Acta Medica Okayama

Volume 37, *Issue* 5 1983 *Article* 1

OCTOBER 1983

Inhibition of Phospholipase A2 and Platelet Aggregation by Grycyrrhizin, an Anti-inframmation Drug

Eiji Okimasu[*] Yasunori Moromizato[†] Sadahiro Watanabe[‡]

Junzo Sasaki[**] Noriyuki Shiraishi[††] Yasuko M. Morimoto[‡‡]

Masanobu Miyahara[§] Kozo Utsumi[¶]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Okayama University,
[††]Okayama University,
[‡‡]Okayama University,
[§]Okayama University,
[¶]Okayama University,

Inhibition of Phospholipase A2 and Platelet Aggregation by Grycyrrhizin, an Anti-inframmation Drug*

Eiji Okimasu, Yasunori Moromizato, Sadahiro Watanabe, Junzo Sasaki, Noriyuki Shiraishi, Yasuko M. Morimoto, Masanobu Miyahara, and Kozo Utsumi

Abstract

We studied the effect of glycyrrhizin, a compound known as an anti-inflammatory and antiallergic drug, on the membrane permeability change induced by phospholipase A2 (PLA2) and on platelet aggregation. Glycyrrhizin was found to inhibit the PLA2-induced carboxyfluorescein (CF) release from D,L-dipalmitoyl phosphatidylcholine (DPPC) liposomes. Part of this inhibitory effect of glycyrrhizin on PLA2 is accounted for by the physical state of the substrate, the DPPC liposome membrane. Glycyrrhizin also inhibited collagen-induced platelet aggregation in a concentration dependent manner, which may in part account for its inhibitory effect on PLA2.

KEYWORDS: phosholipase A2, glycyrrhizin, liposome, platelet aggregation

---

*PMID: 6689106 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 37, (5), 385-391 (1983)

INHIBITION OF PHOSPHOLIPASE A_2 AND PLATELET AGGREGATION BY GLYCYRRHIZIN, AN ANTIINFLAMMATION DRUG

Eiji OKIMASU, Yasunori MOROMIZATO, Sadahiro WATANABE, Junzo SASAKI, Noriyuki SHIRAISHI*, Yasuko M. MORIMOTO*, Masanobu MIYAHARA* and Kozo UTSUMI*

*Department of Anatomy, Okayama University Medical School, Okayama 700 and *Department of Medical Biology, Kochi Medical School, Kochi 781-51, Japan.*

Received April 1, 1983

Abstract. We studied the effect of glycyrrhizin, a compound known as an anti-inflammatory and antiallergic drug, on the membrane permeability change induced by phospholipase A_2 (PLA_2) and on platelet aggregation. Glycyrrhizin was found to inhibit the PLA_2-induced carboxyfluorescein (CF) release from D,L-dipalmitoyl phosphatidylcholine (DPPC) liposomes. Part of this inhibitory effect of glycyrrhizin on PLA_2 is accounted for by the physical state of the substrate, the DPPC liposome membrane. Glycyrrhizin also inhibited collagen-induced platelet aggregation in a concentration dependent manner, which may in part account for its inhibitory effect on PLA_2.

Key words : phospholipase A_2, glycyrrhizin, liposome, platelet aggregation.

Glycyrrhizin, a glycoside obtained from *Glycyrrhizina glaba* roots, shows steroid-like action (1) and has been used as an antiinflammatory and antiallergic agent (2, 3). However, the mechanism of its pharmacological action has not been analyzed.

For the purpose of clarifying the mechanism of the steroid-like action of the drug, it is important to detect phospholipase A_2 (PLA_2) activity since changes in phospholipase A_2 activity play an important role in various inflammatory processes (4, 5) and certain steroid hormones regulate PLA_2 activity indirectly (6). It might be postulated that glycyrrhizin exhibits an inhibitory effect on PLA_2.

