Chemical specificity in short-chain fatty acids and their analogues in increasing osmotic fragility in rat erythrocytes in vitro

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Abstract

We examined the role of the chemical specificity of short-chain fatty acids (SCFAs) and their derivatives in increasing osmotic fragility (OF) in rat red blood cells (RBCs). Except for formic acid, normal SCFAs with 2 to 8 carbons increased the OF in rat RBCs with increasing number of hydrocarbons in a dose-dependent manner. Replacement of the carboxylic group with sulfonic group inhibited, but did not abolish, the SCFA-mediated increase in OF. Introduction of another carboxylic group (dicarboxylic acids) completely abolished the SCFA-mediated increase in OF. Transformation of the hydrocarbon chains in SCFAs from straight to branched or cyclic chains affected the degree of the OF-increasing effect. Introduction of double- or triple-carbon bonds to the hydrocarbon chain in parent SCFAs did not affect the increase in OF. Both hydrophilic (carboxylic group) and hydrophobic elements (hydrocarbons) at opposite sides of a molecule were required to affect the RBC membrane, and the size and form of hydrophobic element were important factors in determining the SCFA-mediated increase in OF. The hydrocarbon chains probably enter the plasma membrane, with the hydrophilic carboxylic base remaining outside of the membrane, and interact with phospholipid in cell membrane and disturb the structure of lipid layer resulting in the increase in OF in the rat RBCs.

Key words, SCFA, RBC, erythrocyte, membrane, osmotic fragility, rat

1. Introduction

Short-chain fatty acids (SCFAs) are the products of microbial fermentation in the digestive tract, and these compounds provide an energy source in a variety of species [1]. SCFAs, which are a group of mono-carboxylic acids that contain a hydrocarbon chain of various lengths, have a variety of biological actions both in vivo [2-5] and in vitro [6-12]. Generally, however, the mechanism of these biological actions on cells or tissues has not been completely clarified. Recently, SCFAs have been reported to induce intracellular Ca release via specific receptors and to activate leukocytes in humans and mice [13, 14].

In our previous report, we showed that the osmotic fragility (OF) in rat red blood cells (RBCs) is a useful indicator for evaluating the interaction of fatty acids and the cell membrane in vitro [15]. We also clarified that SCFAs with 2 (C2) to 5 (n-C5) carbons (including the carbon atoms of the carboxylic group) increase OF in rat erythrocytes. The effect on OF occurred in an SCFA concentration-dependent manner and was also dependent on the number of hydrocarbon chains in the SCFA moiety. Although, in our previous study, the most effective SCFA was n-valeric acid (n-C5), there are no reports available on the effects of carboxylic acid with hydrocarbon chains over 6 carbons on the OF in rat RBCs. Since iso-C4 and iso-C5, both possessing branched hydrocarbon chains, demonstrate weaker effects on OF than do normal n-C4 and n-C5 with straight hydrocarbon chains, the transformation of hydrocarbon chain was confirmed to affect the SCFA-mediated increase in OF. In addition, as the SCFA-mediated increase in OF was not abolished by using trypsin-treated RBC, it is evident that the outer protein in the RBC membrane is not involved in the increase in OF. Thus, SCFAs probably affected the RBC cell membrane directly and resulted in a

decrease in osmotic resistance. We hypothesize that the fatty acids interact with the lipid bilayer of cell membrane via their hydrocarbon chains, resulting in the induction of various biological actions [15]. To clarify the mechanism of the SCFA-mediated increase in OF, further information is required concerning the structure-activity relationships between SCFAs and their effect on OF in rat RBCs.

Thus, we examined the effect of SCFAs with 1 (C1) to 8 carbons (n-C8) and their chemical derivatives, and compared their effects on OF in rat RBCs. RBCs were used as a prototypical cellular model system to examine chemical-mediated effects on the plasma membrane. These determinations have revealed the required elements in SCFA molecules for affecting the lipid bilayer in the cell membrane and increasing OF in rat RBCs.

