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Effect of Various C_{16} Fatty Acids, Isopentadecanoic and Isomargaric Acids, on Stabilization of Rat Erythrocytes and Carrageenin-induced Edema[1]

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Several fatty acids, including 2-hexyldecanoic acid, iso-$C_{15:0}$, iso-$C_{16:0}$, and iso-$C_{17:0}$ as branched series and various types of C_{16} fatty acids, were examined for protection of erythrocytes from heat-induced or hypotonic hemolysis, of heat-induced denaturation of bovine serum albumin (BSA) *in vitro*, and for inhibition of carrageenin-induced edema in hind paw of a rat *in vivo*.

All of fatty acids tested were found to have a protective effect on heat-induced hemolysis of rat erythrocytes and heat-induced denaturation of BSA. Inhibitory effect on hypotonic hemolysis was recognized in all fatty acids except 2-hexyldecanoic acid, and iso-series especially showed greater activity. In regard to carrageenin-induced paw edema, only iso-$C_{15:0}$ and iso-$C_{16:0}$ exhibited inhibitory an effect, and other fatty acids had no effect.

Keywords——C_{16} fatty acids; anti-inflammatory effect; heat-induced hemolysis; hypotonic hemolysis; carrageenin-induced edema; bovine serum albumin heat denaturation; isopentadecanoic acid; isomargaric acid; isopalmitic acid

In the course of screening on anti-inflammatory principle among products of *Streptomyces*, *Streptomyces* sp. F4818, isolated from a soil sample collected in Osaka Prefecture, was found to produce a mixture of fatty acids as an active component in the mycelium.[3] The fatty acid mixture possessed a strong inhibitory activity against carrageenin-induced paw edema in a rat, and contained as straight-chain fatty acids, myristic (n-$C_{14:0}$), pentadecanoic (n-$C_{15:0}$), palmitic (n-$C_{16:0}$), stearic (n-$C_{18:0}$), and oleic (n-$C_{18:1}$) acids, and as branched chain acids, iso- or anteiso-myristic, isopentadecanoic, isopalmitic, and iso- or anteiso-margaric acids.[4] Palmitic acid was identified as the main component in these fatty acids. Protection of erythrocytes from heat-induced or hypotonic hemolysis and of heat-induced denaturation of bovine serum albumin (BSA), and the inhibition for carrageenin-induced edema of the rat paw by oral administration have been investigated on normal series fatty acids, n-$C_{10:0}$—n-$C_{20:0}$, and it has become apparent that only n-$C_{16:0}$ was active on all screening systems for anti-inflammatory agent described above.

The present work shows that iso-fatty acids which were identified as a component in the mycelium of *Streptomyces* sp. F4818, and related C_{16} fatty acids exhibit anti-inflammatory effect.

Materials and Methods

Isopentadecanoic, isopalmitic, and isomargaric acids were supplied by Dr. T. Kikuchi,[5] Research Institute for Wakan-yaku, University of Toyama. 2-Hexyldecanoic acid (purity: 95%) was kindly supplied

1) This work was presented at the 27th Annual Meeting of Kinki Branch, Pharmaceutical Society of Japan, Akashi, Nov. 1977.
2) Location: *133–1, Yamadakami, Suita-shi, Osaka 565, Japan.*
3) T. Mimura, N. Onishi, S. Toyoda, N. Muto, and S. Aonuma, *Yakugaku Zasshi*, **97**, 1240 (1977).
4) T. Mimura, N. Onishi, S. Toyoda, H. Tsujibo, M. Masumoto, and S. Aonuma, *Yakugaku Zasshi*, **97**, 1246 (1977).
5) T. Kikuchi, T. Mimura, Y. Moriwaki, K. Negoro, S. Nakazawa, and H. Ono, *Yakugaku Zasshi*, **93**, 658 (1973).

by the Nikko Chemicals Co., Tokyo. Bovine serum albumin (fraction V, essentially fatty acid free, less than 0.005%) and phenylbutazone were obtained from Sigma Chemical Co., U.S.A., palmitoleic (*cis*-9-hexadecenoic), palmitelaidic (*trans*-9-hexadecenoic), and α-hydroxypalmitic (2-hydroxy-*n*-hexadecanoic) acids (each purity: 99%) from the P–L Biochemicals, Inc., U.S.A.

