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Report

Extracellular Elaidate, a Trans Fatty Acid, Tends to be Incorporated into Triglycerides and Incorporated Elaidate is Released by Lipolysis

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Intake of elaidate, an industrially produced trans fatty acid, is associated with the development of cardiovascular disease. Recently, we revealed that persistent exposure to elaidate impairs the insulin responsiveness of adipocytes. Moreover, extracellular elaidate is incorporated into phospholipids and triglycerides and exists mainly as triglycerides in adipocytes. Because fatty acids in adipocytes are not only used as an energy source but also released as cytokines to regulate cellular function and whole-body metabolism, we hypothesized that elaidate is released from adipocytes. Here, we examined the intracellular behavior of elaidate to explain that incorporated elaidate exists mainly as triglycerides, and whether it is released from adipocytes. Extracellular elaidate was incorporated into triglycerides rather than phospholipids, and elaidate incorporated into triglycerides did not decrease during the study period. Under lipolytic stimulation, incorporated elaidate—together with other fatty acids—was released from adipocytes. These results imply that adipocytes act as a reservoir of elaidate.

Key words trans fatty acid, elaidate, adipocyte, lipolysis

INTRODUCTION

Trans fatty acids (TFAs) are unsaturated fatty acids with non-conjugated trans double bonds. TFAs are produced naturally as well as industrially, and daily dietary intake of industrially produced TFAs is associated with an elevated risk of cardiovascular disease.[1] On this basis, the World Health Organization published a roadmap to food becoming 'trans fat free by 2023.[2]

Intake of industrially produced TFAs has been implicated in the development of insulin resistance and type 2 diabetes mellitus (T2DM),[3–5] and persistent exposure to a physiological concentration of elaidate, an industrially produced TFA, impairs the insulin responsiveness of cultured adipocytes.[6,7] Moreover, extracellular elaidate is incorporated into phospholipids and triglycerides and exists mainly as triglycerides in adipocytes.[8]

Intracellular fatty acids in adipocytes are not only used as an energy source but also released as cytokines to regulate cellular function and whole-body metabolism.[9,10] Therefore, we hypothesized that elaidate incorporated into triglycerides is released from adipocytes and affects the surrounding environment. In this study, we examined the behavior of elaidate in adipocytes to explain that incorporated elaidate exists mainly as triglycerides, and whether it is released from adipocytes following stimulation.

MATERIALS AND METHODS

Cell Culture and Fibroblast Differentiation into Adipocytes 3T3-L1 mouse embryo fibroblasts were purchased from the Japanese Collection of Research Bioresources (JCBR9014; Lot 0125200, Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 mg/L D-glucose (DMEM-high glucose; Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FB-1380; Biosera, Nuaille, France), 50 U/mL penicillin, and 50 µg/mL streptomycin (P7081; Sigma-Aldrich/Merck KGaA, Darmstadt, Germany). 3T3-L1 fibroblasts were seeded at 2.0×10^4/well in 24-well plates and cultured for 4 d, and post-confluent cells were differentiated into adipocytes as described previously.[6–8]

Exposure to BSA-Conjugated Fatty Acids A stock solution of BSA-conjugated elaidate (E4367; Sigma-Aldrich/Merck KGaA), deuterium-labeled stearate-d35 (BX-245; Olbracht Serdary Research Laboratories, Toronto, Canada) or deuterium-labeled stearic-18, 18, 18-d3 acid (stearate-d3; BX-224; Olbracht Serdary Research Laboratories) was prepared as described previously.[6] For temporal exposure to elaidate or stearate-d35, differentiated 3T3-L1 adipocytes were cultured with 10 µM of each fatty acid for 1, 3, or 6 h. For persistent exposure to fatty acids, BSA-conjugated elaidate, stearate-d3, or BSA alone (vehicle) was added to the medium at 10 µM for 10 d prior to induction of differentiation as well as during differentiation for 8 d.[6–8] Because deuterium labeling delays physiological reactions,[11] stearate-d35 was used to determine the amount of stearate incorporated while excluding the effects of metabolism on the amount. To avoid delay of β-oxidation, stearate-d3 was used to analyze the residual incorporated stearate.

