Pharmaceutical Research, Vol. 5, No. 5, 1988

Report

Enhanced Membrane Permeability to Phenol Red by Medium-Chain Glycerides: Studies on the Membrane Permeability and Microviscosity

Kazutaka Higaki,[1] Muneyoshi Kato,[1] Mitsuru Hashida,[1] and Hitoshi Sezaki[1,2]

Received June 29, 1987; accepted January 4, 1988

To clarify the mechanism of the drug absorption enhancement by medium-chain glycerides (MCG), the changes in membrane permeability provoked by MCG were investigated with liposomal uptake experiments. Uptake of phenol red (PR) into liposomes increased with an increase in MCG content in the liposomal membrane, suggesting that PR absorption was enhanced in the "transcellular route." However, the apparent membranous microviscosity obtained in fluorescence depolarization studies tended to increase with the addition of MCG in both the hydrophobic core and the polar head regions of the liposomal membrane. Thus, an enhancement in membrane permeability caused by MCG was not accompanied by a decrease in the apparent membranous microviscosity.

KEY WORDS: enhancement of membrane permeability; uptake into liposomes; membranous microviscosity; medium-chain glyceride; cholesterol; phenol red.

INTRODUCTION

As reported in previous papers, medium-chain glycerides (MCG) have the potential to improve the bioavailability of anionic compounds by affecting the processes of both absorption and biliary excretion (1,2). The enhancing mechanisms of intestinal absorption remained to be elucidated, but the following observations were reported previously: (i) MCG could promote the transport of phenol red (PR) in the water phase from mucosa to serosa in *in vitro* intestinal everted sac experiments (2); (ii) enhancement of PR absorption required the coexistence of MCG (2); and (iii) the activation energies for the release of PR from liposomes were not altered by the addition of MCG or MCG components (3).

In the present study, the effects of MCG on membrane permeability were investigated with liposomal uptake studies. The changes in the apparent membranous microviscosity were monitored for the interior hydrocarbon phase and polar surface of lipid membranes by fluorescent depolarizing studies. The relationship between membrane permeability and microviscosity is discussed herein.

MATERIALS AND METHODS

Materials. PR, 1,6-diphenyl-1,3,5-hexatriene (DPH), and 8-anilino-1-naphthalenesulfonic acid (ANS) magnesium salt were purchased from Nakarai Chemicals (Japan). Polyoxyethylene derivatives of hydrogenated castor oil (HCO-100) and MGK as medium-chain glycerides, a mixture of mono (MG)-, di (DG)-, and tricapryl glyceride (TG) and caprylic acid (CA), were obtained from Nikko Chemicals (Japan). Components of MCG were fractionated with a silica gel column (Wako gel, Wako Pure Chemicals, Japan) as reported previously. Phosphatidylcholine was prepared from egg yolks and purified chromatographically on alumina and silica columns. All other reagents of analytical grade were obtained from Nakarai Chemicals and Wako Pure Chemicals (Japan).

Preparation of Liposomes. For the uptake experiments, liposomes were prepared from a mixture of egg phosphatidylcholine (egg PC), cholesterol (Ch), and MCG or MCG components dissolved in chloroform, according to the method of Bangham *et al.* (4). The thin dry lipid film containing 80 μmol of egg PC was resuspended with 6 ml of pH 6.5 buffered saline, using a Vortex mixer for 10 min. Under nitrogen, the suspension was sonicated (Ohtake Sonicator-150, Japan) for 2.5 min on ice. The obtained liposomes with or without MCG were observed to have a multilamellar structure, with the diameter ranging from 100 to 500 nm as evidenced by electron microscopy. Dialysis experiments using the cellulose tubing showed that 70 to 90% of the MCG added to liposomes existed in the membranes. For fluorescence measurement, the lipid film was resuspended with 0.05 *M* phosphate buffer (pH 6.5) containing 0.1 *M* KCl and the final concentration of egg PC was adjusted to 1 m*M*.

Determination of PR Uptake in Liposomes. The liposomal suspension (5 ml) was mixed with 10 ml of a drug solution in a flask and then the mixture was incubated by shaking at 25°C. Two milliliters of the mixture was taken at a suitable time period and immediately applied to a Sephadex

[1] Department of Basic Pharmaceutics, Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida shimoadachi-cho, Sakyo-ku, Kyoto, 606, Japan.