Effect of Fatty Acid on Stabilization of Rat Erythrocytes——i) Test for Heat-induced Hemolysis: Fresh erythrocytes from a male Sprague–Dawley rat were washed three times with 0.15 M phosphate buffer (pH 7.4) and suspended in the same buffer to make 0.5% hematocrit value. To 2.0 ml of 0.15 M phosphate buffer (pH 7.4) containing 5 μl of EtOH solution of each sample (EtOH as control), 2.0 ml of the erythrocyte suspension was added and mixed throughly. The mixture was kept at 53° for 20 min and then centrifuged at 900×*g* for 5 min. Absorbance of the supernatant at 550 nm was determined as a measure of the amount of hemoglobin released from the hemolyzing erythrocytes.

ii) Test for Hypotonic Hemolysis: To 3.8 ml of buffered hypotonic saline (50—70 mM NaCl in 10 mM phosphate buffer, pH 7.0) 0.2 ml of the fresh rat erythrocyte suspension (5% hematocrit value) was added and mixed throughly. The mixture was kept at 25° for 5 min and then centrifuged at 900×*g* for 5 min. Absorbance of the supernatant at 550 nm was determined. Each sample was dissolved in 5 μl of EtOH and was added to the incubation mixture before addition of erythrocyte suspension.

Effect of Fatty Acid on Heat-induced Denaturation of BSA——Each sample was dissolved in dimethyl sulfoxide (DMSO). To 2.7 ml of BSA solution (0.75% crystalline bovine serum albumin dissolved in 0.25 M phosphate buffer, pH 5.3) 0.3 ml of each sample solution was added and incubated for 15 min at 25°. The mixture was then heated for 10 min at 65° and reimmersed in the 25° bath. After 15 min, the degree of turbidity of the mixture was determined by reading absorbance at 660 nm.

Effect of Fatty Acid on Carrageenin-induced Edema in Rat Paw——According to the method of Van Arman, *et al.*[6] and of Winter,[7] 0.1 ml of 1% carrageenin (Picnin A, Pasco International Corp., Tokyo) in saline was injected subcutaneously into the foot pad of the hind paw of male Sprague–Dawley rats weighing about 150 g. Swelling rate of the edema was determined just after the injection and at 1 hr intervals after 2 hr. Each sample was ground to a fine powder with acacia (final concentration: 5%), suspended in saline and this suspension was administered orally 30 min before carrageenin injection.

Results

Inhibition of Heat-induced Hemolysis by Fatty Acids

Fig. 1 shows comparison of the potency of various fatty acids to inhibit the hemolysis of fresh rat erythrocytes. As shown in Fig. 1, iso-$C_{15:0}$, iso-$C_{16:0}$, and iso-$C_{17:0}$ readily inhibited the heat-induced hemolysis by 50 to 70% in a concentration range of 1.5 to 3.0 μg/ml. 2-Hexyldecanoic acid, a branched fatty acid, showed a slight activity at the same doses. Other C_{16} fatty acids, palmitoleic, palmitelaidic, and α-hydroxypalmitic acids, at similar concentrations showed potencies comparable to that of palmitic acid. All these fatty acids tested inversely, enhanced hemolysis at a higher concentration. As compared with these fatty acids, phenylbutazone required a considerably high concentration (about 700 μg/ml) for 50% inhibition.[3]

Protective Effect of Fatty Acids on Hypotonic Hemolysis

Presence of all fatty acids, except 2-hexyldecanoic acid, at the concentration of 3 or 12 μg/ml in hemolyzing buffered saline (20—160 mM) brought about a distinct shift of the hemolysis to the protection. As illustrated in Fig. 2, it was evident that the active fatty acids showed such a inhibitory activity on hypotonic hemolysis at the osmotic pressure which gave the most protective effect, in 1.5 to 12.0 μg/ml concentration, and iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, and palmitoleic acid exhibited more than 50% inhibition at a concentration of 6 μg/ml. Unsaturated C_{16} fatty acids showed a protective action on hypotonic hemolysis below 6 μg/ml, but did not show inhibition, or rather accelerated hemolysis in a concentration over 12 μg/ml, and α-hydroxypalmitic acid exhibited a protective action only at the concentration less than 3 μg/ml.

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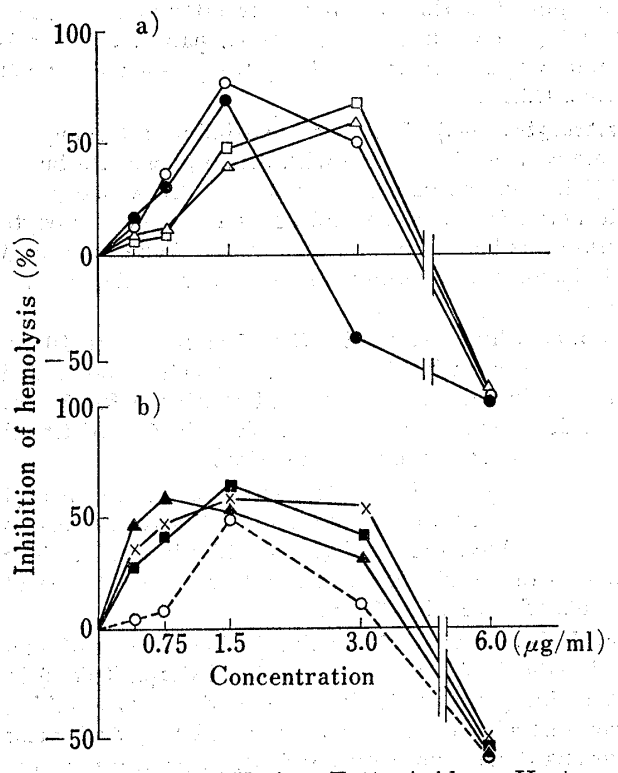

