Biochimica et Biophysica Acta, 775 (1984) 197–202
Elsevier

BBA 72240

INHIBITION OF ADP-INDUCED AGGREGATION OF BOVINE PLATELETS BY SATURATED FATTY ACIDS AND ITS RELATION WITH THE CHANGE OF MEMBRANE SURFACE CHARGE

SHUJI KITAGAWA *, HIROKO NISHITAMA and FUJIO KAMETANI

Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi 1, Tokushima 770 (Japan)

(Received April 2nd, 1984)

Key words: Platelet aggregation; Membrane surface charge; Membrane fluidity; Saturated fatty acids; ADP; Fluorescence; (Bovine blood)

The inhibitory effects of saturated fatty acids with 4 to 18 carbon atoms on ADP-induced aggregation of bovine platelets were investigated. The inhibitory effects of the acids increased with increase of their alkyl chain length up to C_{14}. On the other hand, from C_{16} the inhibitory effects tended to decrease with increase of chain length, and stearic acid (C_{18}) was not inhibitory. There was a linear relationship between the inhibitory effects and alkyl chain lengths up to C_{12}. This linear relation and the slope of the linear regression line suggested that the inhibitory effects of the acids depended on their partition into the membrane. The fatty acids decreased the fluorescence of the surface charge probe 2-*p*-toluidinylnaphthalene-6-sulfonate, indicating that they increased the negative charge on the membrane surface. The relative effects of the acids on the fluorescence were consistent with their relative inhibitory effects on aggregation. These results suggest that the inhibition of platelet aggregation by saturated fatty acids is due to a change in the membrane surface charge of the platelet plasma membrane.

Introduction

A variety of agents have been found to inhibit platelet functions. Among these agents, non-steroidal anti-inflammatory drugs such as aspirin have been shown to act by inhibiting platelet cyclo-oxygenase [1], and drugs such as prostaglandin I_2 by activating adenylate cyclase and increasing the intracellular concentration of cAMP [2]. There are many reports that besides these reagents several amphiphilic substances, such as long-chain unsaturated fatty acids and alcohols, inhibit platelet functions [3–6]. Long-chain unsaturated fatty acids, such as linoleic acid, at concentrations much below their critical micelle concentrations inhibit platelet aggregation induced by ADP or thrombin [3–5]. Their inhibitory effects, like those of alcohols [6], seem to be related with their effects in causing membrane perturbation [4,5].

On the other hand, there are few works on the effects of saturated fatty acids. Only stearic acid and palmitic acid have been reported to have no effects on aggregation of human platelets [4]. However, the effects of fatty acids with carbon numbers of less than 16 are little known.

In this work we examined the inhibitory effects of fatty acids with various alkyl chain lengths and investigated the relationship between their inhibitory effects and their chain lengths. We also tried to clarify the mechanism of the effects of these fatty acids especially in relation to the membrane

* To whom correspondence should be addressed.

Abbreviations: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; ANS, 1-anilinonaphthalene-8-sulfonate.

surface charge or membrane fluidity. This work is important not only with respect to the modification of platelet functions by amphiphilic substances but also with respect to the interaction of alkyl chains with the phospholipid layer of biological membranes in general.

Materials and Methods

Materials. Diphenylhexatriene and the potassium salt of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) were purchased from Sigma Chemical Co. (St. Louis, MO). ADP was from Oriental Yeast Co. (Tokyo, Japan). Fatty acids and all other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nakarai Chemicals Co. (Kyoto, Japan). Fatty acids were prepared as aqueous solutions adjusted to pH 7.4 by adding NaOH (up to C_8) or as methanolic solutions (from C_{10}) just before use.

Measurement of aggregation. Aggregation of bovine (Holstein) blood platelets was measured as described previously [5]. That is, a suspension of platelets separated from plasma protein by centrifugation was mixed with 9 vol. of Na,K-Tris medium (137 mM NaCl, 5.4 mM KCl, 11 mM dextrose, 25 mM Tris-HCl, adjusted to pH 7.4) containing 1 mg/ml fibrinogen and various concentrations of fatty acids; the final platelet concentration was about $9 \cdot 10^4/\mu l$. After addition of $CaCl_2$ at a final concentration of 0.5 mM, the platelet suspension was preincubated with fatty acids for 2 min. Then ADP was added and aggregation was measured at 37 °C in an aggregometer RAM-11 (Rikadenki Kogyo, Co., Tokyo, Japan). The effects of reagents on aggregation were expressed as maximum aggregations with the reagents relative to that without reagents.

