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Effect of trilinolein on cyclic nucleotide formation in human platelets: relationship with its antiplatelet effect and nitric oxide synthesis

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1 Trilinolein, a triacylglycerol with linoleic acid as the only fatty acid residue in all three esterified positions of glycerol, was recently reported to have an inhibitory effect on adrenaline-induced platelet aggregation. In the present study, we found that trilinolein at concentrations ranging from 0.01 to 1 μM increased cyclic GMP formation and decreased cyclic AMP formation in washed human platelets. Both N^G-nitro-L-arginine methyl ester (L-NAME) and methylene blue attenuated the trilinolein-induced increase in cyclic GMP.

2 Adrenaline decreased not only the production of cyclic AMP but also that of cyclic GMP. Trilinolein antagonized the inhibitory effect of adrenaline on cyclic GMP formation, but potentiated the inhibitory effect of adrenaline on cyclic AMP accumulation.

3 Both trilinolein and adrenaline enhanced intracellular calcium but the increment of intracellular calcium induced by them was much less than that produced by thrombin.

4 We propose that the anti-platelet effect of trilinolein is mediated through an increase in cyclic GMP, and that the change in cyclic GMP results from stimulation of nitric oxide synthesis in platelets.

5 We also propose that reduction of both cyclic AMP and cyclic GMP are involved in adrenaline-induced platelet aggregation.

Keywords: Trilinolein; cyclic AMP; cyclic GMP; intracellular calcium; platelets; nitric oxide; adrenaline

Introduction

Trilinolein, a triacylglycerol with linoleic acid as the only fatty acid residue in all three esterified positions of glycerol (Hong *et al.*, 1993b), was recently found to inhibit adrenaline-induced human platelet aggregation (Lai *et al.*, 1994). This inhibition was accompanied by reduced ATP release and thromboxane B_2 formation. However, platelet aggregation induced by collagen, thrombin, ADP or arachidonic acid was not inhibited. Concentration-response curves for the interaction between trilinolein and adrenaline showed that trilinolein was unlikely to be a competitive antagonist of adrenaline. The mechanism for the inhibitory effect of trilinolein on adrenaline-induced platelet aggregation remains to be determined.

Unlike other aggregation inducers such as thrombin which stimulates phospholipase C and increases intracellular calcium, adrenaline acts on the α_2-adrenoceptors inhibiting adenylate cyclase and decreasing adenosine 3':5'-cyclic monophosphate (cyclic AMP) in platelets. It was proposed that a decrease in cyclic AMP is the mechanism through which adrenaline induces platelet aggregation (Brass *et al.*, 1988). However, others have argued that a decrease in cyclic AMP is neither necessary nor sufficient for aggregation, since some intracellular inhibitors of adenylate cyclase do not induce aggregation and aggregation can occur in the presence of increased cyclic AMP (Haslam *et al.*, 1978). The mechanism through which adrenaline induces platelet aggregation is therefore controversial.

Guanosine 3':5'-cyclic monophosphate (cyclic GMP) is also inhibitory in platelets (Mellion *et al.*, 1981). Some aggregating agents such as thrombin also increase cyclic GMP, but this is suggested to be a feedback event, rather than a cause of aggregation (Tremblay & Hamet, 1987). Recently, it was reported that the nitric oxide pathway is present in human platelets (Radomski *et al.*, 1990a); nitric oxide modulates platelet reactivity by increasing cyclic GMP. There has been no report on the effect of triacylglycerol on cyclic GMP production or nitric oxide synthesis.

In an attempt to explore the mechanism by which trilinolein inhibits adrenaline-induced platelet aggregation, in particular whether cyclic AMP or cyclic GMP is involved, we undertook the present study to measure cyclic AMP and cyclic GMP levels in trilinolein-treated washed human platelets.