Analysis of Fatty Acid Composition of Triglycerides, Free Fatty Acids, and Phospholipids The amount of fatty acids was analyzed as described by Ishibashi *et al.,* 2016 and

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2018.[6,8] In brief, total lipids were extracted using the Bligh & Dyer method, and triglycerides, free fatty acids, and phospholipids were separated by thin-layer chromatography (Silica Gel 60, Sigma-Aldrich/Merck KGaA) developed using petroleum ether: diethyl ether: acetic acid (82:18:1, v/v/v) and then hydrolyzed and methylated. The fatty acid content of each lipid was quantitated using a GC-MS-QP2010 Ultra (Shimadzu, Kyoto, Japan) in the selected ion-monitoring mode (*m/z* 55 for palmitoleate, oleate, and elaidate; *m/z* 74 for palmitate; *m/z* 77 for stearate-d35; *m/z* 298 for stearate; *m/z* 301 for stearate-d3). The efficiencies of extraction, isolation, and methylation were normalized to those of an internal standard.

Lipolysis Assay For stimulation with isoproterenol, 3T3-L1 adipocytes (day 7) were washed twice with phosphate-buffered saline and starved under serum-free conditions (DMEM-high glucose containing 0.5% bovine serum albumin [BSA, 017-15146; Fujifilm Wako, Osaka, Japan]) for 24 h (during days 7 to 8). Quiescent cells were washed once with Krebs–Ringer HEPES buffer (KRHB; 20 mM HEPES-NaOH, pH 7.5, 120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 0.6 mM $MgSO_4$, 2 mM $CaCl_2$, and 0.5% BSA [A7030; Sigma-Aldrich/Merck KGaA]). The cells were stimulated without or with 1 µM isoproterenol for 1 h in KRHB. The KRHB was collected and centrifuged at 200 × g for 4 min, and the supernatant was used for fatty acid analysis.

RESULTS

Extracellular Elaidate Tended to be Incorporated into Triglycerides and Remained in Adipocytes To examine the behavior of elaidate in adipocytes, we focused on the directivity of incorporation of extracellular elaidate into lipid classes and the residual amounts of incorporated elaidate. First, to examine the directivity of incorporation of elaidate, it was added into the culture medium and the amounts of elaidate in the lipid classes were measured. As shown in Fig. 1A, because there was little free elaidate, elaidate was immediately incorporated into triglycerides or phospholipids. Elaidate was incorporated more frequently into triglycerides than into phospholipids. Next, we examined the directivity of stearate incorporation using stearate-d35. In contrast to elaidate, stearate-d35 tended to be incorporated into phospholipids, implying that the directivity of elaidate to triglycerides is not a common property of fatty acids.

Next, to examine the residual amounts of incorporated elaidate, elaidate-incorporated adipocytes were cultured in the absence of elaidate for 6 d (Fig. 1B). The fraction of elaidate in phospholipids gradually decreased, but that in triglyceride did not decrease. Moreover, similar findings were obtained using stearate-d3, implying that residuality of elaidate is not specific to elaidate. Therefore, extracellular elaidate tends to be incorporated into triglycerides and not naturally released,