Recently (7) we reported that PLA_2 activity can be determined by the phase transition release technique (8), a very sensitive assay method in which a small amount of carboxyfluorescein (CF) is released from artificial membrane below the phase transition temperature of its constituent lipid (9). Using this technique, it was found that glycyrrhizin inhibited the PLA_2-induced CF release from liposomes just like other PLA_2 inhibitors. In addition to artificial liposome, human platelets whose aggregation is closely correlated to PLA_2 activity (10), were adopted in this study. It was found that glycyrrhizin inhibited collagen-induced platelet aggregation.

These results indicate that the antiinflammatory and steroid-like actions of glycyrrhizin might be related to its inhibitory effect on PLA_2 activity.

MATERIALS AND METHODS

Reagents. D,L-dipalmitoyl phosphatidylcholine (DPPC) and collagen were purchased from Sigma Chemical Co.. Phospholipase A_2 from porcine pancreas (800 U/mg) was purchased from Boehringer. Carboxyfluorescein (CF) was purchased from Eastman Kodak Co. and was purified by the method of Ralston *et al.* (11). All other chemicals used were of analytical grade. Glycyrrhizin was kindly donated by Minophagen Pharmaceutical Co., Tokyo, Japan.

Phospholipid vesicles. The unilamellar vesicles containing CF were prepared by the method of Huang (12). DPPC was dissolved in chloroform-methanol (2: 1) at a concentration of 22 mg/ml. One ml of the lipid solution was evaporated in a concentrator (Taiyo TC-8). Following the addition of 2 ml of 0.1 M NaCl in 20 mM phosphate buffer (pH 6.8) and 0.1 M CF, the tube was agitated with a vortex mixer for 10 min at 50 ℃, and then the vesicles were sonicated for 60 min at 50 ℃ using a Bransonic sonifier (Type 185). The sample became optically clear within 2-5 min, and 1.5 ml aliquots were applied to a Sepharose 4B column (1.5 × 25 cm), eluted at 4 ℃ with a solution of 0.1 M NaCl-20 mM phosphate buffer (pH 6.8), and unilamellar vesicles were collected.

Phase transition release (PTR). The PTR curve was obtained by the method of Weinstein *et al.* (8). The unilamellar liposome suspension was placed in an ice cold small cuvette and transferred to the heated (50 ℃) cuvette holder of a fluorospectrophotometer (Shimazu, Type RF-50). The temperature of the cuvette holder was controlled by a digital temperature thermistor kept over the light-path in the reaction cuvette which had an attached magnetic stirrer. The PTR was monitored at 515 nm and excited at 470 nm. To obtain the total fluorescence intensity achieved by 100 % release of the dye from liposomes, 5 μl of 10 % Triton X-100 was added to the 2 ml of reaction mixture. PLA_2 and glycyrrhizin were added at 0 ℃ in a medium of 0.1 M NaCl-20 mM phosphate buffer (pH 6.8) and 1 mM $CaCl_2$.

Enzyme activity of PLA_2. The activity of pancreatic PLA_2 was assayed by the formation of lysophosphatidylcholine after incubation of the DPPC liposomes (375 μM) with PLA_2 (50 μg/ml) at 10 ℃ for 1 min. Lysophosphatidylcholine and DPPC were separated by thin layer chromatography, and their amounts were determined by the method of Lowry *et al.* (13).

Platelet aggregation. Aggregation of human platelets by collagen (Sigma Type I from Bovine achilles tendon, dissolved in 0.15 M NaCl) was measured by the method of Yamanaka (14). Changes in absorbance at 610 nm were recorded with a spectrophotometer (Shimazu, UV-200) equipped with a 37 ℃ water bath and stirrer.

Protein concentration was determined by the method of Lowly *et al.* (15) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Phase transition release by PLA_2 and its inhibition by glycyrrhizin. It has been reported that the phase transition release (PTR) of CF from DPPC liposome is stimulated in a concentration dependent manner by adding small amounts of pancreatic PLA_2 (7). This technique was applied in the present investigation. A rather large amount of glycyrrhizin also enhanced CF release, and the concentra-

