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Structure-dependent and receptor-independent increase in osmotic fragility of rat erythrocytes by short-chain fatty acids

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Abstract

We examined short-chain fatty acids (SCFAs) with 1 (C1) to 5 (C5) carbon atoms for osmotic fragility (OF) in isolated red blood cells (RBCs) in rats. The RBCs were used as prototypical plasma membrane model. The dense packed RBC was incubated in a phosphate–NaCl buffer solution containing each SCFA at 0 to 100 mM. The RBC suspensions were transferred into the OF test tubes containing NaCl from 0.2 to 0.9%. The hemoglobin concentration was determined and the EC50 in hemolysis was calculated. The OF in RBCs was dose-dependently increased by exposure to SCFAs, except for C1, with an increasing number of carbon atoms. Branched-chain fatty acids (isomers of C4 and C5) have a smaller effect on OF than straight-chain fatty acids (C4 and C5). The SCFA-induced increases in OF were not affected by pretreatment of RBCs with trypsin. The response of the RBC membrane to SCFAs depends on their concentration, carbon chain length and chain structure (straight or branched). The SCFAs probably disturb the lipid bilayer of the RBC membrane and result in a decrease in osmotic resistance. The plasma membrane in rat RBCs could respond to the structure of the SCFAs in detail by using the OF as an indicator.

Keywords: Short-chain fatty acids; Erythrocyte; Membrane; Lipid bilayer; Osmotic fragility; Rat

1. Introduction

Short-chain fatty acids (SCFAs), a mono-carboxylic acid with a number of hydrocarbon chains, are the end-products of microbial fermentation in the forestomach or the large intestine in a variety of species [1]. In addition to acting as a nutritional or energy source, SCFAs have various biological activities both in vivo and in vitro. SCFAs are known to play an important role as humoral factors that stimulate the endocrine and exocrine pancreas in animals [2]. SCFAs also stimulate colonic contraction [3] and Cl^- secretion in rats [4]. SCFAs enhance local anesthesia both in vivo and in vitro [5], and directly affect the cell proliferation cycle in vitro [6]. The mechanism for the above mentioned actions on cells or tissues has not yet been completely clarified.

Recently, SCFAs have been shown to induce intracellular Ca release via specific receptors and activate leukocytes in humans and mice [7]. On the other hand, it has been reported that SCFAs directly affect the cell membrane of nerves and enhance anesthetic drug absorption [8]. Butyric acid has been shown to change the fluidity to the cell membrane of colon cancer cells [9]. These reports indicate the possibility that SCFAs change the phospholipids of the cell membrane so as to allow biological action in cells.

SCFAs have hydrophobic hydrocarbon chain and hydrophilic carboxylic tail in their molecules. SCFAs can function as a detergent and interact with the cell membrane via hydrocarbon chains both in the molecules and in the phospholipid bilayer. Since red blood cells (RBCs) have a basic plasma membrane structure, they are thought to be useful as a model for determining the interaction between SCFAs and cell membrane in vitro. Thus, we examined the effect of SCFAs with 1 (C1) to 5 (C5) carbon atoms on osmotic fragility (OF) in RBCs in vitro. To determine the

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involvement in the outer protein of the RBC membrane, trypsin-treated RBCs were also used. The effect of the hydrocarbon chain structure in the molecules was compared using normal SCFAs and their isomers.

2. Materials and methods

2.1. Animals

This study was approved by the Hokkaido University Animal Committee and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals. Male Sprague–Dawley rats (6 weeks old, Japan SLC, Shizuoka, Japan) were housed in individual stainless-steel metabolic cages. The cages were placed in a room with controlled temperature (22–24 °C), relative humidity (40–60%) and lighting (light 0800–2000 h). The animals had free access to tap water and a solid laboratory diet (CE-2, Japan Clea, Tokyo, Japan) for more than 1 week before the start of the experiments. The rats (250–290 g) were used in the experiments at 7 to 8 weeks old.