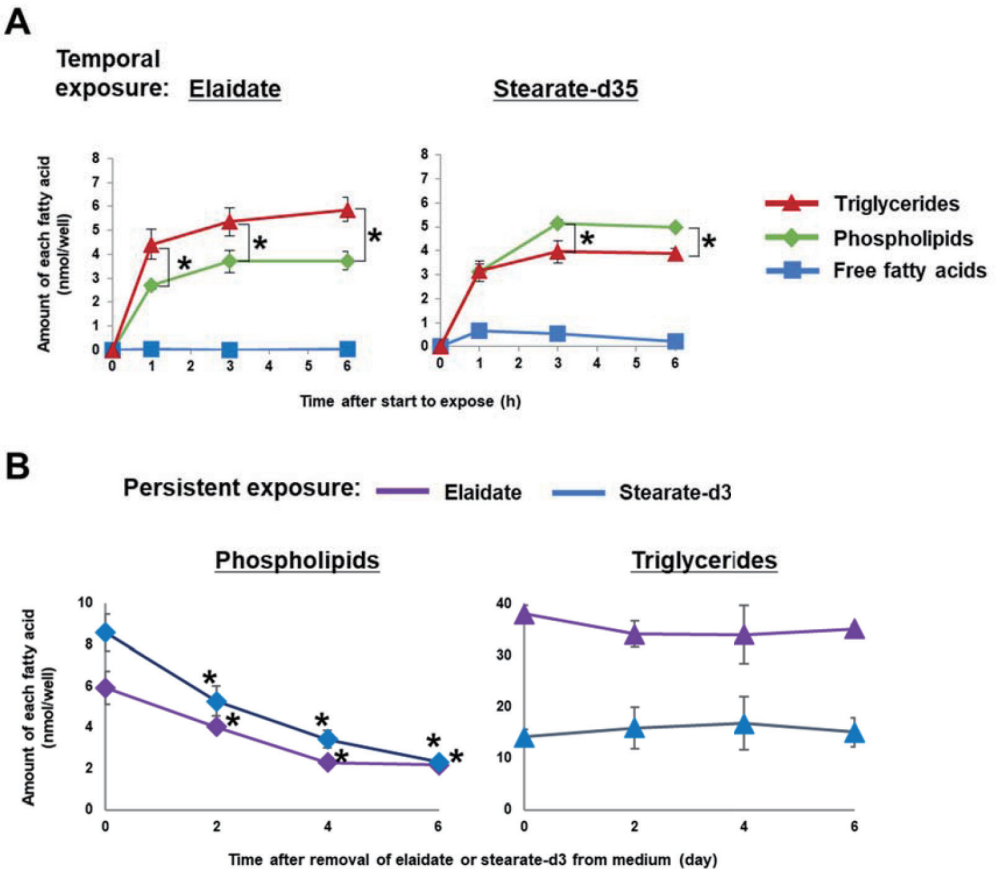

Fig. 1. Extracellular Elaidate Tended to be Incorporated into Triglycerides and Remained in Adipocytes

(A) 3T3-L1 adipocytes were exposed to 10 µM elaidate or stearate-d35 for 1, 3, or 6 h. Values are means ± SD (n = 3). Asterisk indicates significant differences (*$p < 0.05$) by Student's *t*-test. (B) 3T3-L1 adipocytes were persistently exposed to 10 µM elaidate or stearate-d3 before and during differentiation for 18 d. The cells were washed with PBS and administered fresh medium lacking elaidate or stearate-d3, which was refreshed every 2 d. The amount of elaidate or stearate-d3 in triglycerides and phospholipids was measured at the indicated times. Values are means ± SD (n = 3). Asterisks indicate significant differences (*$p < 0.05$; *versus* 0 h) by Student's *t*-test.

