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Research Article

Opposite Effects of Monocarboxylic and Dicarboxylic Acids on Osmotic Fragility in Isolated Pig Red Blood Cells: Comparative Study of Membrane Response to Carboxylic Acids Demonstrated in Red Blood Cells of other Mammalian Species

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ABSTRACT

Lignocellulolytic enzymes are extracellular biocatalysts secreted by filamentous fungi and are involved in the breakdown of recalcitrant lignocellulose plant materials into useful products necessary for fungal growth and development. Even though several studies on filamentous fungi have reported the impact of different substrates on lignocellulolytic enzymes, there is limited information on how mushroom supplements affect secretion of the enzymes, growth, and yield of *Pleurotus ostreatus* when using the alkaline treatment method. In this study, we investigated the influence of cornmeal (T1) and coffee grounds (T2) supplements on lignocellulosic enzymes at different growth stages and the production of *P. ostreatus*. We found that lignolytic enzyme activity was significantly higher in the control (CK) and T2 during mycelial stages, while CK had the lowest hydrolytic enzyme activity during primordia and fruiting. Unlike T1 which had the best biological efficiency, T2 exhibited significantly higher levels of lignolytic enzymes during mycelial stage, whereas CMCase and xylanase activities were higher in supplemented treatments than in the control during primordia and fruiting. Taken together our results demonstrated that cornmeal and coffee ground supplements reduce mycelium growth rate, enhance the production of hydrolytic enzymes during fruiting, and remarkably increase the yield and protein content of *P. ostreatus*.

Keywords: *Pleurotus ostreatus*; Cornmeal; Coffee grounds; Substrate; Supplements; Mushroom production; Biological efficiency

INTRODUCTION

The effects of xenobiotic chemicals on biological tissues are initially observed in the cell membrane. Red blood cells (RBCs), particularly their biomembranes, have been used as a cell model, as they have no nucleus and an extra organelle in the plasma [1,2]. The RBCs of mammalian species are relatively homogeneous and are readily available in large quantities.

As a parameter for the degree of action of various chemicals on the RBC membrane, osmotic fragility (OF) has been widely employed [3,4]. OF is generally determined by the degree of hemolysis induced by changes in osmotic pressure using a NaCl solution. The method is extremely simple and there is little possibility of error associated with the experimental and technical procedure.

We have been investigating the interactions between the cell membrane and carboxylic acids by using isolated RBCs from various animals as a membrane model and OF as an indicator of membrane intensity as mentioned below. The chemicals examined in our series of experiments are monocarboxylic acids, which have a carboxylic group and a hydrocarbon chain with a straight, branched or cyclic structure, and dicarboxylic acids, which have a carboxylic group at each end of a hydrocarbon chain with straight or cyclic structure. We clarified structure-activity relationship of those compounds on RBCs derived from various animals.

The results from our series of experiments revealed that the OF

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responses in RBCs are differ markedly between monocarboxylic acids and dicarboxylic acids. Most monocarboxylic acids, including benzoic acid and its derivatives, increase OF in rat RBCs [5-7] or have no effect on OF in guinea pigs [7,8], sheep [9,10] and cattle RBCs [11,12]. Intra-species differences were recognized in the OF responses to monocarboxylic acids. In contrast, most dicarboxylic acids, including phthalic acid and its isomers, decreased OF in the RBCs derived from all four animal species, rats [7,13], guinea pigs [7,8], sheep [9,10] and cattle [11,12]. No intra-species differences were demonstrated in the OF responses to dicarboxylic acids from the RBCs of different mammalian species tested to date.

In addition, permeation of carboxylic acids into the RBC membrane is thought to be an important factor in inducing changes in OF in the RBC membrane. The partition coefficient is commonly used as a parameter for the permeation of chemicals into the cell membrane [14]. Examination of this parameter for carboxylic acids was performed using artificial [15] or RBC membranes [16]. The partition coefficient for octanol/water has been the most widely utilized as an indicator of the permeation of chemicals into cell membrane [17,18]. Thus, we analyzed the relationship between the change in OF and the degree of permeation of those compounds into the cell membrane by using the octanol/water partition coefficient. A significant positive relationship was observed between the partition coefficients of some kinds of monocarboxylic acids and their effects on OF in rat RBCs [19,20], but not in the RBCs of other animal species [9-12,20].

In the present study, using pig RBCs, we clarified the OF response to monocarboxylic and dicarboxylic acids and compared the data to those from four other animal species. We also tried to analyze the relationship between the OF response to those compounds and their partition coefficients.