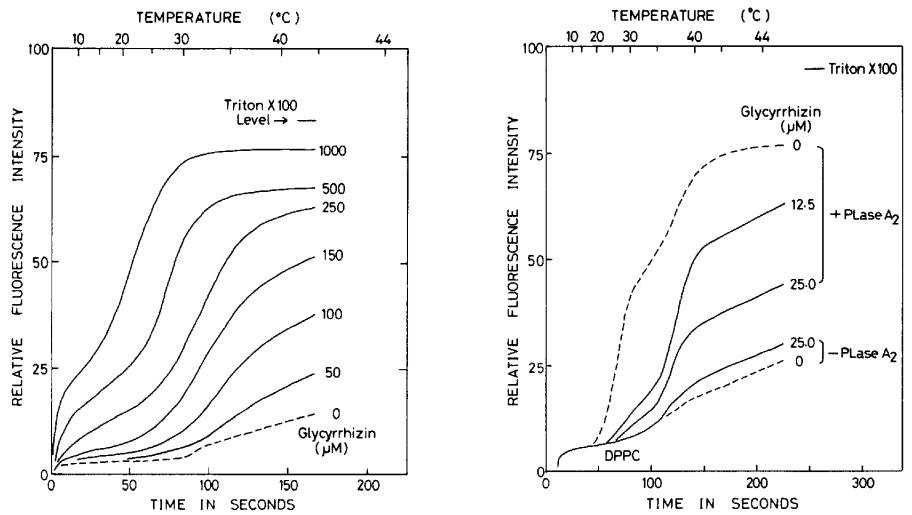

Fig. 1. *(left)* The effect of glycyrrhizin on the phase transition release curve of DPPC. CF-containing unilamellar DPPC liposomes were incubated in a cuvette with various concentrations of glycyrrhizin in a medium of 0.1 M NaCl-20 mM phosphate buffer (pH 6.8)-1 mM $CaCl_2$ at 0 ℃ and transfered to a heated cuvette holder (50 ℃). The temperature in the cuvette increased to above 40 ℃ after 200 seconds as indicated. Fluorescence intensity at 515 nm was monitored by excitation at 470 nm using a Shimazu spectrofluorometer. Total fluorescence intensity of released CF in the medium was measured after addition of 0.0025 % Triton X-100

Fig. 2 . *(right)* The effect of glycyrrhizin on the phase transition release curve of DPPC in the presence of PLA_2. Experimental conditions were as in Fig. 1. PLA_2 (0.1 μg/ml) and various concentrations of glycyrrhizin were added to the medium before incubation. Glycyrrhizin inhibited the release of CF from DPPC liposomes depending on its concentration.

tion required for the maximum release was about 1 mM as shown in Fig. 1. However, the increased CF release by PLA_2 was inhibited by small amounts of glycyrrhizin of about 25 μM (Fig. 2). Similar inhibition of CF release was also observed with certain PLA_2 inhibitors such as trichloroethylene (16) as shown in Fig. 3. Similar inhibitory effects were also obtained with bromophenacyl bromide and indomethacin (17) (data not shown).

Inhibition of PLA_2-induced CF release from DPPC liposomes by glycyrrhizin. When DPPC liposomes were incubated with PLA_2 (0.1 μg/ml) at a constant temperature (22 ℃), the time dependent release of CF induced by PLA_2 was inhibited by adding glycyrrhizin (Fig. 4). But as previously described in Fig. 1, glycyrrhizin itself caused some CF release by its surface active characteristics at rather high concentrations (0.1- 1 mM). Therefore, compensation for this release was made by subtracting the increased fluorescence due to the surface active effect of the drug from the curves of PLA_2-induced CF release inhibited by glycyrrhizin, and the corrected concentration-dependent curve is shown in Fig. 5. The results indicate that the PLA_2-induced CF release is inhibited by glycyrrhizin in a concentration-dependent