[2] To whom correspondence should be addressed at Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida shimoadachi-cho, Sakyo-ku, Kyoto, 606, Japan.

0724-8741/88/0500-0309$06.00/0

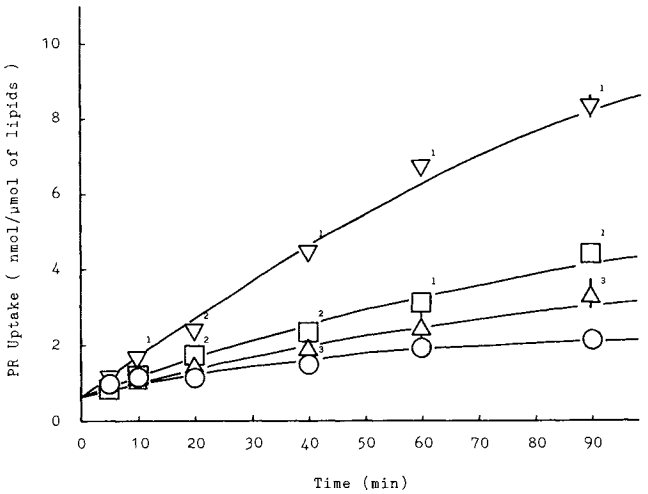

Fig. 1. Effect of MCG on the liposomal uptake of PR. Compositions and symbols for liposomes are as follows: control liposome, egg PC:Ch = 4:1 (○); MCG-containing liposomes, egg PC:Ch:MCG = 4:1:0.5 (△), 4:1:1 (□), and 4:1:2 (▽). Uptake is represented as nanomoles of PR per μmole of lipids. Mean values of more than three experiments are represented and SD values are smaller than the size of each symbol. Statistically significant differences between experimental studies and the control are indicated as follows: (1) $P < 0.001$; (2) $P < 0.01$; (3) $P < 0.02$.

G-50 column (9). Liposomal fractions were completely collected and PR in these fractions was determined spectrophotometrically at 560 nm after alkalinization with 1 *N* NaOH. The uptake rate constant was obtained from the slope of the semilogarithmic plot of the remaining percentage of PR against time.

Fluorescence Labeling of Liposomes. For DPH, a DPH solution (1 m*M*) in tetrahydrofuran was diluted about 1000-fold with a vigorously stirred liposome solution (5,6). For ANS, ANS dissolved in 0.05 *M* phosphate buffer was added to the lipid film to obtain a final concentration of 10 μ*M* ANS in the liposomal suspension (7).

Fluorescence Measurements. Fluorescence polarization and microviscosity were obtained by simultaneous measurement of $I_{//}$ and $I_{\perp}$, parallel and perpendicular to the direction of polarization of the excitation beam, with a Shimazu PR450 spectrofluorophotometer equipped with a Shimazu P/N 204-03290 fluorescence polarizer. The degrees of fluorescence polarization (P) and apparent microviscosity ($\bar{\eta}$) were calculated by the following equations (8):

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

$$\bar{\eta} = \frac{2P}{0.42 - P}$$

In the measurement of fluorescence, excitation and emission wavelengths were 390 and 430 nm for the measurement of DPH and 380 and 470 nm for ANS, respectively. The DPH-labeled liposomal suspension was exposed to the excitation light for less than 10 sec to avoid the irreversible photoisomerization of DPH (6). In all fluorescence measurements, temperature was controlled with a Yamato thermoelite BH-51 (Japan).

Statistical Analyses. Statistical analyses were performed using Student's *t* test.