Methods

Washed human platelets (WHP)

Blood samples were collected from healthy volunteers of either sex, aged between 20 and 40 years old, after overnight fasting. They had not taken any medications for at least 14 days. Venous blood was mixed with 3.8% sodium citrate at a ratio of 9:1 (v/v). After centrifugation at 120 *g* and 37°C for 15 min, platelet rich plasma (PRP) was obtained from the supernatant and washed human platelets (WHP) were prepared from PRP by a method modified from Mustard *et al.* (1972). Briefly, PRP was centrifuged at 1,100 *g* for 20 min and suspended in Tyrode-albumin buffer (composition, mM: NaH_2PO_4 0.02, NaCl 136, KCl 2.68, $NaHCO_3$ 11.9, $MgCl_2$ 1.0, bovine serum albumin 3.5 mg ml^{-1}, glucose 1 g ml^{-1}, pH 7.4) containing heparin (25 units ml^{-1}), apyrase (50 mg l^{-1}) and prostaglandin E (PGE_1, 0.25 μM). After 20 min incubation at 37°C, the platelets were recentrifuged at 1,100 *g* for 20 min and resuspended without the addition of heparin. After incubation for 20 min at 37°C, the platelets were resedimented and resuspended in Tyrode-albumin solution with 2 mM $CaCl_2$ added while apyrase, heparin and PGE_1 were omitted. The final platelet count was adjusted to 1×10^9 cells ml^{-1}.

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Trilinolein effect

Trilinolein was purchased from Sigma (U.S.A.). It was first dissolved and then serially diluted in chloroform. After evaporation of chloroform, it was dissolved in calcium-free Joklik's minimum essential medium (with Earle's salt, L-glutamate and 10× phosphate; Gibco Laboratories, U.S.A.) containing 1% dimethyl sulphoxide (DMSO). Trilinolein solution was used for experimentation within 2 h after it had been prepared. WHP (250 μl) was incubated in a 37°C water bath before 2.5 μl of trilinolein solution was added. The final concentration of DMSO in WHP-trilinolein mixture was 0.01%. At this concentration, DMSO had no effect on platelet aggregation. WHP mixed with DMSO-containing Joklik's medium was used as a control. A preparatory experiment was performed to determine the optimal incubation period for the assessment of trilinolein effect on cyclic GMP formation. Since cyclic GMP production peaked at 2 min the incubation time for WHP-trilinolein mixture was set at 2 min.

Estimation of intracellular cyclic GMP or cyclic AMP content

The method of Karnigulan *et al.* (1982) was followed: 10 mM EDTA was added to the WHP and the mixture was immediately boiled for 5 min. After cooling to 4°C, the precipitated protein was sedimented at 7,000 *g* for 5 min; 200 μl of the supernatant was freeze-dried and the precipitate was dissolved in 100 μl assay buffer solution and 50 μl of this was used to measure the cyclic nucleotide content with an enzyme immunoassay dual range kit (Amersham, U.K.) according to the procedures described by the manufacturer. The acetylation method was used and the cross-reactivity for cyclic GMP and cyclic AMP in this assay is 100% and 0.00016%, respectively.

Interaction between adrenaline and trilinolein

WHP was preincubated with 0.1 μM trilinolein for 2 min before addition of adrenaline. Cyclic GMP and cyclic AMP were measured according to the procedures described above. Adrenaline was freshly prepared and dissolved in distilled water containing 0.5 mM ascorbic acid.

Effect of enzyme inhibitors

WHP was preincubated with 10 μM N^G-nitro-L-arginine methyl ester (L-NAME) or 1 μM methylene blue for 5 min before trilinolein solutions were added. After 2 min incubation, the reaction was terminated with EDTA and the cyclic nucleotide content was measured according to the procedures described above. All enzyme inhibitors were purchased from Sigma, U.S.A.

