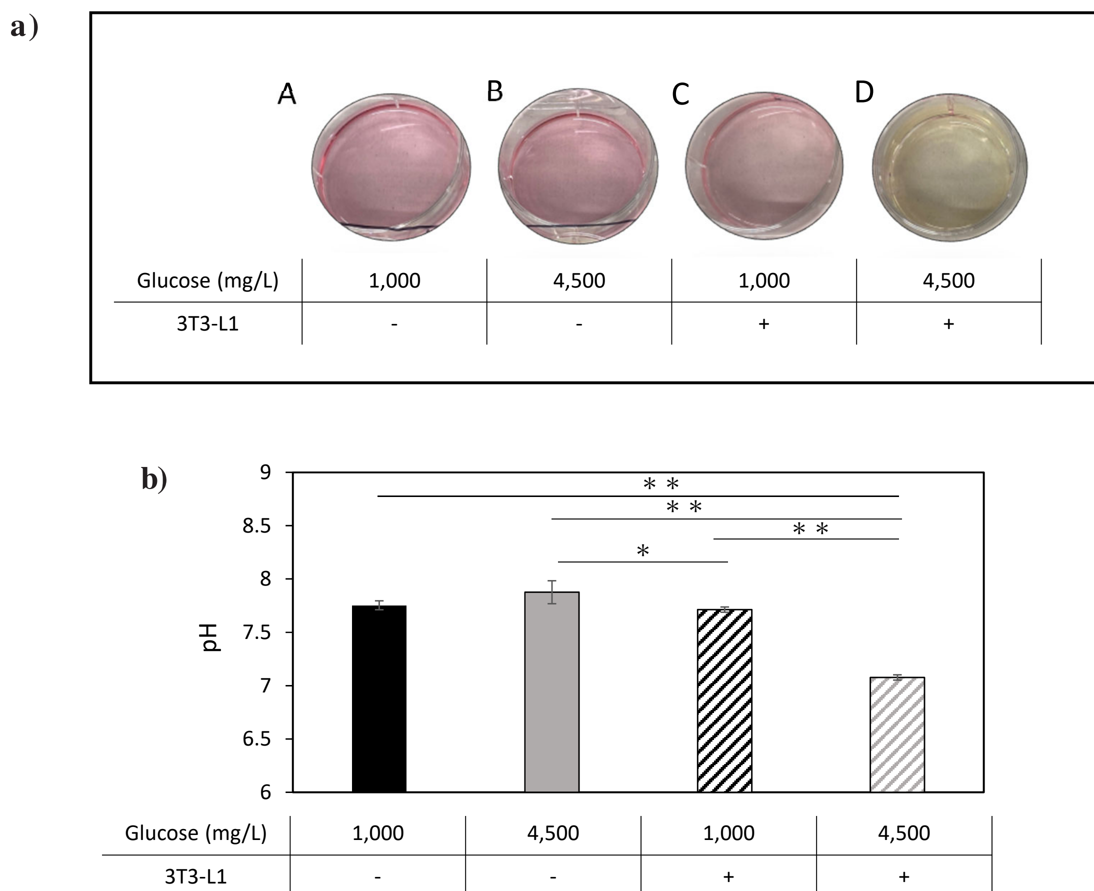
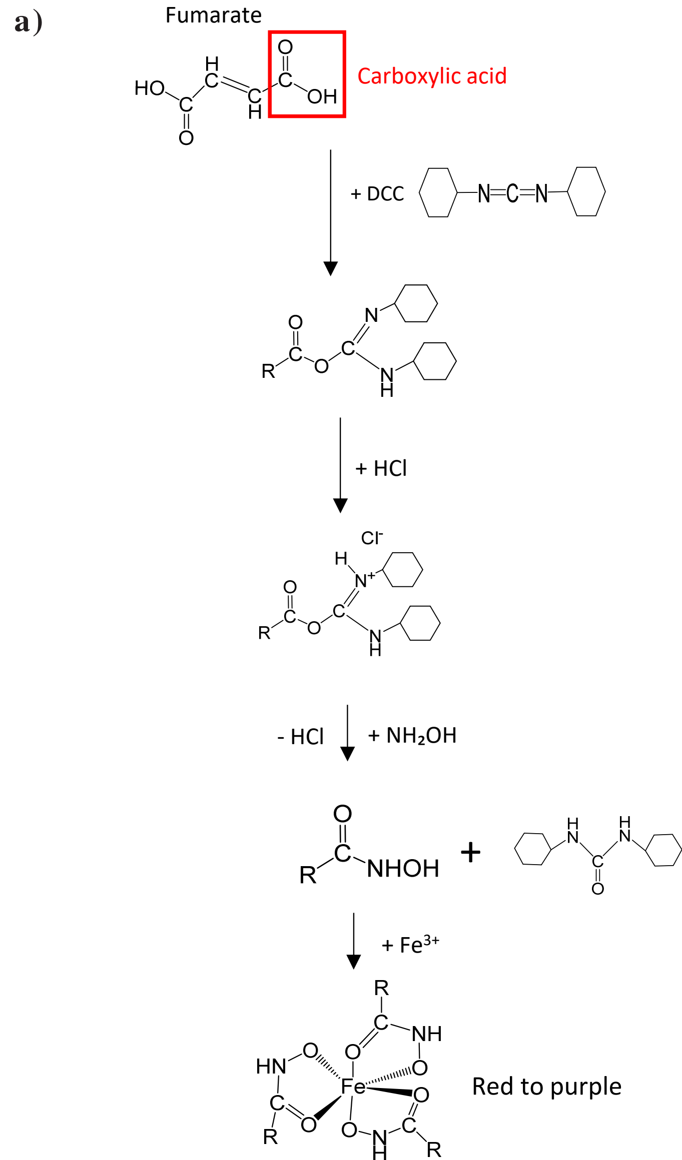