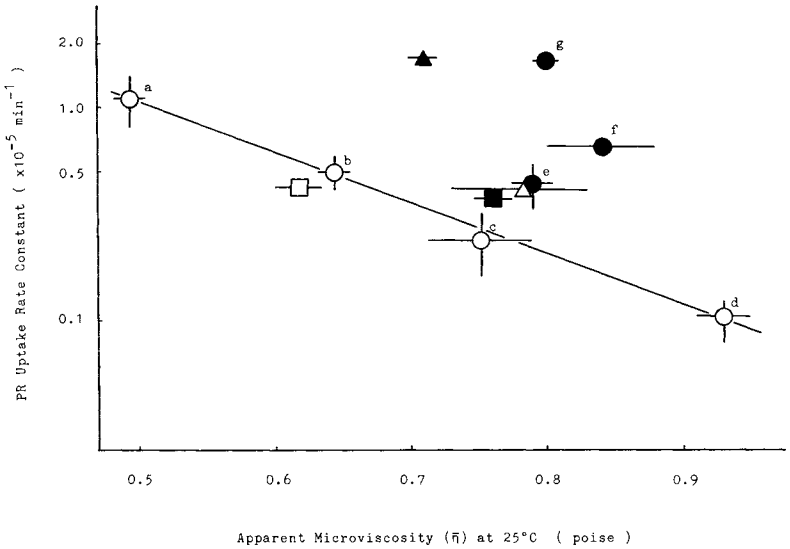

Fig. 2. Relationship between the PR uptake rate constant logarithmic scale and the apparent microviscosity of the liposomal membrane. Uptake rate constants are calculated from the results in Fig. 1. Each datum is represented as the mean ± SD of more than three studies. The straight line was acquired from liposomes consisting of egg PC and Ch by a least-squares method. Compositions and symbols for liposomes are as follows: egg PC:Ch (○) = 4:0 (a), 4:0.5 (b), 4:1 (c), and 4:2 (d); egg PC:Ch:MCG (●) = 4:1:0.5 (e), 4:1:1 (f), and 4:1:2 (g); egg PC:Ch:MG (△) = 4:1:1; egg PC:Ch:DG (▲) = 4:1:1; egg PC:Ch:TG (□) = 4:1:1; and egg PC:Ch:CA (■) = 4:1:1.

Table I. Effect of MCG on the Apparent Microviscosity of the Core Region of the Liposomal Membrane[a]

MCG/egg PC ratio	Apparent microviscosity ($\bar{\eta}$) (P)			
	4°C	15°C	25°C	37°C
Control	1.4171 ± 0.0484	1.0139 ± 0.0217	0.7521 ± 0.0460	0.4752 ± 0.0291
0.025	1.5344 ± 0.0799	1.0398 ± 0.0324	0.7774 ± 0.0086	0.5274 ± 0.0070
0.125	1.3787 ± 0.0413	1.0131 ± 0.0211	0.7890 ± 0.0154	0.4956 ± 0.0089
0.250	1.7893 ± 0.0141*	1.2314 ± 0.0349*	0.8418 ± 0.0411	0.6063 ± 0.0014**
0.500	1.6214 ± 0.0283**	1.0606 ± 0.0290	0.7989 ± 0.0063	0.4775 ± 0.0091

[a] DPH was employed as a fluorescence probe. The composition of the control liposome was egg PC:Ch, 4:1. MCG/egg PC ratio is the molar ratio of adding MCG to egg PC. Data are the means ± SD of more than three experiments.

* Statistically significant difference between experimental studies and the control, $P < 0.001$.

** Statistically significant difference between experimental studies and the control, $P < 0.01$.

RESULTS AND DISCUSSION

In the liposomal preparations containing 10 to 40 μmol of MCG, the uptake of PR was investigated for 90 min (Fig. 1). The amount of PR taken up into liposomes per micromole of lipids increased with the addition of MCG. Liposomes with 40 μmol of MCG showed the highest uptake, about 8.35 μmol/μmol of lipids for 90 min, which was about four times greater than that of the control preparation. These results corresponded with previous findings that PR was released more rapidly with the addition of MCG (3). PR uptake rate constants in the initial phase for liposomes with 10, 20, and 40 μmol of MCG were 4.40 ± 1.17 ($P < 0.1$), 6.53 ± 0.28 ($P < 0.01$), and 16.37 ± 0.10 min^{-1} ($P < 0.01$), respectively (Fig. 2). These constants are two to eight times larger than that of the control (2.39 ± 0.67 min^{-1}).

PR incorporation into liposomes containing each of the MCG components (egg PC:Ch:MCG components, 4:1:1) was also investigated (Fig. 2). DG and TG promoted PR uptake into liposomes. The uptake rate constants for DG and TG were 16.85 ± 0.39 ($P < 0.001$) and 4.20 ± 0.36 ($P < 0.01$) min^{-1}, respectively. These results coincided with the report that phosphatidylcholine (PC) with a shorter fatty acid chain could increase the liposomal membrane permeability (10).