Determination of intracellular calcium ($[Ca^{2+}]_i$)

The method of Pollock & Rink (1986) was followed. Platelets were incubated with 5 μM fura-2/AM (Molecular Probe, Junction City, U.S.A.) at 37°C for 45 min and centrifuged at 500 *g* for 10 min; the resultant pellet was washed with Tyrode solution containing 1 mM EDTA. After recentrifugation at 500 *g* for 10 min, platelets were suspended in the Tyrode solution containing 1 mM Ca^{2+} and incubated for 30 min. The fluorescence of fura-2-loaded cells was measured with a spectrofluorimeter (SLM Instruments Inc. Urbana, Il, U.S.A.) at 37°C with excitation at 340 and 380 nm and emission at 510 nm. $[Ca^{2+}]_i$ was calculated from the ratios of maximum and minimum fluorescence intensities which were determined by the addition of 0.2% digitonin or 40 mM Tris-buffer which contained 20 mM EGTA, respectively (Grynkiewicz *et al.*, 1985).

Data analysis

All data are presented as means ± s.e.mean. Differences among different concentrations of trilinolein were analysed with one-way analysis of variance (ANOVA). Differences among concentration-response curves for platelets that had or had not been pretreated with enzyme inhibitors or trilinolein were analysed with two-way ANOVA. When ANOVA showed a significant difference, a *t* test was then used to determine the concentration of trilinolein at which a significant difference was present. $P<0.05$ was considered to be statistically significant.

Results

Effect of trilinolein on cyclic GMP and cyclic AMP

Figure 1 shows the time course for 0.1 μM and 1 μM trilinolein to stimulate cyclic GMP formation. Cyclic GMP production peaked at 2 min for both concentrations of trilinolein. Figure 2 shows the concentration-response curves for trilinolein to stimulate cyclic GMP and inhibit cyclic AMP formation in platelets after 2 min incubation. The actual values for cyclic GMP and cyclic AMP concentrations in control groups not treated with trilinolein were 0.47 ± 0.05 ($n=10$) and 4.10 ± 0.2 ($n=5$) pmol/10^9 platelets, respectively. Sodium nitroprusside (10 μM) increased cyclic GMP and cyclic AMP to 977 ± 234 and 233 ± 30% of control ($n=5$), respectively. In comparison, the amplitude for the maximal cyclic GMP production induced by trilinolein was only 157 ± 8.6%.

Interaction between trilinolein and adrenaline

Figure 3 shows that 10 μM and 100 μM adrenaline decreased cyclic GMP in platelets (one-way ANOVA followed by *t* test, $P<0.05$, $n=5$). Trilinolein (0.1 μM) reversed the inhibitory effect of adrenaline, the difference between trilinolein and non-trilinolein pretreated groups being statistically significant for each adrenaline concentration (two-way ANOVA, $P<0.05$, $n=5$). In platelets that had been pretreated with trilinolein, there was no significant fall in cyclic GMP level following adrenaline treatment (one-way ANOVA, $P>0.05$).

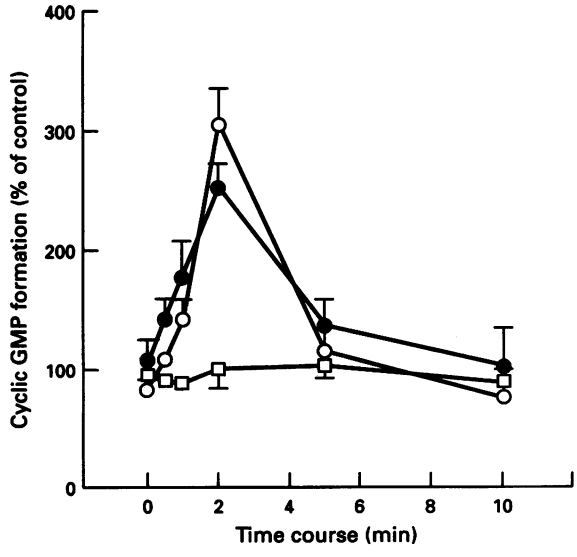

Figure 1 Time course for the stimulatory effect of 0.1 μM (●) and 1 μM (○) trilinolein on cyclic GMP formation in washed human platelets. The increase in cyclic GMP peaked after trilinolein had been mixed with platelets for 2 min. Cyclic GMP concentration in platelets at time 0 was used as a control. Trilinolein-free dimethylsulphoxide containing medium (□) had no effect on cyclic GMP levels. Data are mean ± s.e.mean for 3 separate experiments.