MATERIALS AND METHODS

Reagents

The 22 monocarboxylic acids and 14 dicarboxylic acids examined in this study were the same as those used in our previous report [9]. The chemical structures of all carboxylic acids used in this study are shown in the Results section. All reagents used in the present study, including the carboxylic acids, were purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan) or Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Sampling and treatment of pig blood

Six female parous pigs (Landrace × Large White; 200 ± 10 kg) aged 2 to 3 yeas old were used in this study. They were kept in individual stalls and provided with free access to food and water in the Student-training Farm of Rakuno Gakuen University (Ebetsu, Hokkaido). Blood samples (30 ml) from the animals were collected from the left jugular vein into heparinized tests tube by a veterinarian in Rakuno Gakuen University. The treatment of the blood samples was as follows; the blood samples were transported to the laboratory of Hokkaido Bunkyo University and then kept in refrigerator at 4 °C for about 15 hours. The blood was centrifuged at 2000 g for 15 min (Model 2420, Kubota Inc., Tokyo, Japan) and the plasma and buffy coat were then removed by aspiration. The crude RBCs obtained were immersed in two volumes of cold 0.9% NaCl solution, and the upper layer was then removed by aspiration after centrifugation. This process was repeated three times. The resultant packed RBC suspension was kept in ice-cold water until application to the following procedures.

Experimental procedure

The experimental procedure was the same as that in our previous report [9]. Briefly, the RBC suspension (30 µl) was added 0.6 ml of phosphate-NaCl buffer solution (pH 7.4) containing each of the carboxylic acids at 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 or 100 mM in micro test tubes (1.5 ml of volume, Nichiryo Co., Ltd., Tokyo, Japan). An appropriate amount of NaCl was added to the buffer solution to adjust the osmolality for each chemical tested. The RBC suspensions exposed to the carboxylic acids were incubated by shaking (1 stroke/sec) at 37°C for 1 hr (Shaking Bath TBK 202 DA, Advantec Co., Ltd., Tokyo, Japan). Each tube of acid-treated RBCs was mixed by a mixer (Vortex Genie 2, Model-G560, Scientific Industry, Inc. NY., USA), and each RBC suspension (50 µl) was the transferred into a 96-deep-well microplate (2 ml volume, Whatman Inc., Piscataway, NJ, USA) containing 1 ml of NaCl solution ranging in concentration from 0.1 to 0.8%. The deep-well microplate was then centrifuged at 1300 g (Plate Spin II, Kubota Inc., Tokyo, Japan) for 10 min at room temperature. The supernatants (200 µl) with different hemoglobin concentrations due to the ruptured RBCs were transferred into another 96-well microplate (300 µl volume, Whatman Inc., Piscataway, NJ, USA). The hemoglobin concentration was determined colorimetrically at 540 nm (Microplate Reader Model 680, Bio-Rad Laboratories, Tokyo, Japan).

Statistical analysis

The analytical procedure was also the same as that in our previous report [9]. All values are expressed as means ± S.D. (n=6). Perfect hemolysis of the RBC suspension was induced by a 0.1% NaCl solution and thus the hemoglobin concentration was expressed as 100%. In contrast, no hemolysis was induced by a 0.8% NaCl solution and thus the hemoglobin concentration was expressed as 0%. The NaCl concentration causing 50% hemolysis (EC50) of the RBC suspension was obtained from the hemolysis curve by using a straight-line equation. The EC50 expressed as a NaCl concentration was determined as OF value in the RBCs in this study. The significance of the differences between each concentration (0.1-100 mM) and the control (0 mM) was evaluated by Dunnett's test following one-way ANOVA. The partition coefficients of the tested chemicals used were

taken from the PubChem [21] or ChemSpider [22] websites. The relationship between the ΔEC50 of the RBCs and the partition coefficient of each carboxylic acid was evaluated by regression analysis. Excel Tokei for Windows 2012 (SSRI Co., Ltd., Tokyo, Japan) was used for all statistical analyses. A $p<0.05$ or 0.01 was regard as statistically significant.

RESULTS AND DISCUSSION

Effects of monocarboxylic and dicarboxylic acids possessing straight hydrocarbons

The application of acetic acid (C1; number of carbons in the hydrocarbon chain) at any concentrations from 0.1-100 mM did not affect OF in the pig RBCs (Figure 1A).

In contrast, malonic acid (C2) decreased OF in a dose-dependent manner with a significant decrease in OF induced at 50 and 100 mM ($P<0.05$). Although n-caprylic acid (C7) did not affect OF up to 25 mM and tended to increase OF at 50 mM, these changes were not statistically significant (N.S). As n-caprylic acid at 100 mM induced complete hemolysis in RBCs, no OF value was obtained (Figure 1B). Azelaic acid, a dicarboxylic acid corresponding to n-caprylic acid (C7), decreased OF in a dose-dependent manner at 50 and 100 mM ($P<0.05$). As most changes in OF were induced by monocarboxylic or dicarboxylic acids at 10 to 100 mM, the EC50 values at those 4 concentrations are summarized in Table 1 or Table 2, respectively. For monocarboxylic acids, formic acid (C0) to n-caprylic acid (C7), did not change OF at 10 to 100 mM, except for n-caprylic acid at 100 mM (Table 1). In contrast, all tested dicarboxylic acid (C0 to C7) decreased OF significantly ($P<0.05$) at 25 and/or 50 and 100 mM (Table 2).