Fig. 1. **Color changes in adipocyte culture media.**

Culture medium and culture supernatant from a 4-day culture of 3T3-L1 adipocytes were collected and evaluated for color and pH changes in the medium. (**a**) Photographs of culture medium and culture supernatant. A; DMEM medium (Glucose 1,000 mg/L + FBS), B; DMEM medium (Glucose 4,500 mg/L + FBS) C; 3T3-L1 culture supernatant (Glucose 1,000 mg/L + FBS), D; 3T3-L1 culture supernatant (Glucose 4,500 mg/L + FBS). (**b**) pH value of each medium by pH meter. Data are shown as mean ± SD, *$p < 0.05$; **$p < 0.001$, n = 3. FBS, fetal bovine serum; SD, standard deviation.



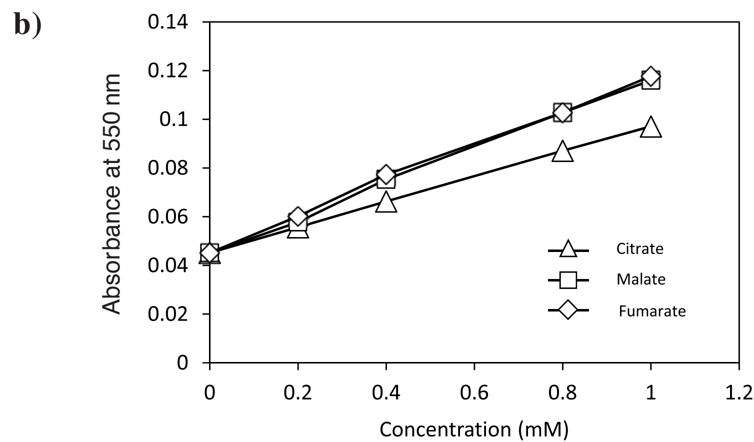


Fig. 2. **Determination of organic acids by qualitative reagents.**

(**a**) Structural changes in the color reaction of carboxylic acids with qualitative reagents. (**b**) Fumaric acid, citric acid, and malic acid were prepared at concentrations of 0.2, 0.4, 0.8, and 1.0 mM, treated ($n = 1$) with qualitative reagents, and measured at 550 nm absorbance. DCC, dicyclohexylcarbodiimide.