Figure 4 shows that 10 μM and 100 μM adrenaline inhibited cyclic AMP formation after it was mixed with platelets. Pretreatment with 0.1 μM trilinolein potentiated the inhibitory effect of adrenaline on cyclic AMP accumulation (two-way ANOVA, $P<0.05$, $n=5$).

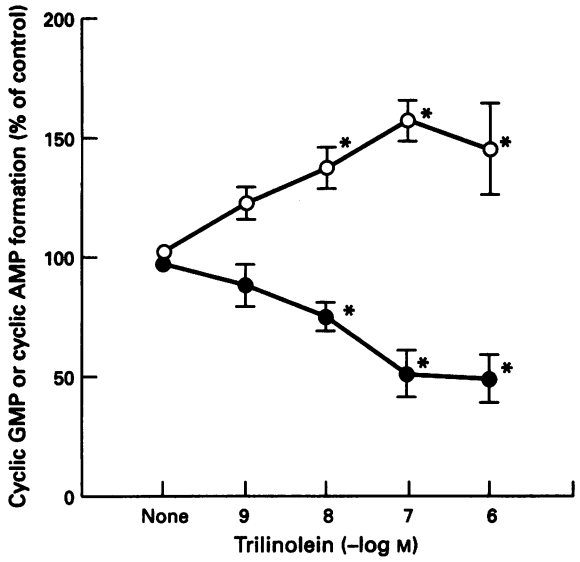

Figure 2 Concentration-response curves for trilinolein to stimulate cyclic GMP (○) and inhibit cyclic AMP (●) formation in washed human platelets. Platelets were mixed with trilinolein solution for 2 min before cyclic nucleotide levels were measured. Cyclic GMP concentrations in platelets that had been incubated with trilinolein-free dimethylsulphoxide containing medium for 2 min was used as a control. *Indicates a significant difference as compared with control (one way ANOVA followed by t test, $P<0.05$, $n=10$ for cyclic GMP and $n=5$ for cyclic AMP).

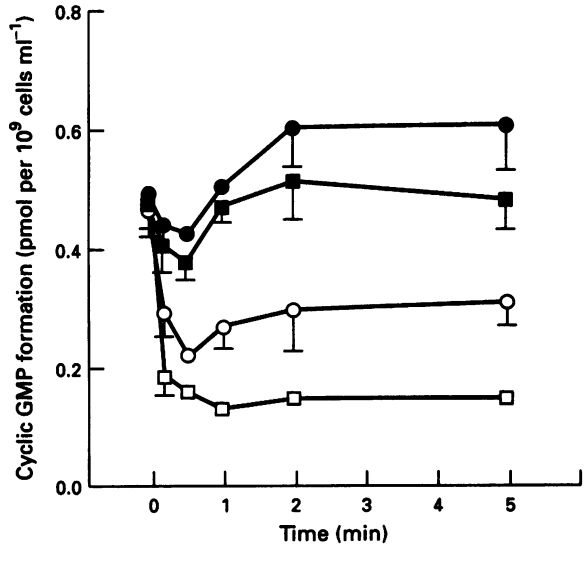

Figure 3 Time course for 0.1 μM trilinolein pretreatment to antagonize the inhibitory effect of 10 μM (○) and 100 μM (□) adrenaline on cyclic GMP formation in washed human platelets. Platelets were pretreated with either trilinolein (●, ■) or trilinolein-free dimethylsulphoxide containing medium (○, □) for 2 min before adrenaline was added. The difference between platelets with and without trilinolein pretreatment was statistically significant at all time points for each adrenaline concentration (two-way ANOVA followed by t test, $P<0.05$, $n=5$).