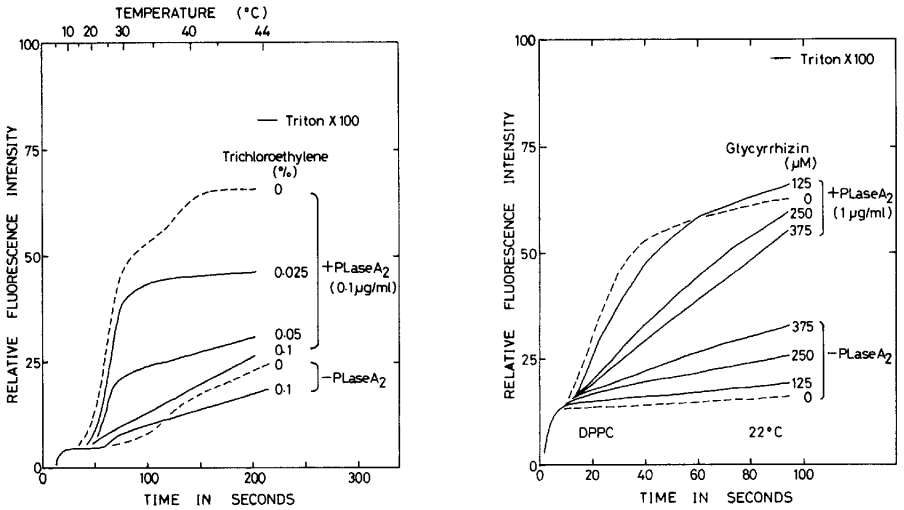

Fig. 3. *(left)* The effect of PLA_2 inhibitor on the phase transition release curve of DPPC liposomes in the presence of PLA_2. Experimental conditions were as in Fig. 2. Trichloroethylene, a membrane modifying reagent, also inhibited the release of CF from DPPC liposomes by decreasing the phase transition point of DPPC.

Fig. 4. *(right)* The effect of glycyrrhizin on the increased CF release of DPPC liposome by PLA_2 at 22 ℃. Unilamellar DPPC liposomes containing CF were incubated with glycyrrhizin in the presence or absence of PLA_2 (1 μg/ml) in a medium of 0.1 M NaCl-20 mM phosphate buffer (pH 6.8)-1 mM $CaCl_2$. The temperature in the cuvette was constant (22 ℃).

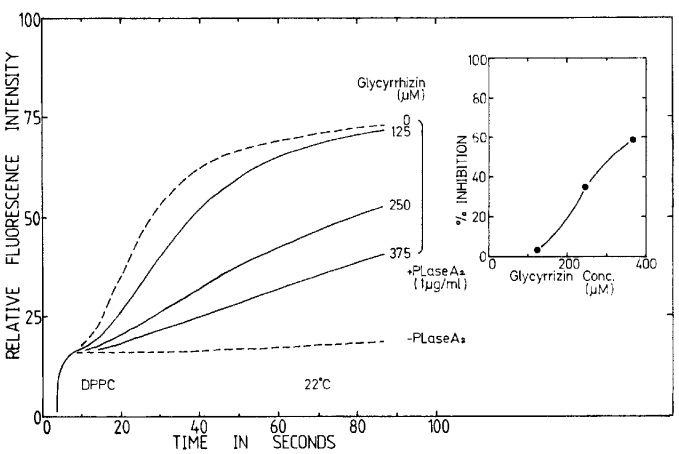

Fig. 5. Concentration dependency of glycyrrhizin in PLA_2 inhibition. The percent inhibition by glycyrrhizin of CF release induced by PLA_2 was obtained from the curve of Fig. 4. The data were normalized as indicated in the text.

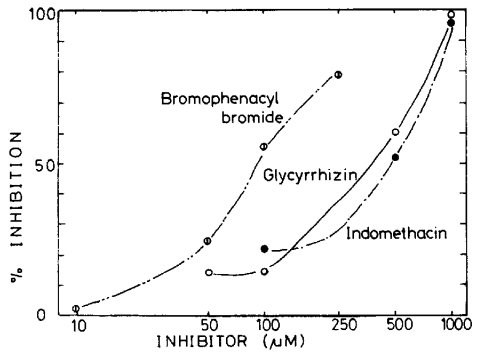

Fig. 6. Inhibition by glycyrrhizin of lysophosphatidylcholine formation induced by pancreatic PLA_2. 375 μM DPPC liposomes were incubated with PLA_2 (50 μg/ml) in the presence or absence of various inhibitors of PLA_2 in a medium of 0.1 M NaCl-20 mM phosphate buffer (pH 6.8)-1 mM $CaCl_2$. The incubation was carried out at 10 ℃ for 1 min. Lysophosphatidylcholine was isolated by thin layer chromatography and the inorganic phosphate content was measured after hydrolysis.