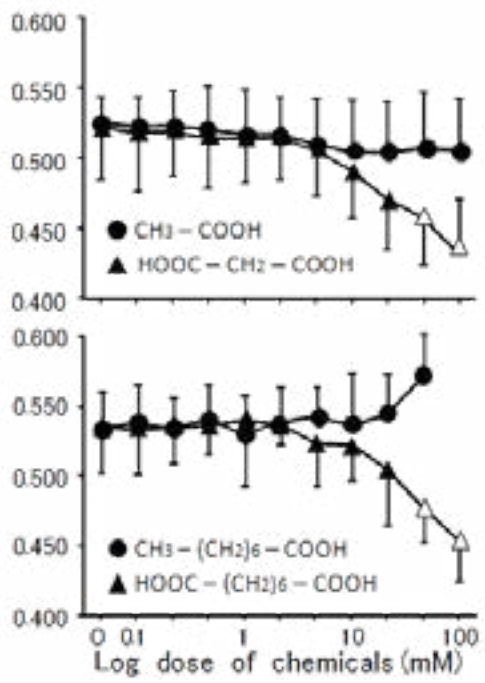


Figure 1: The OF response to monocarboxylic and dicarboxylic acids with straight hydrocarbon chains on OF in pig RBCs. The OF response to acetic and malonic acid (A) and n-caprylic and azelaic acid (B) on OF are presented. Values are the means ± SD (n=6). (●) represents the monocarboxylic acids and (▲) represents the dicarboxylic acids in each panel. Open symbols indicate a significant difference for subsequent concentrations (0.1-100mM) to the control (0mM) analyzed by Dunnett's test ($P<0.05$ including 0.01).

Table 1: Monocarboxylic acids possessing straight hydrocarbons, their chemical structure, partition coefficients and effects on OF in pig RBCs. Values are means ± SD (n=6). The partition coefficients were quoted from the PubChem [21] or ChemSpider [22] website. Asterisks (* and **) indicate a significant difference ($P<0.05$ and $P<0.01$) from subsequent concentration (0.1-100 mM) to the control (0 mM) analyzed by the Dunnett's test. As there were no significant changes for exposure to 0.1-5 mM of all tested monocarboxylic acids, the EC50 values at those doses are omitted and the data for 10, 25, 50 and 100 mM are presented:

No of hydrocarbon	Carboxylic acid	Partition coefficient	Dose (mM)	Change in OF ⊿EC50 (NaCl %)	
0	Formic acid		10	-0.038 ± 0.041	
	H-COOH		25	-0.061 ± 0.007	*
		-0.54	50	-0.075 ± 0.015	**
			100	-0.062 ± 0.022	**
1	Acetic acid		10	-0.021 ± 0.028	
	CH3-COOH		25	-0.022 ± 0.035	
		-0.17	50	-0.019 ± 0.039	*
			100	0.020 ± 0.039	**
2	Propionic acid		10	0.002 ± 0.014	
	CH3-CH2-COOH		25	0.010 ± 0.009	*
		0.33	50	-0.025 ± 0.020	**
			100	-0.011 ± 0.024	**

3	n-Butyric acid		10	0.004 ± 0.015	
	C H 3 - (C H 2) 2 - COOH		25	-0.004 ± 0.014	*
		0.79	50	-0.019 ± 0.012	**
			100	-0.006 ± 0.020	**
4	n-Valeric acid		10	0.006 ± 0.009	
	C H 3 - (C H 2) 3 - COOH		25	-0.002 ± 0.014	
		1.39	50	-0.004 ± 0.014	**
			100	-0.007 ± 0.019	**
5	n-Caproic acid		10	0.002 ± 0.009	
	C H 3 - (C H 2) 4 - COOH		25	0.001 ± 0.014	
		1.92	50	0.001 ± 0.007	*
			100	-0.013 ± 0.019	**
6	n-Enanthic acid		10	0.006 ± 0.020	
	C H 3 - (C H 2) 5 - COOH		25	-0.015 ± 0.021	
		2.42	50	-0.024 ± 0.022	*
			100	-0.061 ± 0.034	**
7	n-Caprylic acid		10	0.002 ± 0.034	
	C H 3 - (C H 2) 6 - COOH		25	0.009 ± 0.019	
		3.05	50	0.009 ± 0.028	**
			100	no data	**

Table 2: Dicarboxylic acids possessing straight hydrocarbons, their chemical structure, partition coefficients and effects on OF in pig RBCs. Values are means ± SD (n=6). The partition coefficients were quoted from the PubChem [21] or ChemSpider [22] website. Asterisks (* and **) indicate a significant difference (P<0.05 and P<0.01) from subsequent concentration (0.1-100 mM) to the control (0 mM) analyzed by the Dunnett's test. As there were no significant changes for exposure to 0.1-5 mM of all tested dicarboxylic acids, the EC50 values at those doses are omitted and the data for 10, 25, 50 and 100 mM are presented.