Measurement of fluorescence polarization. Fluorescence polarization of diphenylhexatriene-labeled bovine platelets was measured as described previously [5]. Platelets at a concentration of about $9 \cdot 10^4/\mu l$ were incubated with 1 μM diphenylhexatriene for 40 min. Diphenylhexatriene fluorescence in platelets was measured in a spectrofluorometer 650-40 (Hitachi Seisakusho Co., Tokyo, Japan) at 37 °C. Fluorescence polarization was determined as described previously [5,6].

Measurement of TNS fluorescence. TNS was employed as a probe to measure the surface potential change in platelet membranes. TNS was added at a final concentration of 5 μM to a platelet suspension as described above. The fluorescence was measured in the spectrofluorometer described above at 37 °C using excitation and emission wavelengths of 323 nm and 433 nm, respectively. After 1-min preincubation with TNS, fatty acids were added to the suspension and the relative fluorescence change was determined.

Results

Effects of fatty acids on ADP-induced aggregation

First we examined the effects of ten saturated fatty acids with carbon numbers of 4 to 18 on 10 μM ADP-induced aggregation. The aggregation was inhibited by all the saturated fatty acids except stearic acid, and results with undecanoic acid are shown in Fig. 1. The inhibitory effects of the saturated fatty acids are plotted against their alkyl chain lengths as shown in Fig. 2; the inhibitory effects of the acids are expressed as their ID_{50} values (i.e., concentrations inducing 50% inhibition of aggregation).

As shown in Fig. 2, the inhibitory effects of saturated fatty acids increased with increase in their alkyl chain length up to C_{14}. Especially the

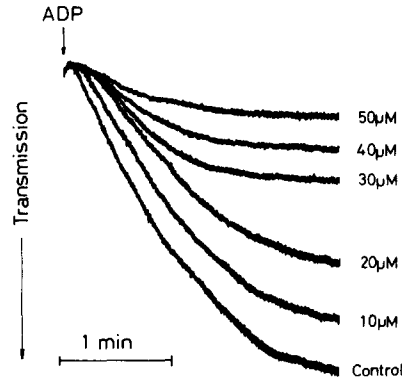

Fig. 1. Effect of undecanoic acid on 10 μM ADP-induced aggregation. The concentrations of undecanoic acid are shown in the figure. A platelet suspension obtained as described in the Materials and Methods (concn. $9 \cdot 10^5/\mu l$) was mixed with 9 vol. of Na,K-Tris medium containing 1 mg/ml fibrinogen. After addition of $CaCl_2$ at a final concentration of 0.5 mM, ADP was added and the aggregation was measured at 37 °C. Aggregation was examined after 2-min preincubation of the platelets with the fatty acid.

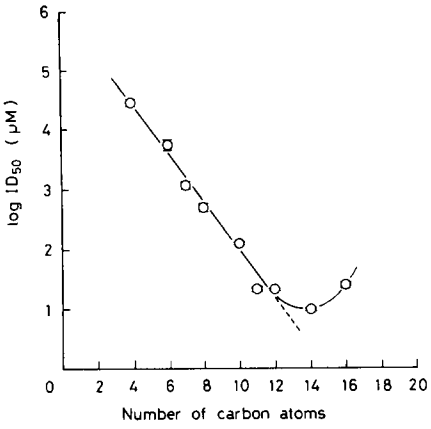

Fig. 2. Relation between the logarithm of the 50% inhibitory concentration of saturated fatty acids, ID_{50}, on 10 μM ADP-induced aggregation and their carbon number. Values are means ± S.D. for three experiments.

data for fatty acids from C_4 to C_{12} fitted a straight line with a negative slope expressed by the following equation:

$$\log ID_{50} = -0.407n + 6.06 \quad (r = 0.992) \qquad (1)$$

where n is the number of carbon atoms of the fatty acid and r is the correlation coefficient. On the other hand, from C_{16}, their inhibitory effects rather decreased with increase of chain length. Stearic acid was not inhibitory even at the concentration of 30 μM, as observed by others with human platelets [4].