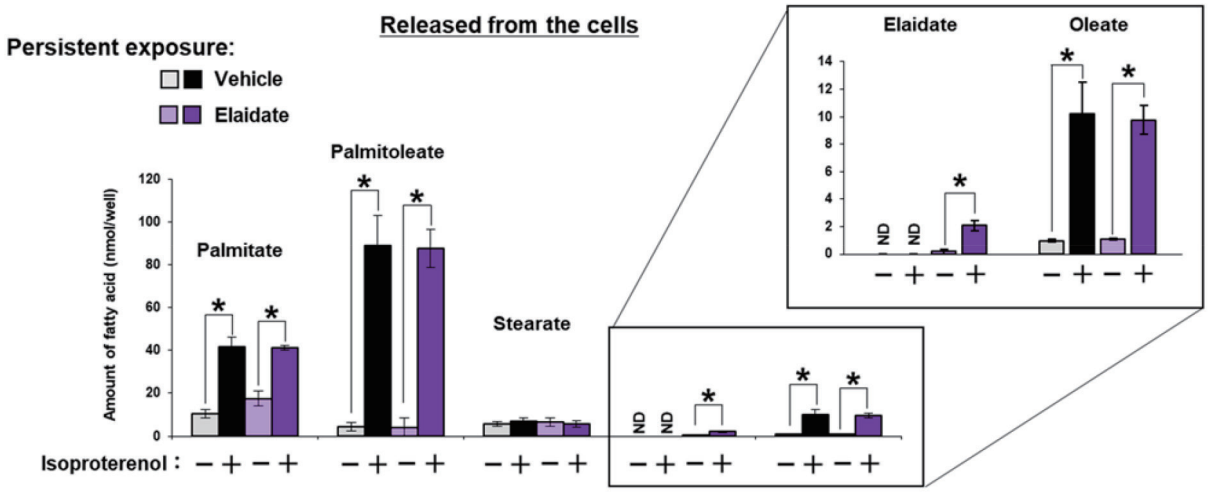

Fig. 2. Elaidate Incorporated into Adipocytes is Released by Lipolytic Stimulation

3T3-L1 adipocytes were persistently exposed to 10 μM elaidate or vehicle before and during differentiation. Quiescent cells were stimulated with isoproterenol in KRHB, and the fatty acid content of the KRHB was quantified. Values are means ± SD (n = 3). ND, not detected. Asterisks indicate significant differences (*$p < 0.05$) by Student's *t*-test.

resulting in intracellular elaidate existing mainly as triglycerides.

Incorporated Elaidate is Released by Lipolytic Stimulation Lipolytic stimulation enhances the release of fatty acids from adipocytes. Therefore, we examined whether lipolytic stimulation enhances the release of elaidate from elaidate-incorporated cells. Stimulation of vehicle-exposed cells with isoproterenol increased the amounts of palmitate, palmitoleate, and oleate in the supernatant (Fig. 2). When elaidate-exposed cells were stimulated with isoproterenol, the amount of elaidate in the supernatant increased. These results imply that elaidate-incorporated adipocytes supply elaidate under lipolytic conditions, such as starvation.

DISCUSSION

Although dietary intake of elaidate has been evaluated and incorporation of elaidate into cells has been revealed, the amount of elaidate incorporated into cells remains unclear. Here, we report that adipocytes may act as a reservoir of elaidate ingestion. Extracellular elaidate tended to be incorporated into triglycerides in adipocytes, and incorporated elaidate was released by lipolytic stimulation. Moreover, the amount of elaidate in triglycerides did not decrease naturally.

Adipocytes isolated from obese individuals release 2.5–4 μmol free fatty acids per hour from 10^7 cells under basal conditions.[12] Because humans have approximately 4 to 8×10^{10} adipocytes,[13] adipose tissue can release 10–20 mmol fatty acids per hour. Given that elaidate accounts for 1.4% of the released fatty acids (Fig. 2), 0.14–0.28 mmol elaidate is released by human adipose tissue, potentially reaching millimolar concentrations in the blood and possibly higher near adipocytes. Macrophages are recruited to adipose tissue under inflammation,[14] and bone-marrow-derived macrophages are activated by 1–100 μM elaidate for 24 h *in vitro*,[15] implying that elaidate released from adipocytes activates macrophages and enhances inflammation.

Elaidate in triglycerides lacks a specific removal system, resulting in accumulation of elaidate in adipocytes, which may be released during starvation. Therefore, in the development of cardiovascular disease and diabetes, the amount of elaidate accumulated in adipose tissue is as important as its ingestion.

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Conflict of interest The authors declare no conflict of interest.

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