Membrane fluidity of the liposomal membrane with or without MCG was investigated. The values summarized in Table I were obtained by utilizing DPH as a fluorescence probe. In all preparations, $\bar{\eta}$ increased with a decrease in temperature, which meant that the membrane fluidity diminished at lower temperatures.

Liposomal preparations containing a MCG/egg PC molar ratio of 0.025 or 0.125 did not show a statistically significant change in microviscosity. Although the preparation with a MCG/egg PC ratio of 0.25 was slightly altered in membrane fluidity from the control at 4, 15, and 37°C, no statistically significant alteration in microviscosity was shown for any preparation at 25°C.

Table II summarizes the apparent microviscosity of liposomal formulations determined using ANS as a polar head region fluorescence probe. At 25°C, preparations with MCG ratios of 0.125 and 0.250 showed a significant increase in microviscosity.

As for MCG components, TG caused a significant decrease in $\bar{\eta}$ in both the hydrocarbon core and the polar head regions ($\bar{\eta}$ = 0.6178 ± 0.0191 and 0.6918 ± 0.0095, respectively) at 25°C (Fig. 2).

To elucidate the relation of membranous permeability and microviscosity, natural logarithms of uptake rate constants calculated from liposomal uptake studies were plotted against $\bar{\eta}$ at 25°C for liposomes with different lipid compositions (Fig. 2). In liposomal preparations with various contents of Ch, the same experiments were carried out as with MCG, and the results are also summarized in Fig. 2. The linear least-squares regression for these data gives a straight line with a regression coefficient of −0.9974. It is well known that Ch decreases the fluidity of the membrane above the transition temperature but increases the fluidity below it (11,12). The increase in membrane fluidity was reported to accompany the increase in membrane permeability to K^+ and Na^+ (13) and to glycerol and erythritol (14). In accordance with these findings, a good correlation was obtained

Table II. Effect of MCG on the Apparent Microviscosity of the Polar Head Region of the Liposomal Membrane[a]

Temp.	Apparent microviscosity ($\bar{\eta}$) (P)				
		MCG/egg PC ratio			
	Control	0.025	0.125	0.250	0.500
25°C	0.7216 ± 0.0058	0.7327 ± 0.0087	0.7609 ± 0.0131*	0.8022 ± 0.0074**	0.7456 ± 0.0130
37°C	0.4994 ± 0.0116	0.5056 ± 0.0085	0.5129 ± 0.0006	0.5759 ± 0.0138***	0.5120 ± 0.0114

[a] ANS was employed as a fluorescence probe. The composition of the control liposome was egg PC:Ch, 4:1. MCG/egg PC ratio is the molar ratio of adding MCG to egg PC. Data are the means ± SD of more than three experiments.

* Statistically signficiant difference between experimental studies and the control, $P < 0.02$.

** Statistically significant difference between experimental studies and the control, $P < 0.001$.

*** Statistically significant difference between experimental studies and the control, $P < 0.01$.

between the effects of Ch on membrane permeability and microviscosity in the present study.

The apparent microviscosity, $\bar{\eta}$, was less affected by MCG addition (Table I and II), although the membrane permeability was manifestly enhanced (Fig. 1). At 25°C, MCG increased the microviscosity not in the region of the acyl chain but in the polar head region. The increase in $\bar{\eta}$ in the polar head region by MCG was very similar to the results of Muranushi *et al.* (15). It was shown that the enhancement of drug absorption by the mixed micelle preparation, i.e., oleic acid or monoolein plus bile acids, should be ascribed to the increasing flexibility of the acyl chain of egg PC by oleic acid or monoolein and to their interaction with the polar head of egg PC. However, monoolein did not alter the order parameters of liposomal and brush border membranes, and they suggested that the "interaction" of monoolein with the polar head group of lipid would destabilize the membrane configuration. Both MCG and monoolein did certainly interact with the polar head region of lipids. It is known that the phase transition temperature and cooperativity of the transition can be influenced by the nature of the head group (16,17). Thus, the changes in the polar head region can modulate the state of the inner hydrocarbon core of a membrane.