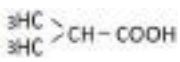
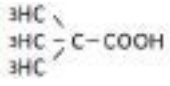
No of hydrocarbon	Carboxylic acid	Partition coefficient	Dose (mM)	Change in OF ⊿EC50 (NaCl %)	
0	Oxalic acid		10	-0.041 ± 0.008	
	HOOC – COOH		25	-0.048 ± 0.027	*
		-0.81	50	-0.078 ± 0.024	**
			100	-0.084 ± 0.011	**
1	Malonic acid		10	-0.030 ± 0.045	
	HOOC – CH2 –COOH		25	-0.051 ± 0.047	
		-0.81	50	-0.064 ± 0.043	*
			100	-0.090 ± 0.049	**
2	Succinic acid		10	-0.026 ± 0.017	
	HOOC – (CH2)2 – COOH		25	-0.041 ± 0.015	*
		-0.59	50	-0.078 ± 0.017	**
			100	-0.091 ± 0.019	**
3	Glutaric acid		10	-0.027 ± 0.026	
	HOOC – (CH2)3 – COOH		25	-0.041 ± 0.013	*
		-0.29	50	-0.081 ± 0.015	**
			100	-0.094 ± 0.028	**

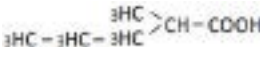
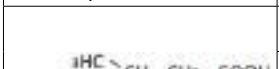
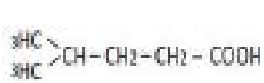
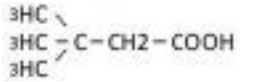
4	Adipic acid		10	-0.023 ± 0.020	
	HOOC – (CH2)4 – COOH		25	-0.028 ± 0.017	
		0.08	50	-0.058 ± 0.041	**
			100	-0.072 ± 0.031	**
5	Pimelic acid		10	-0.019 ± 0.014	
	HOOC – (CH2)5 – COOH		25	-0.028 ± 0.019	
		0.61	50	-0.046 ± 0.031	*
			100	-0.090 ± 0.039	**
6	Suberic acid		10	-0.022 ± 0.011	
	HOOC – (CH2)6 – COOH		25	-0.031 ± 0.017	
		0.8o	50	-0.059 ± 0.025	*
			100	-0.084 ± 0.018	**
7	Azelaic acid		10	-0.013 ± 0.015	
	HOOC – (CH2)7 – COOH		25	-0.032 ± 0.029	
		1.57	50	-0.058 ± 0.024	**
			100	-0.083 ± 0.025	**

Effects of monocarboxylic possessing branched hydrocarbons

Iso-valeric acid (C4) decreased OF significantly (P<0.05) at 25, 50 and 100 mM. (Figure 2). In contrast, 2-mthyl-butyric acid did not affect OF at any of the tested concentrations. Some branched-chain monocarboxylic acids decreased OF dose-dependently at more than 25 mM (P<0.05) and those effects were dependent on the form of the branched hydrocarbons in the moiety (Table 3).

Table 3: Monocarboxylic acids possessing branched hydrocarbons, their chemical structure, partition coefficients and effects on OF in pig RBCs. Values are means ± SD (n=6). The partition coefficients were quoted from the PubChem [21] or ChemSpider [22] website. Asterisks (* and **) indicate a significant difference (P<0.05 and P<0.01) from subsequent concentration (0.1-100 mM) to the control (0 mM) analyzed by the Dunnett's test. As there were no significant changes for exposure to 0.1-5 mM of all tested monocarboxylic acids, the EC50 values at those doses are omitted and the data for 10, 25, 50 and 100 mM are presented.

No of hydrocarbon	Carboxylic acid	Partition coefficient	Dose (mM)	Change in OF ⊿EC50 (NaCl %)	
1	iso-Butyric acid		10	-0.021 ± 0.035	
			25	-0.022 ± 0.036	
		0.94	50	-0.027 ± 0.042	
			100	-0.032 ± 0.038	
2	iso-Valeric acid		10	-0.027 ± 0.014	
			25	-0.036 ± 0.014	**
		1.16	50	-0.047 ± 0.015	**
			100	-0.059 ± 0.014	**
3	2-Methyl-butyric acid		10	-0.012 ± 0.016	
			25	-0.020 ± 0.020	
		1.18	50	-0.022 ± 0.020	
			100	-0.019 ± 0.023	
4	Dimetyl-propionic acid		10	-0.026 ± 0.014	
			25	-0.039 ± 0.020	**
		1.48	50	-0.053 ± 0.018	**
			100	-0.068 ± 0.020	**