Fig. 1. Effect of Various Fatty Acids on Heat-induced Hemolysis

a) —○—, *n*-$C_{16:0}$; —△—, iso-$C_{15:0}$; —□—, iso-$C_{16:0}$; —●—, iso-$C_{17:0}$.
b) ···○···, 2-hexyldecanoic acid; —▲—, palmitelaidic acid; —■—, palmitoleic acid; —×—, α-hydroxypalmitic acid.

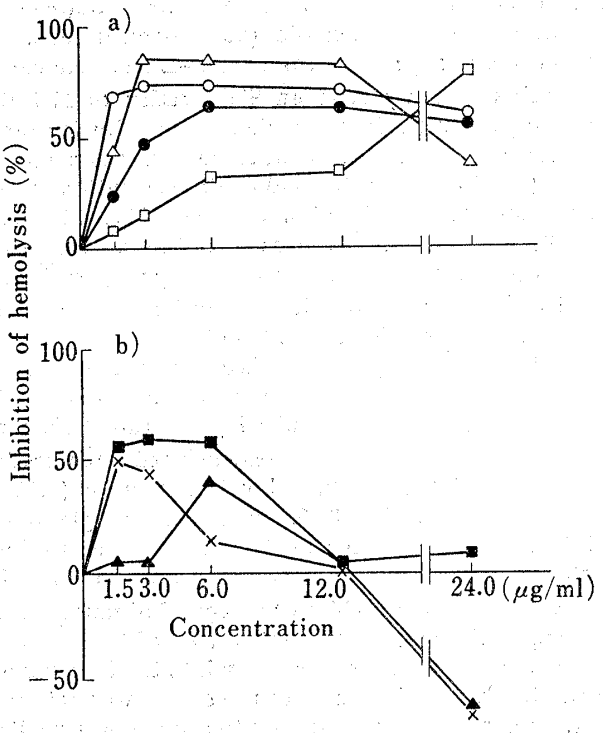

Fig. 2. Effect of Various Fatty Acids on Hypotonic Hemolysis

a) —○—, *n*-$C_{16:0}$ (70 mM NaCl); —△—, iso-$C_{15:0}$ (70 mM NaCl); —□—, iso-$C_{16:0}$ (70 mM NaCl); —●—, iso-$C_{17:0}$ (70 mM NaCl).
b) —▲—, palmitelaidic acid (50 mM NaCl); —■—, palmitoleic acid (60 mM NaCl); —×—, α-hydroxypalmitic acid (60 mM NaCl).

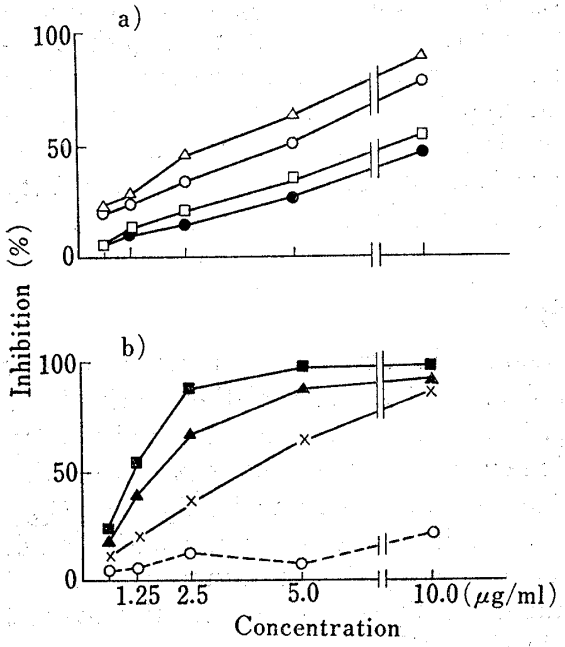

Fig. 3. Effect of Various Fatty Acids on Heat Denaturation of BSA

a) —○—, *n*-$C_{16:0}$; —△—, iso-$C_{15:0}$; —□—, iso-$C_{16:0}$; —●—, iso-$C_{17:0}$.
b) ···○···, 2-hexyldecanoic acid; —▲—, palmitelaidic acid; —■—, palmitoleic acid; —×—, α-hydroxypalmitic acid.