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 (22-24ºC), 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), n-butyric acid (n-C4), n-valeric acid (n-C5), n-caproic acid (n-C6), n-enathic acid (n-C7), n-capric acid (n-C8) and their derivatives are were purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan) or Wako Pure chemical Co., Ltd. (Osaka, Japan). Other reagents used in this study were all biochemical grade.

2.3. Preparation of red blood cells

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

2.4. Experimental procedure

The dense packed cell suspension (0.2ml) was transferred into 2.0ml 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 compare the effect of straight-chain fatty acids (parent chemical) and their derivatives, OF determination was undertaken using RBCs obtained from the same rat. All RBC suspensions used for the SCFA treatment were incubated at 37ºC for 1 hr. The RBC suspension was mixed gently after incubation, and then 0.1ml of volume were transferred into 5-ml OF test tubes containing a NaCl solution ranging from 0.2 to 0.9%. The test tube was immediately

centrifuged at 1300 g for 10 min at room temperature. The supernatant containing various concentrations of hemoglobin derived from the hemolysed RBCs was determined colorimetrically at 540 nm.

2.5 Statistical analysis

Complete hemolysis of the RBC suspension occurred in the 0.2% NaCl solution, for which the hemoglobin concentration was defined as 100%. Hemolysis of the RBCs did not occur in the 0.9% NaCl solution, for which the hemoglobin concentration was defined as 0%. The effective concentration of the NaCl solution inducing 50% hemolysis (EC_{50}) of the applied RBCs was calculated from the hemolysis curve by using straight-line equation between the points immediately adjacent to 50%. To compare the changes in OF induced by different compounds, differences among the control values and those induced by exposure to compounds were obtained as a ΔEC_{50} (difference in %). All values are expressed as means ± SD. Statistical analyses were performed by paired *t*-test (2 groups) or by Bonferroni's test (3 groups or more). A difference with $P < 0.05$ was considered significant.

3. Results

3.1. Osmotic fragility (OF) curve for rat RBCs

Changes in the typical hemolytic curve for rat RBCs exposed to SCFAs are shown in Fig. 1. The curves were shifted right in parallel manner with increases in the numbers of carbon atoms in the SCFAs (Upper panel; C3, n-C5 and n-C7 at 100mM) and the SCFA dose (Lower panel; n-C6 at 1, 10 and 100mM). Fifty percent hemolysis of the added RBCs was defined as EC_{50} and used as a parameter of OF The values

described below were obtained from the hemolytic curve of each application of SCFAs and their derivatives.

3.2. Straight SCFA-induced changes in OF in RBCs

Dose-response relationships between the concentrations of SCFAs with 1 to 8 carbons and their effects on the EC_{50} in rat RBCs are shown in Fig. 2. The OF was increased by exposure to SCFAs in a dose-dependent manner and with increases in number of carbon atoms in their molecules, except for C1. The increase in OF induced by n-C6, n-C7 or n-C8 was larger than that of other SCFAs with a smaller number of carbons. The threshold concentration, inducing significant changes in the OF, varied between SCFAs and was lowest for n-C8 (0.3mM) among the tested SCFAs. The value for n-C8 applied to rat RBCs at 100 mM was not determined, as the RBC hemolyzed immediately after the n-C8 application and before OF determination. Since SCFAs with above 4 carbons strongly enhance increase in OF ($P<0.05$), we therefore used n-C4 to n-C7 and their isomers or derivatives at 30 or 100 mM to evaluate structure-activity relationships.

3.3. Requirement of a carboxylic group

Replacement of the carboxylic group (-COOH) with a sulfonic group ($-SO_2OH$) in n-C5 and n-C7 is shown in Table 1. Replacement with a sulfonic group ($-SO_2OH$) did not abolish the parent SCFA-mediated increase in OF, but did decrease the degree of OF which induced by the application of parent SCFAs. However the effect of the transformation in n-C7 was larger than that in n-C5. Introduction of an additional carboxylic group (dicarboxylic acids) drastically abolished or preferably reduced the parent SCFA-mediated increase in OF (Table 2).