5	2-Mrthy-n-valeric acid		10	-0.020 ± 0.015	
			25	-0.033 ± 0.022	
		1.8	50	-0.050 ± 0.027	*
			100	-0.064 ± 0.036	**
6	3-Mrthy-n-valeric acid		10	-0.005 ± 0.016	
			25	-0.014 ± 0.013	
		1.56	50	-0.017 ± 0.016	
			100	-0.030 ± 0.023	
7	4-Mrthy-n-valeric acid		10	-0.023 ± 0.020	
			25	-0.033 ± 0.017	
		1.66	50	-0.055 ± 0.026	
			100	-0.054 ± 0.027	
8	2-ethyl-n-butyric acid		10	-0.007 ± 0.009	
			25	-0.013 ± 0.014	
		1.66	50	-0.036 ± 0.017	
			100	-0.029 ± 0.013	
9	3,3-Dimethyl-n-butyric acid		10	-0.020 ± 0.036	
			25	-0.040 ± 0.017	*
		1.47	50	-0.061 ± 0.013	**
			100	-0.086 ± 0.022	**

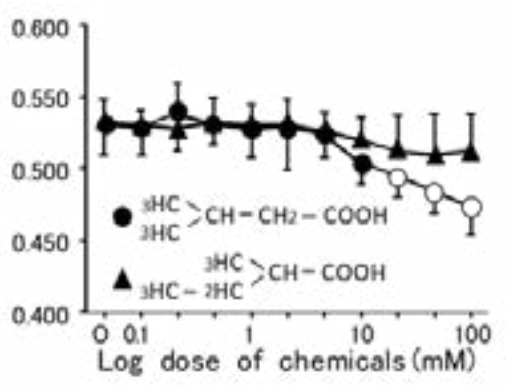


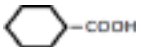
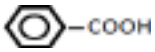
Figure 2: The OF response to monocarboxylic with branched hydrocarbon chains on OF in pig RBCs. The OF response to iso-valeric (•) and 2-methyl-butyric acid (▲) are presented. Values are the means ± SD (n = 6). Open symbols indicate a significant difference for subsequent concentrations (0.1-100mM) to the control (0mM) analyzed by Dunnett's test (P<0.05 including 0.01).

Effects of monocarboxylic possessing cyclic hydrocarbons

Cyclopropane carboxylic acid (C3) did not affect OF at any of tested concentrations in this experiment (Figure 3). In contrast, cyclopentane carboxylic acid (C5) decreased OF in a dose dependent manner at 25, 50 and 100 mM (P<0.05). For monocarboxylic acids possessing cyclic hydrocarbons, although cyclopentane (C5) and cyclohexane carboxylic acids (C6), as well as benzoic acid (C6) decreased OF at 100 mM, the other carboxylic acids (C3, C4) did not affect OF in the pig RBCs (Table 4).

Table 4: Monocarboxylic acids possessing cyclic hydrocarbons, their chemical structure, partition coefficients and effects on OF in pig RBCs. Values are means ± SD (n=6). The partition coefficients were quoted from the PubChem [21] or ChemSpider [22] website. Asterisks (* and **) indicate a significant difference (P<0.05 and P<0.01) from subsequent concentration (0.1-100 mM) to the control (0 mM) analyzed by the Dunnett's test. As there were no significant changes for exposure to 0.1-5 mM of all tested monocarboxylic acids, the EC50 values at those doses are omitted and the data for 10, 25, 50 and 100 mM are presented.

No of hydrocarbon	Carboxylic acid	Partition coefficient	Dose (mM)	Change in OF ⊿EC50 (NaCl %)	
	Cyclopropane-carboxylic acid		10	-0.011 ± 0.018	
			25	-0.017 ± 0.018	
3		0.08	50	-0.014 ± 0.006	
			100	-0.026 ± 0.004	

	Cyclobutane-carboxylic acid		10	-0.005 ± 0.017	
			25	-0.010 ± 0.016	
4		0.65	50	-0.000 ± 0.013	
			100	-0.017 ± 0.009	
	Cyclopentane-carboxylic acid		10	-0.033 ± 0.031	
			25	-0.048± 0.028	*
5		1.21	50	-0.061 ± 0.019	**
			100	-0.069 ± 0.018	**
	Cyclohexane-carboxylic acid		10	-0.014 ± 0.008	
			25	-0.032 ± 0.013	
6		1.96	50	-0.055 ± 0.014	**
			100	-0.067 ± 0.021	**
	Benzoic acid		10	-0.009 ± 0.022	
			25	-0.013 ± 0.022	
6		1.87	50	-0.025 ± 0.015	
			100	-0.056 ± 0.022	*

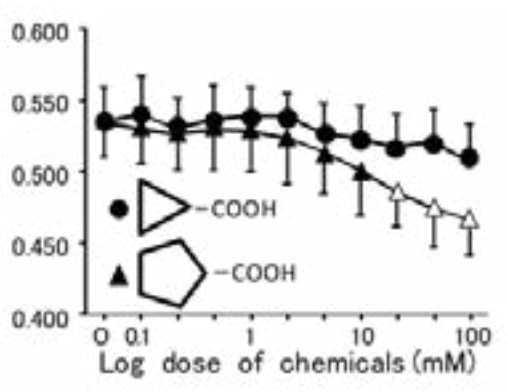
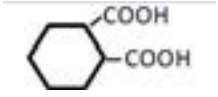


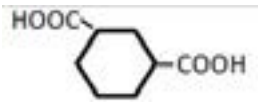
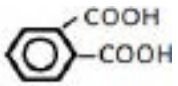
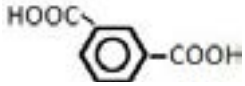
Figure 3: The OF response to monocarboxylic with cyclic hydrocarbons in pig RBCs. The OF response to cyclopropane- (•) cyclopentane-carboxylic acid (▲) are presented. Values are the means ± SD (n = 6). Open symbols indicate a significant difference for subsequent concentrations (0.1-100mM) to the control (0mM) analyzed by Dunnett's test (P<0.05 including 0.01).