Levine *et al.* (18) suggested that the glycerol group is likely to provide the main permeability barrier of the bilayer. Information on the changes in mobility of the glycerol moiety should also be necessary to evaluate the effect of MCG. In any case, the mobility of the membrane was less influenced by MCG than Ch, suggesting differences in the mechanism regulating membrane permeability between these two compounds.

Recently the contribution of proteins to the enhancement of drug absorption has been disputed (19,20). It was shown that the effect of a protein fraction on the membrane fluidity was comparatively large (21) and that the fluidity also influenced the configuration of the proteins in the membrane (22). Probably MCG can influence the membrane proteins when administered in the form of an emulsion in *in situ* absorption studies.

Although the contribution of the changes in protein fractions to promoting the action of MCG must be investigated hereafter, it was clarified that MCG influenced the lipid fraction of the membrane and enhanced the membrane permeability to PR.

ACKNOWLEDGMENT

The authors are grateful to Dr. H. Komatsu, Pola Laboratories, for his helpful suggestion and discussion of liposomal experiments.

REFERENCES

1. K. Higaki, I. Kishimoto, H. Komatsu, M. Hashida, and H. Sezaki. *J. Pharmacobio-Dyn.* **9**:532–539 (1986).
2. K. Higaki, I. Kishimoto, H. Komatsu, M. Hashida, and H. Sezaki. *Int. J. Pharm.* **36**:131–139 (1987).
3. K. Higaki, N. Takechi, M. Hashida, and H. Sezaki. *Chem. Pharm. Bull.* (in press).
4. A. D. Bangham, M. M. Standish, and J. C. Watkins. *J. Mol. Biol.* **13**:238–252 (1965).
5. B. R. Lentz, Y. Barengolz, and T. E. Thompson. *Biochemistry* **15**:4521–4528 (1976).
6. M. Shinitzky and Y. Barenholz. *J. Biol. Chem.* **249**:2652–2657 (1974).
7. J. Romer and M H. Bickel. *Biochem. Pharmacol.* **28**:799–805 (1979).
8. M. Shinitzky and Y. Barenholz. *Biochim. Biophys. Acta* **515**:367–394 (1978).
9. N. Muranushi, Y. Nakajima, M. Kinugawa, S. Muranishi, and H. Sezaki. *Int. J. Pharm.* **4**:281–290 (1980).
10. J. De Gier, J. G. Mandersloot, and L. L. M. Van Deenen. *Biochim. Biophys. Acta* **150**:666–675 (1968).
11. B. Bloj and D. B. Zilversmit. *Biochemistry* **16**:3943–3948 (1977).
12. R. A. Demel and F. B. Dekruyff. *Biochim. Biophys. Acta* **457**:109–132 (1976).
13. D. Papahadjopoulos, S. Nir, and S. Ohki. *Biochim. Biophys. Acta* **266**:561–583 (1971).
14. R. N. Mcelhaney, J. De Gier, and E. C. M. Van Der Neut-Kok. *Biochim. Biophys. Acta* **298**:500–512 (1973).
15. N. Muranushi, N. Takagi, S. Muranishi, and H. Sezaki. *Chem. Phys. Lipids* **28**:269–279 (1981).
16. J. L. Browning. In *Research Monographs in Cell and Tissue Physiology, Vol. 7. Liposomes,* Elsevier/North-Holland, Amsterdam, 1981, pp. 189–242.
17. J. D. Esko, J. R. Gilmore, and M. Glaser. *Biochemistry* **16**:1881–1890 (1977).
18. Y. K. Levine, N. J. M. Birdsall, A. G. Lee, and J. C. Metacalfe. *Biochemistry* **11**:1416–1421 (1972).
19. T. Nishihata, T. Higuchi, and A. Kamada. *Life Sci.* **34**:427–436 (1984).
20. H. Kajii, T. Horie, M. Hayashi, and S. Awazu. *Life Sci.* **37**:523–530 (1985).
21. T. Brasitus. *Biochim. Biophys. Acta* **728**:20–30 (1983).
22. A. A. Spector and M. A. Yorek. *J. Lipid Res.* **26**:1015–1035 (1985).