Effect of enzyme inhibitors

Figure 5 shows that preincubation of WHP with either 10 μM L-NAME or 1 μM methylene blue for 5 min before trilinolein was added attenuated the stimulatory effect of trilinolein on

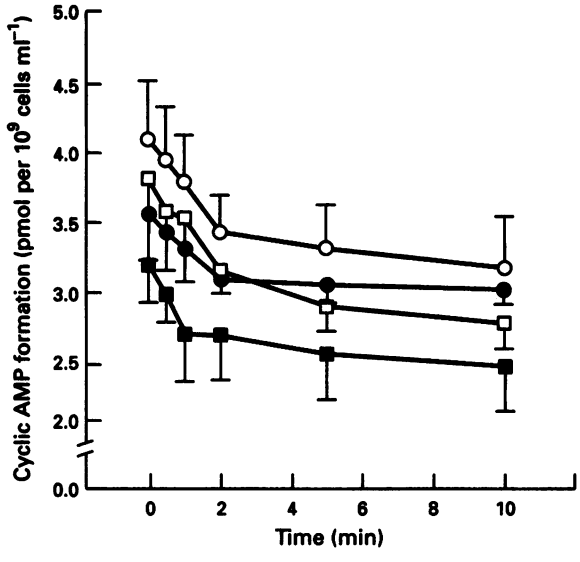

Figure 4 Time course for 0.1 μM trilinolein pretreatment to potentiate the inhibitory effect of 10 μM (○) and 100 μM (□) adrenaline on cyclic AMP formation in washed human platelets. Platelets were pretreated with either trilinolein (●, ■) or trilinolein-free DMSO-containing medium (○, □) for 2 min before adrenaline was added. The difference between platelet with and without trilinolein pretreatment was statistically significant (two-way ANOVA, $P<0.05$, $n=5$).

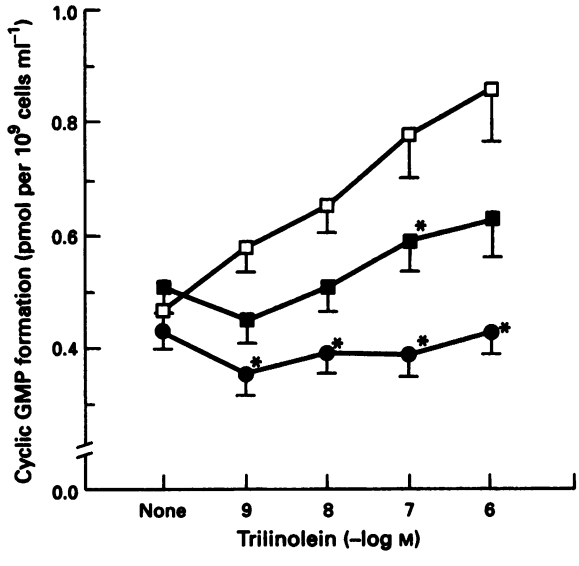

Figure 5 Concentration-response curves for N^G-nitro-L-arginine methyl ester (L-NAME, 10 μM) and methylene blue (1 μM) to attenuate the trilinolein stimulated cyclic GMP formation in washed human platelets. L-NAME (■), methylene blue (●) or drug-free medium (□) were preincubated with platelets for 5 min before trilinolein was mixed and then cyclic GMP was measured 2 min later. *Indicates a significant difference between L-NAME or methylene blue-treated platelets and medium-treated ones at specific trilinolein concentrations (two-way ANOVA followed by t test, $P<0.05$, $n=10$).