Effects of dicarboxylic acid possessing cyclic hydrocarbon

1,2-Cyclohexane dicarboxylic acid decreased OF dose-dependently with significant decrease induced at 100 mM (P<0.05) (Figure 4). Phthalic acid also decreased OF dose-dependently with significant decreases induced at 25, 50 and 100 mM. Other dicarboxylic acids possessing cyclic hydrocarbons, including a benzene ring, also decreased OF (P<0.05 or 0.01) at more than 25 mM (Table 5).

Table 5: Dicarboxylic acids possessing cyclic hydrocarbons, their chemical structure, partition coefficients and effects on OF in pig RBCs. Values are means ± SD (n=6). The partition coefficients were quoted from the PubChem [21] or ChemSpider [22] website. Asterisks (* and **) indicate a significant difference (P<0.05 and P<0.01) from subsequent concentration (0.1-100 mM) to the control (0 mM) analyzed by the Dunnett's test. As there were no significant changes for exposure to 0.1-5 mM of all tested dicarboxylic acids, the EC50 values at those doses are omitted and the data for 10, 25, 50 and 100 mM are presented.

No of hydrocarbon	Carboxylic acid	Partition coefficient	Dose (mM)	Change in OF ⊿EC50 (NaCl %)	
	1,2-Cyclohexane-dicarboxylic acid		10	0.001 ± 0.014	
1			25	-0.006 ± 0.013	
		0.64	50	-0.031 ± 0.028	
			100	-0.050 ± 0.025	**

	1,3-Cyclohexane-dicarboxylic acid		10	-0.015 ± 0.011	
2			25	-0.032 ± 0.017	**
		0.46	50	-0.041 ± 0.018	**
			100	-0.063 ± 0.023	**
	1,4-Cyclohexane-dicarboxylic acid		10	0.007 ± 0.012	
3			25	-0.023 ± 0.023	
		0.83	50	-0.031 ± 0.013	
			100	-0.058± 0.032	**
	Phthalic acid		10	-0.033 ± 0.023	
4			25	-0.050 ± 0.016	*
		0.73	50	-0.076 ± 0.016	**
			100	-0.103 ± 0.018	**
	Isophathalic acid		10	-0.002 ± 0.015	
5			25	-0.017 ± 0.014	
		1.66	50	-0.031 ± 0.013	
			100	-0.056 ± 0.013	*
	Terephthalic acid		10	-0.010 ± 0.017	
6			25	-0.029 ± 0.014	
		2	50	-0.041 ± 0.009	*
			100	-0.059 ± 0.016	**

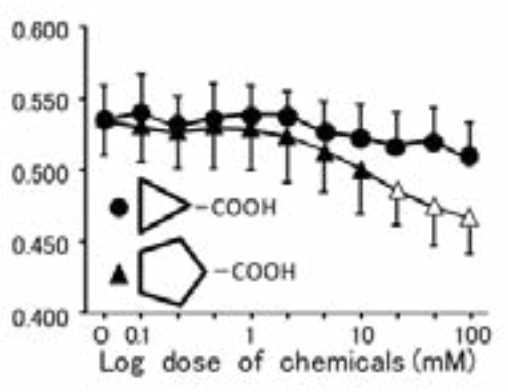


Figure 4: The OF response to dicarboxylic with cyclic hydrocarbons in pig RBCs. The OF response to 1,2-cyclohexane-dicarboxylic acid (●) and phthalic acid (▲) are presented. Values are the means ± SD (n=6). Open symbols indicate a significant difference for subsequent concentrations (0.1-100mM) to the control (0mM) analyzed by Dunnett's test (P<0.05 including 0.01).

Relationship between the partition coefficients of the acids and their effect on OF

The regression analysis for the monocarboxylic acids tested in this study revealed that there was no clear relationship between the partition coefficient of the compounds and their effects on OF in the pig RBCs at 10 to 100 mM (Table 6). On the other hand, the regression analysis for the dicarboxylic acids revealed a significant negative relationship (P<0.05) between the partition coefficients of the compounds and their effects on OF at 10, 25 and 50 mM, but not 100 mM.