2.2. Reagents

Biochemical grade formic acid (C1), acetic acid (C2), propionic acid (C3), butyric acid (C4), *iso*-butyric acid (*iso*-C4), valeric acid (C5) and *iso*-valeric acid (*iso*-C5) were purchased from Wako Pure Chemical (Osaka, Japan). Trypsin (EC3.4.21.4, Type II-S from the porcine pancreas) was purchased from Sigma (USA) and used for the digestion of protein on the surface of the RBCs. Other reagents used were all biochemical grade.

2.3. Preparation of RBCs

On the day of the experiment, the rats were anesthetized with pentobarbital sodium (30 mg/kg). Blood (8–10 ml) from the abdominal aorta was collected into a heparinized test tube. The RBCs were separated from plasma by centrifugation at $2000 \times g$ for 15 min at 4 °C. The crude RBCs were then washed three times with 2 volumes of cold 0.9% NaCl solution. A packed cell suspension was obtained and thereafter kept in ice-cold water until the following treatment.

To evaluate the trypsin treatment, 1.5 ml of the dense packed cell suspension was transferred into a 3.0 ml phosphate–NaCl buffer solution (pH 7.4) containing trypsin at 0.2 mg/ml (T (+)). The same packed cell suspension incubated without trypsin was used as control (T (−)). Both RBC suspensions were incubated 37 °C for 30 min in a water bath. The RBCs were collected by centrifugation at $1300 \times g$ for 10 min at room temperature. The dense packed cell suspension was kept in ice cold water until the next SCFA treatment.

2.4. Experimental procedure

The dense packed cell suspension (0.2 ml) was transferred into 2.0 ml of a phosphate–NaCl buffer solution (pH7.4) containing each SCFA at 0, 0.1, 0.3, 1, 3, 10, 30 and 100 mM. The osmolarity was regulated by the amount of NaCl added into the buffer solution when each SCFA was applied. To evaluate the interaction with the surface protein of the membrane, the RBC suspensions treated with or without trypsin were incubated with 100 mM C4 or 30 mM C5. To compare the effects of straight- and branched-chain SCFA, the osmotic resistance was tested after treatment with C4 and *iso*-C4 treatment, and C5 and *iso*-C5. All RBC suspensions used for SCFA treatment were incubated at 37 °C for 1 h. The RBC suspension was mixed gently, after incubation, and then 0.1 ml of volume was transferred into 5-ml OF test tubes containing an NaCl solution ranging from 0.2 to 0.9%. The test tube was immediately centrifuged at $1300 \times g$ for 10 min at room temperature. The supernatant containing various concentrations of hemoglobin derived from the hemolytic RBCs was determined calorimetrically at 540 nm.

2.5. Statistical analysis

Complete hemolysis of the RBC suspension occurred in the 0.2% NaCl solution, in which hemoglobin concentration was defined as 100%. Hemolysis of the RBCs did not occur in a 0.9% NaCl solution, and was defined as 0%. The effective concentration of NaCl solution inducing 50% hemolysis (EC50) of the applied RBCs was calculated from the OF curve by using straight-line equation between the adjacent below and above points to 50% of hemolysis. All values are expressed as means ± SEM. Statistical analysis was performed by two-way or three-way ANOVA followed by Turkey's HSD test. A difference with $P < 0.05$ was considered significant.

3. Results

Typical OF curves were shown for untreated RBCs (control) and the RBCs treated with 30 or 100 mM C4 (Fig. 1). The EC50 of hemolysis was increased by the exposure of RBCs to C4 in rats. The EC50 of hemolysis in rat RBCs was 0.406 ± 0.005 for the control, 0.437 ± 0.005 for 30 mM C4, and 0.462 ± 0.005 for 100 mM C4 treatment, respectively. The OF curve was shifted to the right with increase in C4 concentration.