1

2 3.4. Effects of the transformation of hydrocarbon chains

3 Effects of the transformation of the hydrocarbon chain are summarized in Table 3,
4 4, and 5. SCFAs possessing more than 3 carbon atoms in their hydrocarbon chains have
5 branched-chain isomers of the straight-SCFAs. All of the isomers examined in this
6 experiment increased OF in the rat RBCs (Table 3). All of the isomers with 3 or 4
7 hydrocarbons increased OF to a lesser degree than did their parent SCFAs. Although,
8 most of the isomers with 5 hydrocarbons had a lower impact on OF than did their parent
9 SCFA (n-C6), 4-Methyl-n-valeric acid induced a larger in increase in OF than did n-C6.
10 Exposure to 100 mM 4-Methyl-n-valeric acid induced hemolysis of rat RBCs in 0.9%
11 NaCl solution but no EC_{50} value was obtained. Cyclic SCFAs with ring-like
12 hydrocarbon chains of 3 to 6 carbons atoms in length also increased OF (Table 4).
13 However the degree of increase in OF induced by cyclic SCFAs with 3 to 5
14 hydrocarbons was lower than those of their respective parent SCFAs. Cyclic SCFAs
15 with 6 hydrocarbons and Benzoic acid, which is a cyclic SCFA with a double carbon
16 bond (benzene ring), also showed an ability to increase OF. Although
17 cyclohexane-carboxylic acid increased OF to a greater degree than did straight SCFA
18 (n-C7), benzoic acid was less active than n-C7. Unsaturated mono-carboxylic acids
19 possessing a double carbon bond, such as crotonic and 3-butenoic acid with 3 carbons
20 or tetrolic acid with a triple carbon bond in a hydrocarbon chain, also increased OF
21 (Table 5). Though effect of 100mM tetrolic acid was slightly larger than that of its
22 parent SCFA (n-C4), there were no other significant differences between the parent
23 SCFA (n-C4) and unsaturated SCFAs with 3 hydrocarbons

24

25 Discussion

In our previous report, we found that exposure to SCFAs (C1 to n-C5) changes osmotic resistance in rat RBCs in a carbon number-dependent manner [15]. The changes in the OF were determined by the degree of hemolysis induced by the changes in osmotic pressure using a step-up protocol with increasing concentrations of NaCl. The method is extremely simple and there is little possibility of error based on experimental and analytical procedure. Thus, we proposed that OF in rat RBCs could be used as an indicator of the interaction between SCFAs and the biomembrane [15]. In this experiment, we determined the structural requirements of SCFAs (C1 to n-C8) to induce an increase in OF in rat RBCs by using a wide range of SCFAs and derivatives

In the present study, straight chain fatty acids with 2 to 8 carbon atoms increased the OF in rat RBCs. This increase in OF was dependent on the concentration of the SCFA applied and on the number of carbons in the SCFA molecules (Fig. 2). Application of n-C8 at 100mM immediately induced hemolysis and no OF value could be determined (EC_{50} for hemolysis obtained by various concentrations of NaCl solution). These results indicate that the membrane of rat RBC is also be affected by the number of carbon atoms when there are more than 6 carbons in the molecules.

The replacement of a carboxylic group (-COOH) with a sulfonic group (-SO_2OH) decreased but did not abolish the increase in OF induced by n-C5 and n-C7 (Table.1). The differences between compounds having carboxylic and sulfonic groups were larger in n-C7 compounds than in n-C5 compounds. The effect of carboxylic group replacement was greater in SCFAs with longer hydrocarbon chains. The physico-chemical or structural balance between the hydrocarbon chain and opposite group in SCFA molecules may be an important factor in determining the ability of the compound to increase OF. Dicarboxylic acids, which possess two carboxylic groups

at each end of a hydrocarbon chain, had no effect on OF. This indicates that a structure in which a hydrocarbon chain and a carboxylic group are located on opposite sides of the molecule is needed to induce an increase in OF.