Table 6: Correlation between the change in EC50 during hemolysis in pig RBCs and the partition coefficients of carboxylic acids. Values were calculated by regression analysis (mean value of each carboxylic acid; n=6) between the partition coefficients and change in EC50 during hemolysis induced by each dose of the monocarboxylic and dicarboxylic acids, with benzoic acids, phthalic acid and its isomers included or not. Correlation efficient "r" and significance "P" are shown. A P value <0.05 is defined as statistically significant in the present study.

Substances	n	mM	Intersept	Slope	r	P
	22	10	-0.0207	0.0053	0.4003	0.0649
Monocarboxylic acid	22	25	-0.0307	0.0072	0.3542	0.1058
	22	50	-0.0402	0.0083	0.271	0.2224
	21	100	-0.0247	-0.0117	0.3141	0.1656
	14	10	-0.0239	0.0087	0.6568	0.0107
	14	25	-0.0366	0.0081	0.5708	0.033
Dicarboxylic acid	14	50	-0.0617	0.0133	0.644	0.0129
	14	100	-0.0815	0.0093	0.4948	0.072

This series of experiments has clarified the effects of the application of monocarboxylic and dicarboxylic acids at 0.1 to 100 mM for one hour on OF in pig RBCs. Among the monocarboxylic acids possessing straight hydrocarbons tested, acetic acid (C1), propionic acid (C2), n-butyric acid (C4), n-valeric acid (C5) and n-caproic acid (C5) did not affect OF in pig RBCs at any concentration. Formic acid (C1) and n-enanthic acid (C7) decreased OF in a dose-dependent manner and induced significant decreases ($P<0.01$) in OF at 25, 50 and 100 mM, and 100 mM, respectively. n-Caprylic acid tended to increase OF dose-dependently and induced hemolysis at 100 mM. Some monocarboxylic acids possessing branched or cyclic hydrocarbons, including benzene ring, decreased OF dose-dependently ($P<0.05$ or 0.01), with the degree of changes in OF dependent on the monocarboxylic acid structures. On the other hand, all dicarboxylic acids possessing straight and cyclic hydrocarbons tested in this study decreased OF in a dose-dependent manner ($P<0.05$ or 0.01). The degree of change in OF was dependent on the dicarboxylic acid structures.

The OF responses in pig RBCs to monocarboxylic and dicarboxylic acids were basically similar to those in guinea pig [7], sheep [8] and cattle RBCs [11] and much different to those in rat RBCs [7].

In our previous reports, we found that the OF response to monocarboxylic acids was classified into 2 types, and we proposed these 2 types be referred to as the rat type and guinea pig type, respectively [9]. In rat-type RBCs, OF was increased by monocarboxylic acids, with the OF response dependent on the length of hydrocarbons in their moieties. To date, such responses to monocarboxylic acids have only been observed in rat RBCs [7]. In contrast, in guinea pig-type RBCs, OF was not affected or rather decreased by monocarboxylic acids. Such OF responses were observed in guinea pig, sheep and cattle RBCs [7,8,11]. The present experiments revealed that pig RBCs can be classified into the guinea pig type in terms of the OF response to monocarboxylic acids.

We believe that the cause of the difference in OF response between the 2 types due to the nature of the RBC membrane, particularly the composition of acyl-chains in the phospholipid layer. The acyl-chains in the phospholipids are derived from fatty acids and are composed of a carboxylic group and a hydrocarbon chain. Based on previous reports, we consider arachidonic acid (AA) to be a crucial fatty acid, and it thought to be a candidate for the dual nature of OF responses to monocarboxylic acids.

The first point to be considered is the quantitative view of AA in the RBC membrane. It was shown that the proportion of AA is the highest among fatty acids contained in phospholipid layer in rat RBCs [23]. In addition, the proportion of AA in rat RBCs is higher than those in guinea pig [23], sheep, cattle and pig RBCs [24]. The second point to be considered is thee physiological roll in AA in the cell or RBC membrane. It was reported that the release in AA from the intact rat aorta and cultured smooth muscle cells, which is induced by exogenous bovine serum albumin, decrease membrane fluidity in the cells in both tissues [25]. The relationship between the rate constant of Rb+(K+) efflux and the percentage of AA in the RBC membrane was examined in six mammalian species (cows, horses, pigs, rabbits, rats and humans) [26]. The results showed that rat RBCs possessed the highest rate constant of Rb+(K+) efflux as well as the highest percentage of AA among the RBC membranes of the tested mammalian species, excluding humans. Another report demonstrated that K+ leakage from human RBCs is increased by partial replacement of native fatty acid in the phospholipids by AA [27].

These reports indicate that endogenous AA increases the fluidity and permeability of substances in the cell membrane. It is thought that a large amount of AA probably prevents the formation of firm bonds between acyl-chains and enlarges the spaces present in the phospholipid layers in the RBC membrane. We speculated that the cause of the different OF responses to monocarboxylic acids, which were showed in the RBCs derived from various animal species, could involve the proportion of AA in phospholipid layers in the RBC membrane.