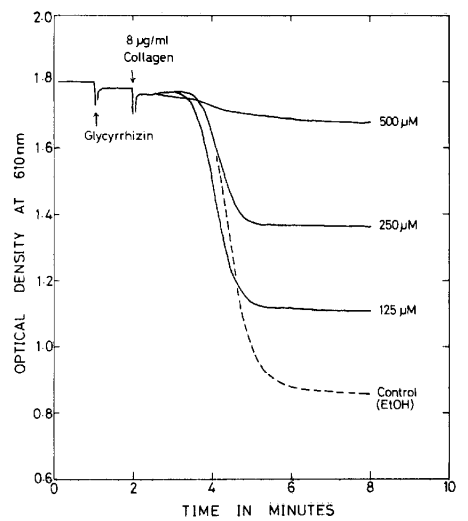

Fig. 7. The effect of glycyrrhizin on collagen-induced platelet aggregation. Human platelet rich plasma was incubated at 37 ℃, and aggregation was induced by collagen in the presence or absence of glycyrrhizin. Ethanol was added to the platelet rich plasma in the control experiment.

manner.

Inhibition of lysophosphatide formation by glycyrrhizin. The inhibitory action of glycyrrhizin on PLA_2 activity was also confirmed by measuring lysophosphatidylcholine formation after incubation of the DPPC liposomes with the enzyme in the presence of various concentrations of glycyrrhizin as shown in Fig. 6. The concentration of the drug required for the inhibition of PLA_2 was quite high compared to that measured by the PTR technique. This may be due to the high concentration of PLA_2 required for detection of the inhibitory action of glycyrrhizin in the short incubation period (18). Of course, the initial velocity of lysophosphatidylcholine formation was very high at such high concentrations of PLA_2. Therefore, low temperature incubation was also required for detection of the inhibitory action of the drug (7). Similar inhibition of lysophosphatidylcholine formation was observed with standard inhibitors of PLA_2 such as bromophenacyl bromide and indomethacin (17, 19) under the same conditions (Fig. 6).

Inhibition by glycyrrhizin of collagen-induced platelet aggregation. It has been reported that the activity of membrane associated PLA_2 is stimulated by collagen, thrombin and ADP and that collagen-induced platelet aggregation is inhibited by some PLA_2 inhibitors (10). Since glycyrrhizin inhibited PLA_2-induced CF release and PLA_2 activity, it was expected that the drug would also inhibit collagen-induced platelet aggregation. In fact, as shown in Fig. 7, the drug inhibited collagen-induced platelet aggregation in a dose dependent manner, though the concentration required was very high. Concerning this inhibitory action of glycyrrhizin on platelet aggregation, it was reported recently that the drug also inhibited cyclooxygenase activity and inhibited the formation of prostaglandins (20). Therefore, it is considered that the inhibition of platelet aggregation by glycyrrhizin is the result of its inhibition of PLA_2 and cyclooxygenase.

The details of the inhibitory mechanism of glycyrrhizin are still obscure, but there is a possibility that glycyrrhizin causes changes in the phase transition point of DPPC liposomes. In fact, there are several inhibitors of PLA_2 activity which modify membrane fluidity and lower the transition temperature, such as ethrane, halothane and trichloroethylene (16).

These results indicate that the antiinflammatory and steroid hormone-like actions of glycyrrhizin might be attributable at least in part to the drug's inhibitory effect on PLA_2 activity. A more detailed investigation of the mechanism of glycyrrhizin's action on membrane events in inflammatory processes is currently in progress.