Transformation of hydrocarbon chains between straight, branched or cyclic forms change the OF in rat RBCs. In the present study, most isomers of n-C4, n-C5 or n-C6 increased OF to a lesser degree than did their parent SCFAs, though 4-Methyl-n-valeric acid induced a much larger increase in OF than did its parent SCFA with a straight chain (n-C6). Although the increase in OF induced by cyclic compounds was generally lower than that induced by the parent SCFAs (n-C4, n-C5 and n-C6), cyclohexane-carboxylic acid produced a larger increase in OF than did straight SCFA (n-C7). Benzoic acid possessing a benzene ring produced a smaller increase than did n-C7. As the benzene ring is much smaller than a cyclohexane ring, it was clarified that not only the length of the fatty acid chain but the size or form of the hydrocarbon chain is also an important factor in SCFA-mediated increases in OF in rat RBCs.

There have been many reports on SCFAs, including mono-carboxylic acids, and their biological actions, and it has been shown that the number of carbons in the hydrocarbon chain is an important factor in determining the biological activity of mono-carboxylic acids [3,10,11,16,17]. In addition, the transformation of the hydrocarbon chain in mono-carboxylic acids affects biological activity [2,3,10,11,16-19]. It was also reported that the presence of one carboxylic group is an essential element in the induction of these biological activities [3,10,11,16]. The increase in OF induced by SCFAs and their derivatives, and the biological activities of mono-carboxylic acids are very similar. There is a possibility that common receptive mechanism exists on the plasma membrane of the cell and is activated by these chemicals

Recently, SCFAs have been shown to induce intracellular Ca release via specific receptors and activate leukocytes in humans and mice [13]. The receptors to mono-carboxylic acids, or G-protein coupled receptors (GPR) 41 and GPR43, have been identified and characterized [14]. It was reported that trypsin treatment reduces octanoic acid–induced amylase release from the pancreatic fragment in sheep and goats [12]. In our previous study, however, the pre-treatment of RBCs with trypsin did not affect the degree of OF increase induced by exposure to SCFAs [15]. This result indicates that the surface protein on the RBC membrane is not involved in the SCFA-induced increase in OF. In general, the physiological or pharmacological effect of SCFAs on cells or tissues has been considered to be occurred via specific SCFA receptor. However, the present study has presented the possibility that the biological effects of SCFAs occur via direct action on the lipid layer of cell membrane.

On the other hand, there have been some reports that mono carboxylic acids directly affect the plasma membrane of tissues and induce biological actions. Organic acids, including SCFAs, were shown to affect the axonal membrane in crayfish and to accelerate procaine absorption into the lipid bilayer [18]. n-Butyric acid, a mono carboxylic acid, changes the fluidity of the cell membrane of colon cancer cells [20]. Salicylic acid derivatives, which are also derivatives of benzoic acid, affect the plasma membrane and change the shape, stiffness and relaxation time of isolated RBCs within 2 min [17]. Free fatty acids and their related compounds have been reported to affect the fluidity of membranes [21, 22] and reduce osmotic resistance in RBCs [23,24]. These results raise the possibility that SCFAs and mono-carboxylic acids also change the structure and strength of the cell membrane, and thereby induce biological activity in the individual cell. With respect to their physico-chemical characteristic, these compounds are amphipathic, being composed of a hydrocarbon and carboxylic base

The SCFAs and their derivatives tested in the present study had a hydrophobic hydrocarbon chain and a hydorphilic carboxylic or sulfonic tail in their molecules. We hypothesized that the hydrocarbon chains enters the RBC membrane with hydrophilic carboxylic base remaining outside of the membrane, and interact with the phospholipids present in the outer layer of the plasma membrane. The mono-carboxylic acids including SCFAs probably disturb the lining of the phospholipid layer on the RBC membrane, and weaken the osmotic pressure from inside the cells. The degree of disturbance on cell membrane probably depends on the length, size and/or form of the hydrophobic hydrocarbon chain in the molecules. In dicarboxylic acids, the hydrocarbons located between two carboxylic groups probably could not structurally interact with the phospholipid layer in RBC membrane.