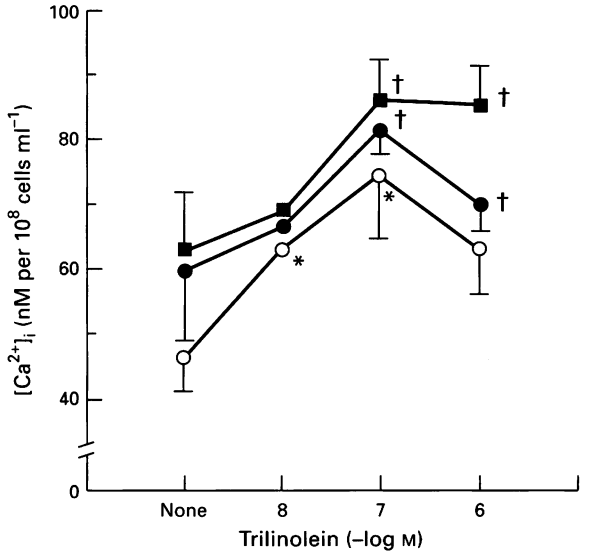

Figure 6 Intracellular calcium content ($[Ca^{2+}]_i$) in platelets was significantly increased by 0.01 and 0.1 μM trilinolein (○) (*, one-way ANOVA followed by t test, $P<0.05$, $n=5$). Adrenaline 10 μM (●) or 100 μM (■), but not buffer (○), further increased $[Ca^{2+}]_i$ in platelets pretreated with 0.1 or 1 μM trilinolein (†, two-way ANOVA followed by t test, $P<0.05$, $n=5$). Adrenaline also increased $[Ca^{2+}]_i$ in platelets not pretreated with trilinolein (t test, $P<0.05$, $n=5$).

cyclic GMP formation. The effect of L-NAME was statistically significant at a concentration of 0.1 μM trilinolein, while that of methylene blue was significant at all trilinolein concentrations.

Determination of intracellular calcium

Figure 6 shows that the addition of 0.01 or 0.1 μM trilinolein to a suspension of fura-2-loaded washed human platelets resulted in a significant increase of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) (one-way ANOVA followed by t test, $P<0.05$, $n=5$). $[Ca^{2+}]_i$ in platelets treated with buffer, 0.01 or 0.1 μM of trilinolein was 46 ± 4.8, 63.2 ± 1.8 and 74.3 ± 9.8 nM, respectively. Addition of adrenaline to the platelets pretreated with 0.1 and 1 μM trilinolein further increased $[Ca^{2+}]_i$ (two-way ANOVA followed by t test, $P<0.05$, $n=5$). In the absence of trilinolein, 10 μM and 100 μM adrenaline increased $[Ca^{2+}]_i$ to 59.4 ± 10.5 and 64.2 ± 9.4 nM, respectively. Since 0.2 unit ml^{-1} thrombin increased $[Ca^{2+}]_i$ to 343.3 ± 114.6 nM ($n=5$), it is much more potent in increasing $[Ca^{2+}]_i$ than trilinolein and adrenaline.

Discussion

Cyclic AMP is a well known inhibitory secondary messenger in platelets. A decrease in cyclic AMP formation was proposed as the mechanism mediating adrenaline-induced platelet aggregation (Brass *et al.*, 1988). Since trilinolein also inhibited cyclic AMP formation, cyclic AMP is unlikely to be the mediator responsible for the inhibitory effect of trilinolein on adrenaline-induced platelet aggregation.

Cyclic GMP is another inhibitory secondary messenger in platelets. Although some aggregators such as thrombin also increase cyclic GMP, such an increase was considered as a feedback event rather than a cause of aggregation (Tremblay *et al.*, 1987). Sodium nitroprusside and organic nitrates were reported to be converted to nitric oxide and inhibit platelet aggregation in conjunction with an increase in cyclic GMP (Mellion *et al.*, 1981). We found that 10 μM sodium nitroprusside increased cyclic GMP to 977 ± 234% of control. The maximal amplitude for trilinolein-stimulated cyclic GMP level shown in Figure 2 was 157 ± 8.6% of control, much less than that induced by sodium nitroprusside.

We previously showed that trilinolein antagonized adrenaline-induced platelet aggregation (Lai *et al.*, 1994). In the present study, we demonstrated that adrenaline reduced the level of cyclic GMP and this reduction is prevented by trilinolein. We therefore suggest that cyclic GMP is involved in adrenaline-induced platelet aggregation and that an increase in cyclic GMP could be the mechanism by which trilinolein antagonizes adrenaline-induced platelet aggregation. Although some early studies claimed that cyclic GMP was transiently increaesd 10 s after platelets had been mixed with adrenaline (Jakobs *et al.*, 1974), we could not confirm such a stimulatory effect of adrenaline on cyclic GMP. In other tissues, cyclic GMP was found to be increased by adrenaline in gastric mucosa (Ruoff, 1977) amd glioma cells (Schwartz, 1976), while it was not changed in thyroid cells (Brandi *et al.*, 1983), enterocytes (Guandalini *et al.*, 1982) or adrenal cells (Percehellet & Sharma, 1980).