The dose–response relationships were presented between SCFA concentrations and OF values in rat RBCs (Fig. 2). Significant effects were obtained in SCFAs ($P < 0.001$), doses ($P < 0.001$) and their interaction (SCFAs × doses, $P < 0.001$) by using two-way ANOVA. Significant increases in OF were induced at more than 100 mM for C2, 30 mM for C3 and C4, and 10 mM for C5 ($P < 0.05$,

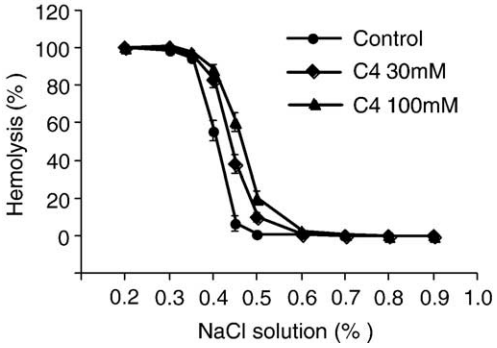

Fig. 1. Osmotic fragility (OF) curve of rat RBCs treated with butyrate (C4) in rat RBC. The OF curve of rat RBCs was shifted right by exposure to C4. (Data in low concentrations are not shown.) Values are means ± SEM ($n=6$).

Turkey's HSD test). Significant change in OF was not induced by C1 application up to a dose of 100 mM. The treatment with SCFAs decreased osmotic resistance in rat RBCs in a dose-dependent manner. The OF induced by

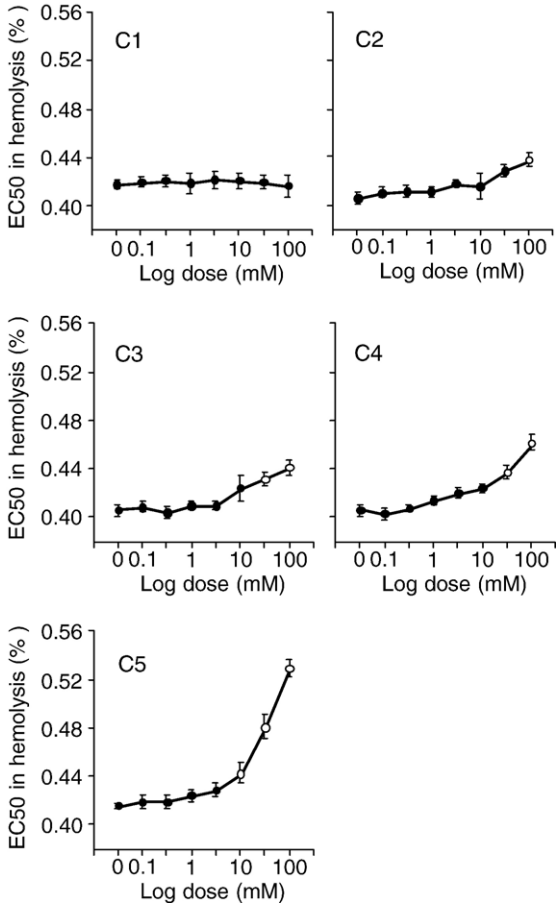

Fig. 2. Changes in OF in rat RBCs by application of SCFAs with 2 to 5 carbons. C1, formic acid; C2, acetic acid; C3, propionic acid; C4, butyric acid; C5, valeric acid. Values are means ± SEM ($n=6$). Significant effects were obtained in the effects of SCFAs ($P<0.001$), doses ($P<0.001$) and their interaction (SCFAs × doses, $P<0.001$) by two-way ANOVA. Open symbols indicate a significant difference from the control value in (0 mM SCFA) by using Turkey' HDS test ($P<0.05$).