The changes in OF were determined on the basis of the degree of hemolysis induced by the changes in osmotic pressure using a step-up protocol with increasing concentrations (0.2-0.9%) of NaCl. In the present study, the incubation of dense packed RBC suspension (0.2 ml) into 2 ml of phosphate-NaCl buffer solution dose not correspond to situations that can occur in vivo. The absence of plasma albumin, which is important for fatty acids transport in blood, also should be taken in consideration when extrapolating data to in vivo condition. High concentrations of SCFAs or derivatives (30 or 100 mM) were needed to induce that kind of destructive change in the RBC membrane in this experiment. In general, high concentration (30 or 100 mM) of SCFAs in blood or body fluid will not occur even when the production of SCFAs increases in the gastrointestinal tract by microbial fermentation. In future experiments, we will need to use a method for evaluating minute changes in the lipid layer induced by much lower concentrations of SCFAs. In addition, the biological activities of mono-carboxylic acids may be dependent not only on their chemical structure but also

on the characteristics of the lipid layer on the action sites. The composition of the phospholipids in the cell membrane was reported to be various between tissues in the same species [25] and between the same tissues among different species [26]. We have to clarify whether the phenomenon observed in this study can be extrapolated to the cell membrane of other tissues or the RBCs of other species. In the present study, any intracellular signals related to RBC functions were not determined. Thus, it is needed to evaluate the changes in intracellular signals after the recognition of SCFAs on cell membrane in the next stage. In addition, we have to clarify the problem whether the OF in RBC can be a useful model for effects of SCFAs on some physiological functions in other cells.

In conclusion, mono carboxylic acids with certain forms of hydrocarbon chains, including SCFAs and their derivatives, act to increase OF in isolated rat RBCs. A hydrocarbon chain with a certain number of carbon atoms (hydrophobic portion) and a carboxylic or sulfonic group (hydrophilic tail) are necessary for the induction of an increase in OF in rat RBCs. The plasma membrane in rat RBCs can distinguish slight differences in the molecular structure of SCFAs, especially the structure of the hydrocarbon chains, such as length, size and/or form, attached to the carboxylic base. Thus, the RBC membrane is probably a useful model for studying interactions between SCFAs, including mono-carboxylic acids, and biological membranes composed of a lipid bilayer.

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Table 1. Effect of the replacement of a carboxylic group (-COOH) with a sulfonic group (-SO_2OH) on OF in rat RBCs

No of hydrocarbon	Compound	Dose (mM)	Change in OF _EC50 (NaCl %)	No of observation
4	n-C5;n-Valeric acid	30	0.053±0.006	5
4	Butane-sulfonic acid	30	0.022±0.008**	5
4	n-C5;n-Valeric acid	100	0.086±0.009	5
4	Butane-sulfonic acid	100	0.072±0.005*	5
6	n-C7; n-Enanthic acid	30	0.170±0.021	6
6	Hexane-sulfonic acid	30	0.058±0.005**	6
6	n-C7; n-Enanthic acid	100	0.217±0.036	6
6	Hexane-sulfonic acid	100	0.081±0.007**	6

Values are the mean ± SD. Statistical analysis of equivalent doses of the parent SCFAs (n-C5 and n-C7) and their derivatives were undertaken by paired-*t* test (*; $P<0.05$, **; $P<0.01$).