In contrast to monocarboxylic acids, most of dicarboxylic acids commonly decreased OF in the RBCs derived from rats [7], guinea pigs [7], sheep [8] and cattle [11] in our previous reports, and pigs in the present series of experiments. It is difficult to explain the cause of the shared OF responses simply by differences in phospholipid composition in the RBC membrane of different animal species. We proposed a "wedge-like effect" to explain the phenomenon of the OF-lowering effect by dicarboxylic acids [7,9,11,13]. In brief, when dicarboxylic interact with the cell membrane, hydrophilic hydrocarbon chains, which form a U or V type conformation, enter the acyl-chain layer of the phospholipids and the two hydrophobic carboxylic groups are directed to the outside and their moieties remain in the water/lipid interface. The dicarboxylic acids positioned on the surface of cell membrane are, therefore, thought to make rigid bonds with

the head element and root of the acyl-chains in the phospholipids, stabilizing the RBC membrane leading to decreases in OF.

Among the monocarboxylic acids, formic acid (C0) and n-enanthic acid (C7) with straight hydrocarbon chains, iso-valeric (C4), dimethyl-propionic (C5), 2-methyl-n-valeric (C5), 4-methyl-n-valeric (C5), and 3,3-dimethyln-butyric acids (C5) with branched hydrocarbon chains, and cyclopentane-carboxylic (C5), cyclohexane-carboxylic (C6) and benzoic acids (C6) decreased OF in a dose-dependent manner ($P<0.05$ or 0.01). Although the decrease in OF induced by the above monocarboxylic acids was similar to those induced by dicarboxylic acids, which can be explained by the wedge-like effect, the mechanism of the OF-lowering effect may differ between the two types of carboxylic acids.

It has been reported that some detergents [28,29] or amphiphilic chemicals [30] induce a biphasic effect on the RBC membrane; a protective effect on the cell membrane at low concentrations and a fragile or hemolytic effect on the cell membrane at high concentrations. These biphasic effects on the RBC membrane are not necessarily observed for all detergents and are dependent on the type of detergent [31]. Monocarboxylic acids have a hydrophilic element (carboxylic group) and a hydrophobic element (hydrocarbons) in the moieties. Therefore, they possess amphiphilic characteristics like as many kinds of detergent. Thus, the OF-lowering effect by monocarboxylic acids may be due to the protective effect on the RBC membrane similar to that demonstrated by some detergents.

Two mechanisms have been proposed to explain the stabilizing effect of amphiphilic

chemicals. Various kinds of amphiphilic chemicals, such as detergents [32] or anesthetics [33], are known to increase the membrane area and cell volume in the RBCs. The increase in cell dimension is thought to be one reason for the observed protection against cell burst and hemolysis. In contrast, the anti-hemolytic effect of amphiphilic chemicals is reported to occur without an increase in the volume of RBCs [30]. The penetration of amphiphilic chemicals into the RBC membrane is thought to induce changes in ion distribution between the inside and outside of the cell membrane [30]. The cell lysis induced by the increase in cell volume or changes in ion balance is assumed to be dependent on the type of chemicals applied to the cells. In further experiments in our laboratory, it is necessary to determine the changes in size or volume of RBCs to clarify the mechanism of carboxylic acids on the RBC membrane in greater detail.

The partition coefficient is a physicochemical parameter which indicate the delivery of chemical compounds between different two solvents [14]. The values of octanol/water partition coefficient for chemicals have been used as an indicator of permeation into the membrane mainly composed of phospholipids [17,18]. There was, however, no significant relationship between the partition coefficient of monocarboxylic acids and their effect on the degree of changes in OF. In contrast, a positive significant relationships were observed between the partition coefficient of dicarboxylic acids and their effect on the degree of decreases in OF at 10, 25 and 50 mM. However, the slope in the equation obtained by regression analysis was very small or close to 0. This result indicates that the degree of change in OF was not affected by the type of dicarboxylic acid tested or the doses, and was not affected by the partition coefficient value. It is, therefore, difficult to use the partition coefficient as a comprehensive indicator for estimating the degree of changes in OF in pig RBCs.

CONCLUSION

We were able to demonstrate the possibility that the fatty acid concentrations, especially the content or proportion of AA, in the phospholipid layers provides a clue to determining the type of OF response (rat or guinea pig type) to monocarboxylic acids in the RBCs of various mammalian animals. In addition, we were also able to propose the wedge-like effect as a membrane-stabilizing action induced by dicarboxylic acids. These two concepts are demonstrated in our previous report [11]. Although, a guinea-pig type OF response was demonstrated in RBCs from guinea pigs, sheep, cattle, and now pigs, a rat type OF response was only shown in RBCs from rats. Further experiments are needed to find rat-type OF response in RBCs from other animal species other than rats, by using the proportion of AA in the RBC membrane as an indicator. This approach would prove our hypothesis that AA in the RBC membrane is the main factor in determining the type of OF response to monocarboxylic acids. this would also prove the wedge-like effect of dicarboxylic acids as a concept commonly observable the RBCs from various animal species.

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