REFERENCES

1. Kumagai, S., Yano, S., Otomo, M. and Takeuchi, K.: Study on the corticoid action of glycyrrhizin and the mechanism of its action. *Endocrinol. Jpn.* **4,** 17-27, 1957.
2. Yamamoto, S., Miyamoto, A., Awachi, Y. and Hiroshima, Y.: Antiinflammatory activity of strong neo-minophagen C by granulation tissue test. *J. Therapy* **45,** 157-158, 1963 (in Japanese).
3. Kuroyanagi, T. and Sato, M.: Effect of predonisolone and glycyrrhizin on passive transfer of experimental allagic encephalomelitis. *Allergy* **15,** 67-75, 1966.
4. Faherty, J.J. and Ward, P.A.: Chemotactic factors and the neutrophil. *Semi. Hematol.* **16,** 163-174, 1979.
5. Hirata, F., Schiffman, E., Venkatasubamanian, K., Salomon, D. and Axelrod, J.: A phospholipase A_2 inhibitor protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. USA* **77,** 2533-2536, 1980.
6. Hirata, F.: The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. *J. Biol. Chem.* **254,** 7730-7733, 1981.
7. Okimasu, E., Shiraishi, N., Kobayashi, S., Morimoto, M.Y., Miyahara, M. and Utsumi, K.: Permeability changes of phospholipid liposomes caused by pancreatic phospholipase A_2: analysis by means of phase transition release. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **145,** 82-86, 1982.
8. Weinstein, J.N., Klausner, R.D., Innerarity, T., Ralston, E. and Blumenthal, R.: Phase transition release, a new approach to the interaction of proteins with lipid vesicle. *Biochim. Biophys. Acta* **647,** 270-289, 1981.

9. Menashe, M., Lichtenberg, D., Gutierrez-Merino, C. and Biltonen, R.L.: Relationship between the activity of pancreatic phospholipase A_2 and the physical state of the phospholipid substrate. *J. Biol. Chem.* **256,** 4541-4543, 1981.
10. Billak, M.M., Lapetina, E.G. and Cuatrecasas, P.: Phospholipase A_2 and phospholipase C activities of platelets. Differential substrate specificity, Ca^{++} requirement, pH dependence, and cellular localization. *J. Biol. Chem.* **255,** 10227-10231 ,1980.
11. Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.H. and Blumenthal, R.: Carboxyfluorescein as a probe for liposome-cell interactions. Effect of impurities and purification of the dye. *Biochim. Biophys. Acta* **649,** 133-137, 1981.
12. Huang, C.: Studies of phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry* **8,** 344-352, 1969.
13. Lowry, O.H., Roberts, N.R., Leiner, K.Y., Wu, M.L. and Farr, A.L.: The quantitative histochemistry of brain. 1. Chemical method. *J. Biol. Chem.* **207,** 1-17, 1954.
14. Yamanaka, M.: Platelet aggregation. *Jpn. J. Clin. Pathol. (Special Tissue)* **27,** 15-18, 1976 (in Japanese).
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275, 1951.
16. Vigo, C., Lewis, G.P. and Piper, P.J.: Mechanism of inhibition of phospholipase A_2. *Biochem. Pharmacol.* **29,** 623-627, 1980.
17. Smolen, J.E. and Weissman, G.: Effect of indomethacin, 5,8,11,14-icosatetraynoic acid, and bromophenacyl bromide on the lysosomal enzyme release and superoxide anion generation by human polymorphonuclear leukocytes. *Biochem. Pharmacol.* **29,** 533-538, 1980.
18. Op Den Kamp, J.A.F., Oe Gier, J. and Van Deenen, L.L.M.: Hydrolysis of phosphatidylcholine liposomes by pancreatic phospholipase A_2 at the transition temperature. *Biochim. Biophys. Acta* **345,** 253-256, 1974.
19. Kaplan, L., Weiss, J. and Elsback, P.: Low concentration of indomethacin inhibit phospholipase A_2 of rabbit polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA* **75,** 2955-2958, 1978.
20. Ohuchi, K., Kamada, Y., Levine, L. and Tsurufuji, S.; Glycyrrhizin inhibits prostaglandin E_2 production by activated pertoneal macrophage from rat. *Prostaglandin Med.* **7,** 457-463, 1981.