Table 2. Effect of the introduction of another carboxylic group (dicarbonic acids) on OF in rat RBCs

No of hydrocarbon	Compound	Dose (mM)	Change in OF _EC50 (NaCl %)	No of observation
2	C3; Propionic acid	30	0.032±0.015	4
2	Maleic acid	30	− 0.010±0.002**	4
2	C3; Propionic acid	100	0.039±0.010	4
2	Maleic acid	100	− 0.021±0.005**	4
3	n-C4; n-Butyric acid	30	0.037±0.016	4
3	Succinic acid	30	− 0.010±0.004**	4
3	n-C4; n-Butyric acid	100	0.062±0.017	4
3	Succinic acid	100	− 0.025±0.003**	4
4	n-C5; n-Valeric acid	30	0.067±0.019	4
4	Glutalic acid	30	− 0.002±0.005**	4
4	n-C5; n-Valeric acid	100	0.112±0.020	4
4	Glutalic acid	100	− 0.009±0.005**	4
5	n-C6; n-Caproic acid	30	0.154±0.036	4
5	Adipic acid	30	− 0.011±0.003**	4
5	n-C6; n-Caproic acid	100	0.245±0.054	4
5	Adipic acid	100	− 0.018±0.008**	4

Values are the mean ± SD. Statistical analysis of equivalent doses of the parent SCFAs (n-C4, n-C5 and n-C6) and dicarboxyric acids were undertaken by paired-*t* test (**; $P<0.01$).

Table 3. Effect of branched SCFAs (isomers) on OF in rat RBCs

No of hydrocarbon	Compound	Dose (mM)	Change in OF _EC50 (NaCl %)	No of observation
3	n-C4; n-Butyric acid	30	0.012±0.003	6
3	iso-Butyric acid	30	0.004±0.008	6
3	n-C4; n-Butyric acid	100	0.037±0.007	6
3	iso-Butyric acid	100	0.017±0.009*	6
4	n-C5;n-Valeric acid	30	0.062±0.016	6
4	iso-Valeric acid	30	0.033± 0.015*	6
4	2-Methyl-butyric acid	30	0.030±0.015**	6
4	Dimethyl-propionic acid	30	0.011±0.014**	6
4	n-C5;n-Valeric acid	100	0.110±0.015	6
4	iso-Valeric acid	100	0.053± 0.022**	6
4	2-Methyl-butyric acid	100	0.052±0.013**	6
4	Dimethyl-propionic acid	100	0.068±0.028*	6
5	n-C6;n-caproic acid	30	0.172±0.040	6
5	2-Methyl-n-valeric acid	30	0.079± 0.047**	6
5	3-Methyl-n-valeric acid	30	0.096±0.042	6
5	4-Methyl-n-valeric acid	30	0.373±0.040**	6
5	2-Ethyl-n-butyric acid	30	0.045±0.038**	6
5	3,3-Dimethyl-butiryic acid	30	0.068± 0.049**	6
5	n-C6;n-caproic acid	100	0.267±0.056	6
5	2-Methyl-n-valeric acid	100	0.137±0.075*	6
5	3-Methyl-n-valeric acid	100	0.160±0.053	6
5	4-Methyl-n-valeric acid	100	Not detetermined	6
5	2-Ethyl-n-butyric acid	100	0.075±0.071**	6
5	3,3-Dimethyl-butiryic acid	100	0.083±0.055*	6

Values are the mean ± SD. Statistical analysis of equivalent doses of the parent SCFAs and their isomers were undertaken by paired-*t* test (n-C4 and isomer) or by Bonferroni's test (n-C5, n-C6 and their isomers) (*; $P<0.05$, **; $P<0.01$).

Table 4. Effect of cyclic SCFAs and benzoic acid on OF in rat RBCs

No of hydrocarbon	Compound	Dose (mM)	Change in OF _ EC_{50} (NaCl %)	No of observation
3	n-C4; n-Butyric acid	30	0.008±0.004	5
3	Cyclopropane-carboxylic acid	30	0.001±0.004*	5
3	n-C4; n-Butyric acid	100	0.024±0.009	5
3	Cyclopropane-carboxylic acid	100	0.007±0.005**	5
3	n-C5;n-Valeric acid	30	0.048±0.009	5
3	Cyclobutane-carboxylic acid	30	0.006±0.009**	5
3	n-C5;n-Valeric acid	100	0.076±0.007	5
3	Cyclobutane-carboxylic acid	100	0.023±0.011**	5
4	n-C6; n-caproic acid	30	0.172±0.040	6
4	Cyclopentane-carboxylic acid	30	0.083±0.049**	6
4	n-C6; n-caproic acid	100	0.267±0.056	6
4	Cyclopentane-carboxylic acid	100	0.162±0.087**	6
5	n-C7;Enanthic acid	30	0.174±0.034	4
5	Cyclohexane-carboxylic acid	30	0.268±0.017**	4
5	Benzoic acid	30	0.050±0.013**	4
5	n-C7;Enanthic acid	100	0.254±0.013	4
5	Cyclohexane-carboxylic acid	100	0.433±0.024**	4
5	Benzoic acid	100	0.140±0.018**	4