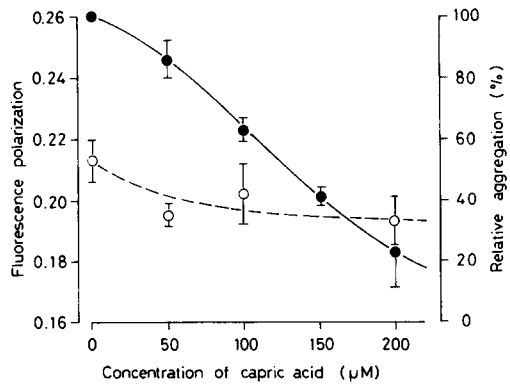

Fig. 3. Effect of capric acid on fluorescence polarization of diphenylhexatriene-labeled platelets (○- - -○) and 10 μM ADP-induced aggregation (●———●). Fluorescence intensities of 1 μM diphenylhexatriene-labeled platelets were detected through a polarizer oriented parallel and perpendicular to the direction of the polarized excitation beam in the presence or absence of the fatty acid. Fluorescence polarization was calculated as described previously [5,6]. The effect of capric acid on aggregation was examined after 2-min preincubation of platelets with capric acid. Data on fluorescence polarization are means ± S.D. for eight to twelve experiments and data on aggregation are those for three experiments.

Effects of fatty acids on membrane fluidity

To determine the inhibitory mechanisms of saturated fatty acids, we next examined their effects on membrane fluidity as their effects on fluorescence polarization of diphenylhexatriene-labeled platelets, because their analogs, such as alkyl alcohols and unsaturated fatty acids, seem to inhibit platelet aggregation by causing membrane perturbation [4–6]. As shown in Fig. 3 for capric acid, addition of the fatty acids slightly decreased the fluorescence polarization, indicating that they increased membrane fluidity to a slight degree [7,8]. However, the extents of decrease were considerably less than with unsaturated fatty acids [5] or alcohols [6].

Effects of fatty acids on membrane surface charge

The membrane surface charge or surface potential has been suggested to have important roles in membrane stability and cellular functions [9,10]. Since fatty acids might transfer a net negative charge to the membrane surface, we then examined the effects of saturated fatty acids on the membrane surface charge by measuring the fluorescence of TNS, which is a probe of the membrane surface charge or surface potential probe, like its analog ANS [11–13]. These probes are

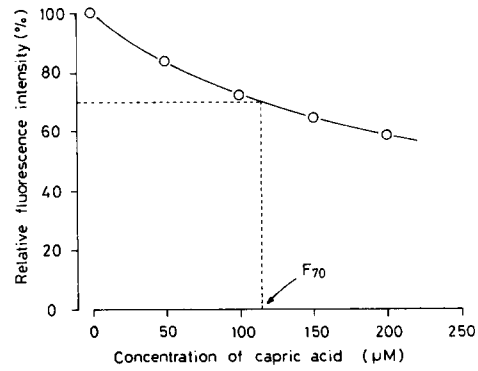

Fig. 4. Example of the effect of capric acid on TNS fluorescence. The F_{70} value was determined as the concentration inducing decrease of TNS fluorescence to 70% of the control value.

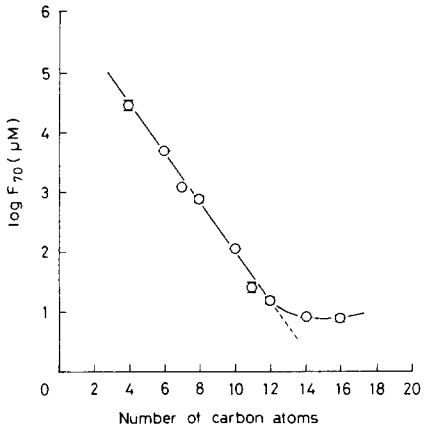

Fig. 5. Relation between the logarithms of the F_{70} values of saturated fatty acids and their number of carbon atoms. Data are means ± S.D. for duplicate experiments.

thought to bind mainly with phospholipids in the membrane [11,14]. As shown in Fig. 4. for capric acid, addition of the fatty acids decreased the fluorescence of TNS, indicating that they increased the negative charge on the membrane that repels the organic anion TNS from the membrane. The fluorescence decreased with increase in the fatty acid concentration. With capric acid, a concentration that caused 50% aggregation inhibition decreased the fluorescence to about 70% from the control value. Other fatty acids caused similar decrease in fluorescence.