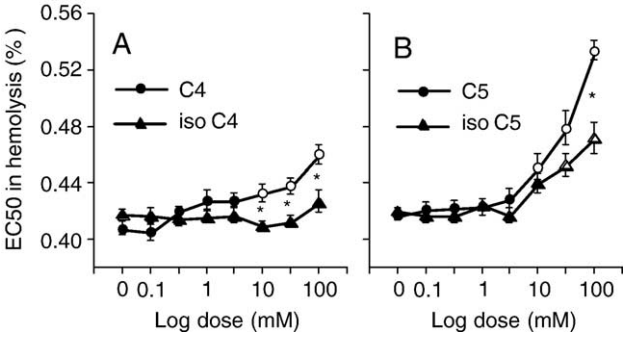

Fig. 3. Effect of straight and branched SCFAs on OF in RBC. (A) C4 and *iso*-C4, (B) C5 and *iso*-C5. Values are means ± SEM ($n=5$). Significant effects were obtained in SCFAs ($P<0.001$), form of hydrocarbon chain ($P<0.001$), doses ($P<0.002$), carbon number × form of hydrocarbon chain ($P<0.001$), carbon number × doses ($P<0.001$), form of hydrocarbon chain × doses ($P<0.001$) and carbon number × form of hydrocarbon chain × doses ($P<0.001$) by three-way ANOVA. Open symbols indicate a significant difference from the control value in (0 mM SCFA) by using Turkey's HDS test ($P<0.05$). Asterisks represent a significant difference in OF between in straight and branched SCFAs having the same number of carbons by using Turkey's HDS test ($P<0.05$).

SCFA exposure was increased in a chain-length dependent manner from C2 to C5.

The effects of straight-chain and branched-chain SCFAs on OF in rat RBCs were compared (Fig. 3A and B). Significant effects were obtained in SCFAs ($P<0.001$), form of hydrocarbon chain ($P<0.001$), doses ($P<0.002$), carbon number × form of hydrocarbon chain ($P<0.001$), carbon number × doses ($P<0.001$), form of hydrocarbon chain × doses ($P<0.001$) and carbon number × form of hydrocarbon chain × doses ($P<0.001$) by using three-way

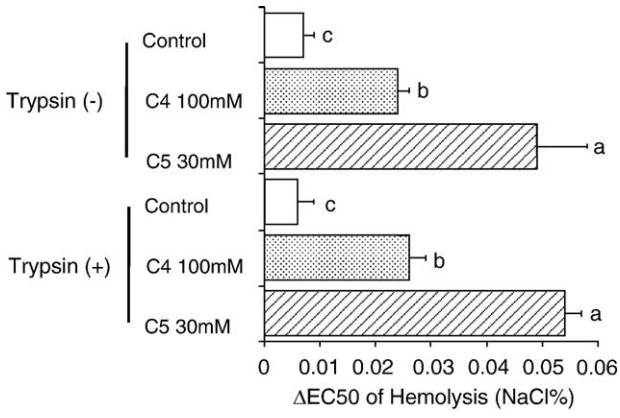

Fig. 4. Effect of pretreatment with trypsin on the SCFA-induced increase in OF in rat RBCs. Values are means ± SEM ($n=5$). The increment in OF (ΔEC50) was calculated as the difference between the value before trypsin treatment ((+) or (−)) and the value after trypsin treatment ((+) or (−), control) followed by exposure to SCFAs (100 mM C4 or 30 mM C5). Two-way ANOVA was used to detect the effects of trypsin treatment, exposure to SCFAs (control, 100 mM C4 and 30 mM C5) and their interaction (trypsin treatment × exposure to SCFAs). Significant effect was obtained in exposure to SCFAs ($P<0.001$), but not in trypsin treatment ($P=0.604$) or their interaction ($P=0.709$). Mean values not sharing a common letter are significantly different among groups according to Turkey's HSD test ($P<0.05$).

ANOVA. Dose–response curves indicated that the effects of normal C4 and C5 were larger than those of *iso*-C4 and *iso*-C5 ($P<0.05$, Turkey's HSD test). Thus, the branched-chain acids appear to exhibit weaker activity for increasing OF in rat RBCs.

Applying 100 mM C4 and 30 mM C5 induces an increased OF in RBCs (Fig. 4). The increment in OF (ΔEC50) was calculated as the difference between the value before trypsin treatment ((+) or (−)) and the value after trypsin treatment ((+) or (−), control) followed by the exposure to SCFAs (100 mM C4 or 30 mM C5). Two-way ANOVA was used to detect the effects of trypsin treatment, exposure to SCFAs (control, 100 mM C4 and 30 mM C5) and their interaction (trypsin treatment × exposure to SCFAs). A significant effect was obtained in exposure to SCFAs ($P<0.001$), but not in trypsin treatment ($P=0.604$) or their interaction ($P=0.709$). Trypsin treatment itself did not affect the OF in rat RBCs in control. The OF in rat RBCs after exposure to 100 mM C4 or 30 mM C5 was also not affected by pre-treatment of trypsin.