Values are the mean ± SD. Statistical analysis of equivalent doses of the parent SCFAs and cyclic SCFAs were undertaken pared-*t* test (n-C4, n-C5, n-C6 and their derivatives) or by Bonferroni's test (n-C7 and its derivatives). (*; $P<0.05$, **; $P<0.01$).

Table 5. Effect of SCFAs with double (crotonic acid and 3-butenoic acid) or triple carbon bonds (tetrolic acid) in their hydrocarbons on OF in rat RBCs

No of hydrocarbon	Compound	Dose (mM)	Change in OF _EC50 (NaCl %)	No of observation
3	n-C4; n-Butyric acid	30	0.020±0.007	4
3	Crotonic acid	30	0.011±0.008	4
3	3-Butenoic acid	30	0.013±0.009	4
3	Tetrolic acid	30	0.021±0.010	4
3	n-C4; n-Butyric acid	100	0.035±0.005	4
3	Crotonic acid	100	0.033±0.007	4
3	3-Butenoic acid	100	0.034±0.006	4
3	Tetrolic acid	100	0.050±0.003*	4

Values are the mean ± SD. Statistical analysis of equivalent doses of the parent SCFAs and their derivatives were undertaken by Bonferroni's test (*; $P<0.05$).

Figure Legend

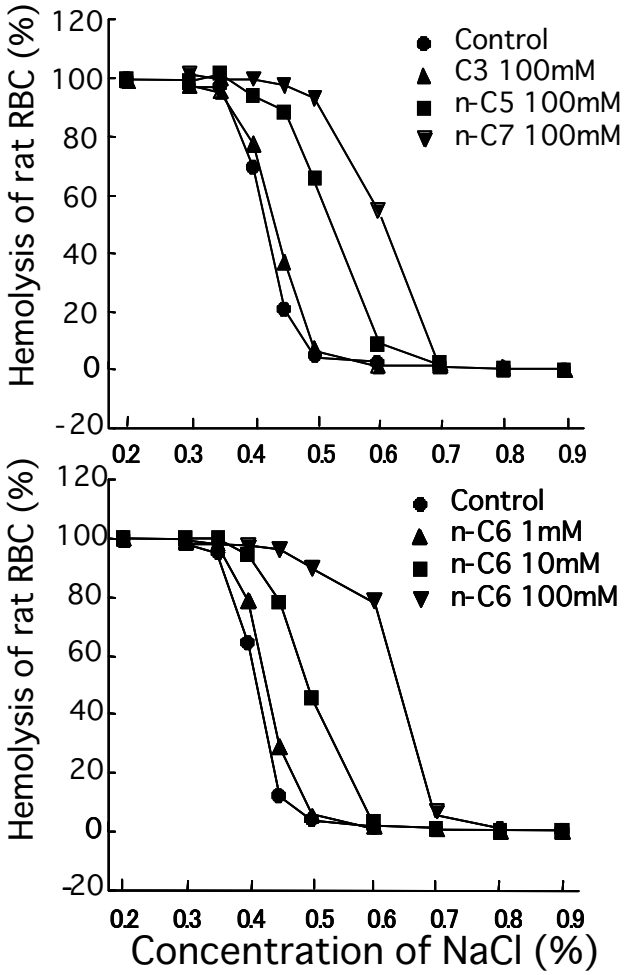
Fig. 1. Hemolytic curves in rat RBCs exposed to SCFAs