To examine the relation between the effects of fatty acids on TNS fluorescence and their alkyl chain length quantitatively, we plotted the F_{70} values of the fatty acids (i.e., the concentrations inducing a decrease in fluorescence to 70%) against their carbon number. As shown in Fig. 5, the data for fatty acids of C_4 to C_{12} also fitted a straight line of negative slope which was expressed by the following equation:

$$\log F_{70} = -0.415n + 6.14 \qquad (r = 0.997) \qquad (2)$$

This result is consistent with the results on the inhibitory effects of the acids shown in Fig. 2 and Eqn. 1, although the effect of palmitic acid in decreasing fluorescence was more than its effect in inhibiting aggregation. Stearic acid did not cause decrease in fluorescence to 70% at concentrations below 30 μM.

Discussion

Our results on the effects of fatty acids on aggregation and the fluorescence of TNS suggest that the inhibition of aggregation by saturated fatty acids is due to their effect in changing the surface charge of the platelet plasma membranes. Although saturated fatty acids also slightly increased the membrane fluidity as revealed here by fluorescence experiments using diphenylhexatriene, their effects were considerably less than those of unsaturated fatty acids [5] and alcohols [6]. Therefore, it is more probable that their effects in inhibiting aggregation were mainly due to their effects on the surface charge. The slight increase in the membrane fluidity by saturated fatty acids may also be related with the change in the surface charge.

Although fatty acids are membrane permeable substances [15], they are thought to be located mainly in the outer leaflet of the membrane [16,17], because of the asymmetrical distribution of phospholipids in the membrane. That is, acidic phospholipids such as phosphatidylserine have been found to be present mostly in the inner leaflet of the membrane, whereas in the outer leaflet of platelet membranes neutral phospholipids such as phosphatidylcholine and sphingomyelin are mainly located [18] as well as of other biological membranes [19]. Therefore, fatty acids are thought to interact mainly with choline groups of phospholipids on the outer surface, thus increasing the net negative charge on the outer surface and changing the membrane surface potential.

Change in the membrane surface charge or surface potential is suggested to result in change of shape of erythrocytes [9] and change of various membrane-associated functions such as enzymatic activities [10] and ionic transport [20]. It is unknown why change in surface charge on addition of fatty acids should affect platelet function. Since normal shape of platelets or normal ionic transport is suggested to be important for platelet functions [21–23], possibly as a result of their changes, it affects construction of the cytoskeletal network, because reorganization of cytoplasmic contractile and structure proteins is known to be essential for platelet activation [24,25]. The inhibitory effects of inorganic anions such as SCN^- and ClO_4^- on

platelet aggregation which we revealed previously [26] may also be explained by a similar mechanism.

Our study also revealed a linear relationship between the inhibitory effects of saturated fatty acids of C_4 to C_{12} on platelet aggregation and their alkyl chain lengths. This linear relation and its slope suggest that the inhibitory effects of saturated fatty acids depend on their partition into the membrane, because partition of the acids into biological membranes such as the erythrocyte membrane exhibit a similar relation suggesting partitioning of the acids into the lipid layer [27]. Similar results have been obtained for the inhibitory effects of alkyl alcohols on platelet aggregation [6], and for the hemolytic effects of alkyl ammonium derivatives [28].

Our results on the effects of fatty acids on the fluorescence of TNS probably reflect the partitioning of the acids into the membrane, resulting in change of membrane surface charge, because almost the same linear relation was observed between fluorescence changes by saturated fatty acids and their alkyl chain lengths. Therefore, the inhibitory effects of saturated fatty acids seem to be determined by their amounts incorporated into the membrane, irrespective of their nature.

The inhibitory effects of saturated fatty acids of more than C_{12} deviated from the linear regression line. This was probably due to a difference in the extents of their partition into the membrane, because fluorescence experiments using TNS suggested a change in the partitioning behavior of saturated fatty acids with carbon numbers of more than 12. This deviation from the linear regression in the relationship between the partition coefficients of saturated fatty acids and their alkyl chain lengths has already been demonstrated in other biological membranes such as the erythrocyte membrane [27]. Moreover, a similar deviation was observed for the hemolytic effects of alkyl ammonium derivatives [28]. As suggested by Klein and Ellory [28], there may be a physical effect of the length of the alkyl chain, and kinking of a very long alkyl chains may be necessary for its entry into the ordered acyl chains of the membrane phospholipids.