4. Discussion

We have demonstrated that SCFAs with 2 to 5 carbons, except for C1, decreased osmotic stability in rat erythrocytes in vitro. The increase in OF depended on the number of carbon atoms in the SCFA molecules. Transformations of the hydrocarbon chain influenced the OF in rat RBCs. Branched-chain acids (*iso*-C4 and *iso*-C5) had a smaller effect on OF than the straight-chain acids (C4 and C5). The SCFA-induced increases in OF were not affected by pretreatment RBCs with trypsin.

With regard to the carbon-number dependency of the biological activity in SCFAs, the endocrine [10] and exocrine [11] response to SCFAs also depends on the number of carbon atoms in their molecules, as does intracellular Ca release in leukocytes [7]. SCFAs possessing more than four carbons have branched-chain isomers. The difference in biological response to SCFAs with straight- or branched-hydrocarbon chains has also been observed in endocrine pancreatic response to SCFAs in ruminant animals [12].

Specific recognition sites for SCFAs are hypothesized to exist on the surface of the exocrine cells of the pancreas in sheep, goats [13] and guinea pigs [14]. Since trypsin treatment abolished octanate (C8)-induced amylase release from the pancreatic fragment in guinea pigs, the surface protein in the exocrine cell is probably involved in the biological interaction with SCFAs [14]. Yajima showed that a receptor site for SCFAs existed on the epithelium and that this mechanism played a role in controlling the electrolyte secretion and motility of the hindgut in rats [15]. Recently, SCFA receptors, or G-protein coupled receptors (GPR) 41 and GPR43, have been identified and characterized [16].

In our experiment, trypsin treatment did not affect the SCFA-induced increases in OF. The dose of trypsin used in this study was two-fold of dose that abolished C8-induced amylase release from the pancreatic fragments in sheep and goats [14]. This result means that the surface protein, including specific SCFA receptor, in the RBC membrane is not involved in this increase in OF. SCFAs directly affect the cell membrane of nerves and enhance anesthetic drug absorption [8]. Butyric acid changes the fluidity to the cell membrane of colon cancer cells [9]. The possibility, therefore, arises that SCFAs affect the phospholipids of the cell membrane so as to allow biological action in cells.

The change in osmotic resistance is probably due to the detergent-like characteristics of SCFAs with a hydrophobic hydrocarbon chain and a hydrophilic carboxylic tail. The hydrocarbon chains in SCFAs can enter into the RBC membrane and interact with phospholipids in outer membrane layer. The strength of the RBC membrane probably changes after exposure to SCFAs, and results in a decrease in osmotic resistance. In addition, the present results indicate that not only the length, but also the form (straight or branched-chain) of hydrocarbon chain in the SCFA molecules also affect the phospholipids in the RBC membrane.

In a previous report, lactic acid, which has a monocarboxylic acid in the molecule, was shown to affect the osmotic resistance in RBCs [17]. Long-chain fatty acids (palmitic, stearic, linolenic or arachidonic acids) also affect osmotic resistance in RBCs [18,19]. The temperature during incubation periods also influences these effects [19]. Fatty acid concentration in the blood affects the fatty acid composition in the membrane and results in changes in the OF in RBCs [20]. These reports indicate that lipids in the plasma affect the phospholipids of RBC membrane and result in the changes in osmotic resistance.

The response of the RBC membrane to SCFAs depends on their concentration, carbon chain length and chain structure (straight or branched). Thus, the RBC membrane is probably a useful model for studying interactions between SCFAs or their related materials, and the outer cell membrane, since the structure of hydrocarbon chain obviously affected the interaction with the RBC membrane.

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