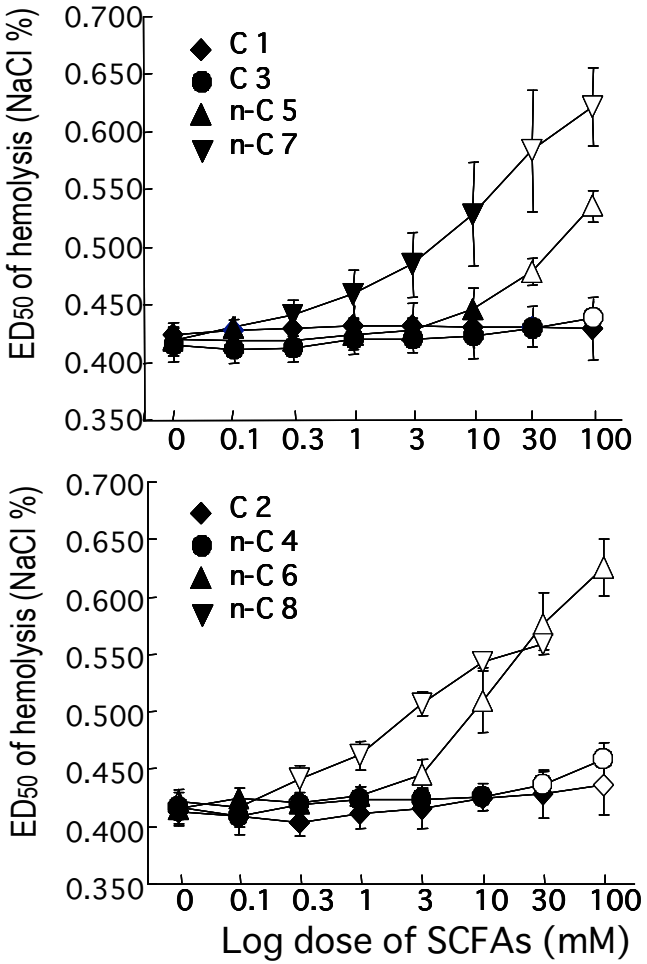
Values are means (n=4). Hemolytic curves in rat RBCs were obtained after exposure to each SCFA at each dose for 1 hr. Curves were determined for the kind (upper panel; C1, C3 and n-C5 at 100mM) or dose of SCFA (lower panel; 1, 10 and 100 mM n-C6). The EC_{50} value for hemolysis (concentration in NaCl %) were obtained by using straight-line equation between the points immediately above and below 50%. The value obtained was used as a parameter of OF.

Fig.2. Dose-response relationship between concentrations in straight-SCFAs with 1 to 8 carbons and their effect on OF in rat RBC

Values are the mean±SD (n=4 to 14). Upper panel; Formic acid (C1), Propionic acid (C3), n-Varelic acid (n-C5), n-Enanthic acid (n-C7) Lower panel; Acetic acid (C2), n-Butyric acid (n-C4), n-Caproic acid (n-C6) , n-Capric acid (n-C8) Open symbols indicate that there was a significant difference between the control (0mM SCFA) and subsequent doses (0.1-100mM) on the basis of Bonferroni's test ($P<0.05$).

Fig 1. Mineo H


Hemolysis of rat RBC (%)
120
100
80
60
40
20
0
-20
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Control
C3 100mM
n-C5 100mM
n-C7 100mM
Hemolysis of rat RBC (%)
Control
n-C6 1mM
n-C6 10mM
n-C6 100mM
Concentration of NaCl (%)


0.700
0.650
0.600
0.550
0.500
0.450
0.400
0.350
ED50 of hemolysis (NaCl %)
C 1
C 3
n-C 5
n-C 7
0 0.1 0.3 1 3 10 30 100
C 2
n-C 4
n-C 6
n-C 8
Log dose of SCFAs (mM)