The partitioning effects of fatty acids [27] and the biological activities such as hemolytic activities of alkyl ammonium derivatives [28] have been reported to have approximately constant values beyond a certain chain length. However, the inhibitory effects of the fatty acids on platelets decreased markedly with increase in the carbon numbers above 14. This was at least partly due to a difference in the partitioning behavior of the acids in the platelet membrane, because stearic acid, which did not inhibit aggregation, also caused a smaller decrease in TNS fluorescence than other long-chain fatty acids such as myristic acid, although palmitic acid caused a relatively larger decrease in fluorescence than inhibition of aggregation. Other effects of long-chain fatty acids besides their effects on surface charge may also influence their effects on platelet aggregation.

Acknowledgement

This work was supported in part by a grant from the Japanese Ministry of Education, Science and Culture.

References

1 Cerletti, C., Livio, M. and De Gaetano, G. (1981) Biochim. Biophys. Acta 714, 122–128
2 Gorman, R.R., Fitzpatrick, F.A. and Miller, O.V. (1978) Adv. Cyclic Nucleotide Res. 9, 597–609
3 Hoak, J.C., Spector, A.A., Fry, G.L. and Warner, E.D. (1970) Nature 228, 1330–1332
4 Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L., Dawidwicz, E.A., McIntyre, D.E., Salzman, E.A. and Klausner, R.D. (1982) Ann. N.Y. Acad. Sci. 401, 61–75
5 Kitagawa, S., Endo, J. and Kametani. F. (1984) Biochim. Biophys. Acta 798, 210–215
6 Kitagawa, S., Shinohara, T. and Kametani, F. (1984) J. Membrane Biol. 79, 97–102
7 Giraud, F., Claret, M., Bruckdorfer, K.R. and Chailley, B. (1981) Biochim. Biophys. Acta 647, 249–258
8 Fukuzawa, K., Chida, H., Tokumura, A. and Tsukatani, H. (1981) Arch. Biochem. Biophys. 206, 173–180
9 Fortes, P.A.G. and Ellory, J.C. (1975) Biochim. Biophys. Acta 413, 65–78
10 Wojtczak, L. and Nalecz, M.J. (1979) Eur. J. Biochem. 94, 99–107
11 Radda, G.K. and Vanderkooi, J. (1972) Biochim. Biophys. Acta 265, 509–549
12 Azzi, A. (1975) Q. Rev. Biophys. 8, 237–316
13 Chiu, V.C.K., Mouring, D., Watson, B.D. and Haynes, D.H. (1980) J. Membrane Biol. 56, 121–132
14 Rubalcava, B., De Muñoz, D.M. and Gitler, C. (1969) Biochemistry 8, 2742–2747

15 Aubert, L. and Motais, R. (1975) J. Physiol. 246, 159–179
16 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4457–4461
17 Sheetz, M.P. and Singer, S.J. (1976) J. Cell Biology 70, 247–251
18 Chap, H.J., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–164
19 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96
20 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
21 Kanaho, Y. and Fujii, T. (1982) Biochem. Biophys. Res. Commun. 106, 513–519
22 Kanaho, Y., Sato, T. and Fujii, T. (1982) Cell Struct. Function 7, 39–48
23 Kitagawa, S., Endo, J., Kubo, R. and Kametani, F. (1983) Biochem. Biophys. Res. Commun. 111, 306–311
24 Jennings, L.K., Fox, J.E.B., Edwards, H.H. and Phillips, D.R. (1981) J. Biol. Chem. 256, 6927–6932
25 Pribluda, V. and Rotman, A. (1982) Biochemistry 21, 2825–2832
26 Kitagawa, S., Hongu, Y. and Kametani, F. (1982) Biochem. Biophys. Res. Commun. 104, 1371–1375
27 Sallee, V.L. (1978) J. Membrane Biol. 43, 187–201
28 Klein, R.A. and Ellory, J.C. (1980) J. Membrane Biol. 55, 123–131