Effect of Fatty Acids on Heat-induced Denaturation of BSA

Data in Fig. 3 showed that, at a low concentration, all the fatty acids except 2-hexyldecanoic acid readily protected denaturation of BSA induced by heat. Lower concentrations were less effective in general, although C_{16} unsaturated fatty acids showed over 50% inhibition at the concentration of 2.5 μg/ml. At concentrations below 0.75 μg/ml, every fatty acids was inactive.

Inhibitory Effect of Fatty Acids on Carrageenin-induced Paw Edema in Rats

Anti-inflammatory effect of each sample of fatty acids was tested for carrageenin-induced edema in hind paw of a rat, and its results are shown in Table I. For comparison, phenylbutazone was tested. The oral administration of 100 mg/kg of iso-$C_{15:0}$ or iso-$C_{16:0}$ and the same dose of phenylbutazone showed similar strength of inhibition on carrageenin-

induced hind paw edema in a rat. No inhibition was observed by oral administration of other fatty acids at the same dose.

Table I. Effect of Various Fatty Acids by Oral Administration on Carrageenin-induced Paw Edema in Rats

Sample	No. of experiments	Swelling (%) 2 hr[d]	3 hr	4 hr	5 hr	6 hr	7 hr
1) Control	8	19.0±2.2	38.2±2.0	58.0±1.7	60.8±1.7	55.5±1.5	54.2±1.4
Iso-$C_{15:0}$	4	14.9±1.7	24.7±2.6[b]	43.9±4.4[b]	47.2±4.7[b]	44.9±4.2[b]	44.0±4.7[a]
Iso-$C_{16:0}$	4	12.3±1.8	24.5±4.0[b]	42.8±4.0[b]	48.1±2.1[b]	46.4±2.7[a]	46.9±3.3[a]
Iso-$C_{17:0}$	4	16.9±1.3	35.2±2.4	61.2±2.3	64.4±2.2	59.4±1.5	59.4±1.5
2) Control	6	17.8±1.9	37.3±2.7	50.5±2.1	58.4±2.2	57.6±3.0	51.8±2.5
Palmitelaidic acid	6	30.2±4.1	50.7±3.4	53.1±2.7	54.3±3.1	50.2±3.2	45.8±3.0
Palmitoleic acid	6	23.7±3.4	43.8±5.0	53.1±3.4	57.2±2.4	56.0±3.7	49.0±3.6
α-Hydroxypalmitic acid	6	19.7±2.7	39.3±6.0	47.5±5.5	53.9±4.1	54.3±4.5	50.0±3.8
3) Control	8	40.7±1.8	51.5±1.3	57.8±1.6	58.9±1.9	52.8±2.1	46.7±1.7
2-Hexyldecanoic acid	6	37.1±1.6	48.3±1.6	54.2±1.4	55.3±1.8	51.4±1.9	46.7±1.1
Phenylbxtazone	6	14.9±2.7[c]	23.7±2.7[c]	28.7±3.0[c]	35.1±3.1[c]	35.7±2.8[c]	34.9±2.4[c]

All values represent mean±s.e.
Dose: 100 mg/kg, *p.o.*
Significantly different level from control group; *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$, *d*) Time after injection of carrageenin.

Discussion

In the present work, iso-fatty acids, unsaturated, and α-hydroxyl-C_{16} fatty acids were examined for their anti-inflammatory effect. In C_{16} series, palmitic and isopalmitic acids without a double bond were active on every screening system, but *n*-$C_{16:1}$, palmitoleic, and palmitelaidic acids, had no effect on carrageenin-induced edema as shown in Table II, and biological activity was reduced by α-hydroxylation of *n*-$C_{16:0}$.

Table II. Activities of C_{16}- and Iso-Fatty Acids in Various Experimental Systems

Sample	Screening system: Heat-induced hemolysis	Hypotonic hemolysis	BSA denaturation	Carrageenin-induced edema[a]
Palmitic acid	+	+	+	+
Isopentadecanoic acid	+	+	+	+
Isopalmitic acid	+	+	+	+
Isomargaric acid	+	+	+	−
2-Hexyldecanoic acid	+	−	−	−
Palmitoleic acid	+	+	+	−
Palmitelaidic acid	+	+	+	−
α-Hydroxypalmitic acid	+	+	+	−

a) 100 mg/kg, *p.o.*

Although normal pentadecanoic acid was not effective on carrageenin-induced edema,[4] iso-$C_{15:0}$ showed a strong inhibition in all experiments. These results show that the terminal dimethyl group in iso-$C_{15:0}$ functions largely as an active site *in vivo*.

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