It was proposed that adrenaline induces platelet aggregation by acting on α_2-adrenoceptors, inhibiting adenylate cyclase, and decreasing cyclic AMP. A decreased cyclic AMP is considered as the mechanism for adrenaline to induce platelet aggregation (Brass *et al.*, 1988). However, some studies argue that a decrease in cyclic AMP is neither necessary nor sufficient for aggregation, since some intracellular inhibitors of adenylate cyclase do not induce aggregation and aggregation can occur in the presence of increased cyclic AMP (Haslam *et al.*, 1978). Our previous study showed that trilinolein inhibited only platelet aggregation induced by adrenaline, not that induced by collagen, thrombin, ADP or arachidonic acid (Lai *et al.*, 1994). It could be proposed that a decrease in the levels of both cyclic AMP and cyclic GMP is required for adrenaline to induce platelet aggregation, and that the trilinolein-induced increase in cyclic GMP overcomes the decrease in cyclic AMP and inhibits platelet aggregation. However, in aggregation induced by other aggregators such as thrombin, either cyclic GMP is already increased or the trilinolein-induced increase in cyclic GMP is not potent enough to overcome other aggregatory pathways such as a marked increased in cytosolic free calcium, so trilinolein could not antagonize platelet aggregation induced by these potent aggregators.

Synthesis of nitric oxide from L-arginine by nitric oxide synthase is known to occur in a number of tissues. Recently, it was demonstrated that human platelets contain nitric oxide synthase and release nitric oxide as a result of the activation of nitric oxide synthase which modulates platelet reactivity by increasing cyclic GMP (Radomski *et al.*, 1990a). It is also recognized that nitric oxide synthase releases NO and increases cyclic GMP which can be blocked by L-NAME (Radomski *et al.*, 1990b) and methylene blue (Trovati *et al.*, 1994; Jaraki *et al.*, 1994). The mechanism for trilinolein to increase cyclic GMP could be a stimulation of nitric oxide synthase because this effect of trilinolein can be inhibited by both L-NAME and methylene blue. The NO synthase in platelets is Ca^{2+}-dependent (Moncada *et al.*, 1991). We showed that the increases in $[Ca^{2+}]_i$ induced by trilinolein (0.1 μM) and adrenaline (100 μM) are 74.3 ± 9.8 and 64.2 ± 9.4 nM, respectively. Such a small increase may not be high enough to induce platelet aggregation, but enough to activate NO synthase (Nathan *et al.*, 1994). The increase of $[Ca^{2+}]_i$ induced by thrombin in our study was 343.3 ± 114.6 nM. It was not only higher than with trilinolein or adrenaline, but also enough to trigger platelet activation (Rink *et al.*, 1982).

Trilinolein was purified by our laboratory from *Panax pseudoginseng*, a medicinal herb widely used for cardiovascular disorders in traditional Chinese medicine (Hong *et al.*, 1993b). It is a rare type of triacylglycerol since most of the naturally occurring triacylglycerols are of the mixed type which contain different fatty acyl residues in three esterified positions of glycerol (Mayes, 1983). In addition to an inhibitory effect on platelet aggregation, this compound improved the deformability of washed erythrocytes. Recently, it was also found to improve the deformability of erythrocytes in whole blood which had been subjected to cardiopulmonary bypass (Tsai *et*

al., 1994). Two other triacylglycerols, namely triolein and tripalmitolein, improved erythrocyte deformability (Hong *et al.*, 1993a). The mechanism by which these triacylglycerols improve erythrocyte deformability has not been established. Whether it is related to an increase in nitric oxide and cyclic GMP in erythrocytes awaits further investigation. Structure-activity analysis is also required to determine the relationship between fatty acyl profile of a triacylglycerol with its effect on nitric oxide synthesis and cyclic nucleotide formation.

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