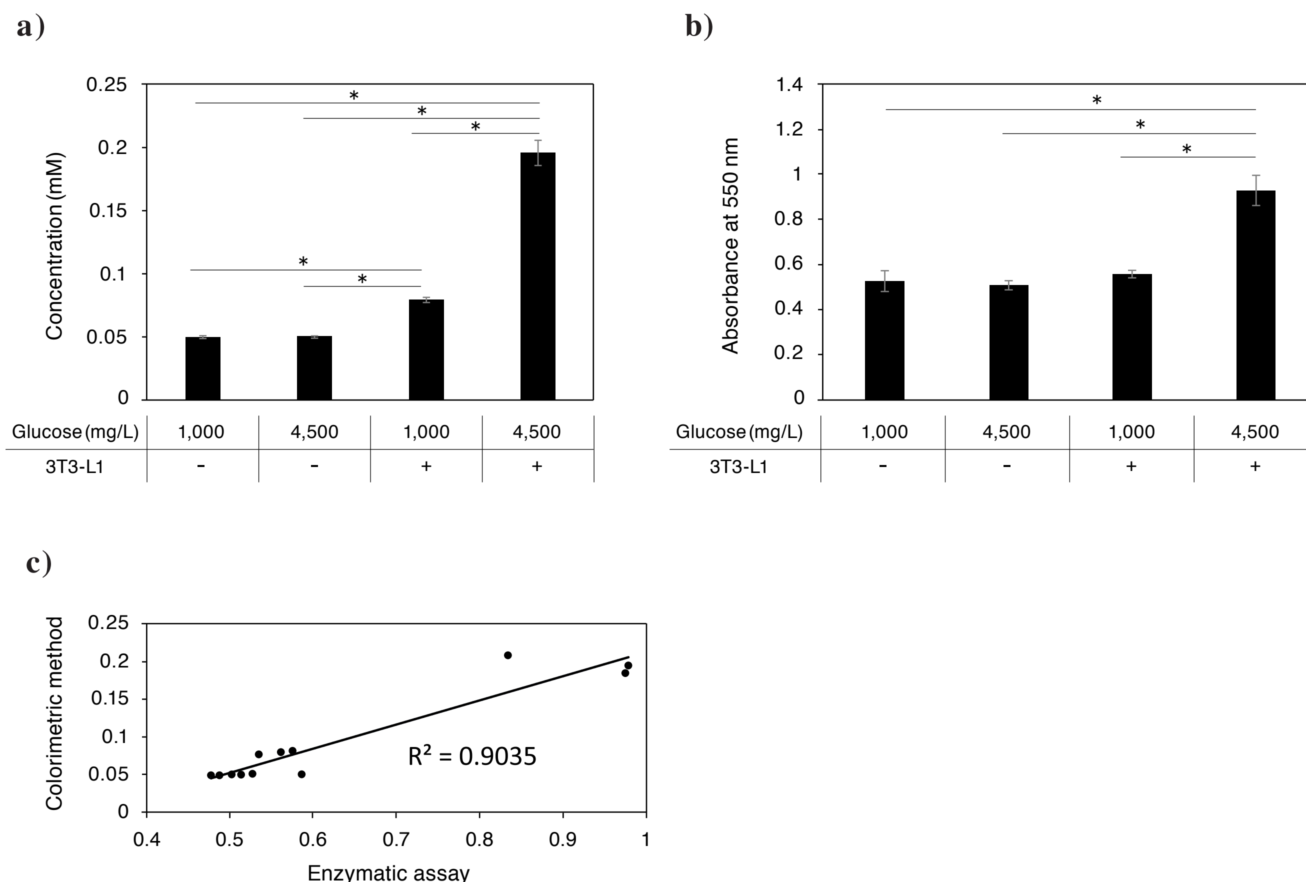


Fig. 3. **Comparison of accuracy of fumaric acid determination.**

Fumarate levels were determined in culture supernatants of 3T3-L1 cells grown in normal medium and 3T3-L1 cells differentiated and cultured for 4 days. (**a**) Fumarate determination by Fumarate assay kit. (**b**) Absorbance of color reaction using a qualitative reagent. Data are shown as mean ± SD, *$p < 0.001$, $n = 3$. SD, standard deviation. (**c**) Correlation of fumarate levels between the enzymatic assay kit and the colorimetric method.

Conclusion

The present study revealed that carboxylic acids in the medium are qualitatively measured by a colorimetric method. In order to explore the inhibition of fumaric acid production, it is necessary to culture adipocytes with high glucose and evaluate the changes in the concentration of fumaric acid in the culture medium. Previously, we derivatized fumaric acid and quantified it by GC-MS, but pretreatment and GC-MS measurements were difficult for multi-sample analysis [2]. In addition, the measurement of fumaric acid by the enzymatic method is expensive, so development of a simpler method is required. However, the evaluation based on the color change of the culture medium alone is unreliable, since the color change is also affected by bacterial infection and other factors. Therefore, we used an organic acid coloring reagent to evaluate fumaric acid production, and found a correlation between the color change of the medium and the change in fumaric acid produced by the enzymatic method. In the future, this measurement system will facilitate the search for compounds that inhibit the production of fumaric acid.

Conflict of interest statement

None.

Research grant

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (JSPS KAKENHI #20K05895).

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