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Antiplatelet Effect of Gingerol Isolated from *Zingiber officinale*

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Abstract

The purpose of this investigation was to determine the antiplatelet mechanism of gingerol.

Gingerol concentration-dependently (0·5–20 μM) inhibited the aggregation and release reaction of rabbit washed platelets induced by arachidonic acid and collagen, but not those induced by platelet-activating factor (PAF), U46619 (9,11-dideoxy-9α,11α-methano-epoxy-PGF$_{2\alpha}$) and thrombin. Gingerol also concentration-dependently (0·5–10 μM) inhibited thromboxane B_2 and prostaglandin D_2 formation caused by arachidonic acid, and completely abolished phosphoinositide breakdown induced by arachidonic acid but had no effect on that of collagen, PAF or thrombin even at concentrations as high as 300 μM.

In human platelet-rich plasma, gingerol and indomethacin prevented the secondary aggregation and blocked ATP release from platelets induced by adenosine 5′-diphosphate (ADP, 5 μM) and adrenaline (5 μM) but had no influence on the primary aggregation. The maximal antiplatelet effect was obtained when platelets were incubated with gingerol for 30 min and this inhibition was reversible.

It is concluded that the antiplatelet action of gingerol is mainly due to the inhibition of thromboxane formation.

Platelet aggregation is the main mediator involved in haemostasis and thrombosis formation. Thromboxane A_2 formed from arachidonic acid derived from membrane phospholipids of stimulated platelets (Hamberg et al 1975; Moncada & Vane 1979) is a potent platelet-aggregating agent and vasoconstrictor (Moncada & Vane 1979) and is thought to be responsible for the recruitment of further platelets to the initial aggregate (Svensson et al 1976). Cyclo-oxygenases and thromboxane synthase are the chief enzymes converting arachidonic acid to thromboxane A_2. In the past few years, many pharmacologists have devoted themselves to finding the inhibitors of cyclo-oxygenases and thromboxane synthase (Gresele et al 1984; Verstraete et al 1985).

Gingerol isolated from *Zingiber officinale* is classed as a flavour and used as a condiment and as a carminative (Fig. 1). The purpose of this investigation is to determine the antiplatelet mechanism of gingerol.

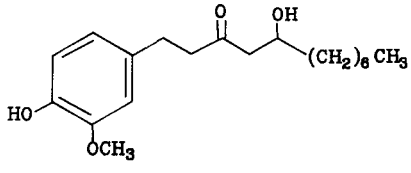

FIG. 1. Chemical structure of gingerol.

Materials and Methods

Platelet preparation

Blood was collected from the rabbit marginal ear vein, anticoagulated with sodium citrate (3·8%, 1 : 14) and centrifuged for 10 min at 90 *g* at room temperature (21°C); platelet-rich plasma (PRP) was obtained from the upper portion. The platelet suspension was obtained from EDTA-anticoagulated PRP according to the washing procedure described previously (Teng et al 1987). Platelet numbers were counted using a Coulter Counter (Model ZM) and adjusted to $4{\cdot}5\times 10^8$ platelets mL^{-1}. The platelet pellets were suspended in Tyrode solution containing calcium (1 mM) and bovine serum albumin (0·35%). All glassware was siliconized.

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Platelet aggregation and ATP release

PRP or the platelet suspension was stirred at 900 rev min^{-1} for 1 min and the aggregation inducer was added to trigger the platelet aggregation. Aggregation was measured by the turbidimetric method (O'Brien 1962). The absorbance of PRP or the platelet suspension was taken as 0% aggregation and that of platelet-poor plasma or platelet-free Tyrode solution as 100% aggregation. ATP released from platelets was detected by bioluminescence (DeLuca & McElory 1978). ATP at a known concentration was used to calibrate the intensity of bioluminescence. Both the aggregation and release of ATP were simultaneously measured by a Lumi-aggregometer (Model 1020, Payton, Canada) connected to two dual-channel recorders.

Thromboxane B_2 and prostaglandin D_2 assay

Six minutes after the challenge of platelets with the aggregation inducer, 2 mM EDTA and 50 μM indomethacin were added. After centrifugation in an Eppendorf Centrifuge (Model 5414) for 2 min, the supernatant was obtained, and thromboxane B_2 and prostaglandin D_2 were assayed using radioimmunoassay kits according to the procedures described by the manufacturers.

Labelling of membrane phospholipids and measurement of the production of [^{3}H]inositol phosphate

This method was modified from those of Huang & Detwiler (1986) and Neylon & Summers (1987). EDTA-PRP was centrifuged at 500 *g* for 10 min, the platelet pellets were suspended in 700 μL Ca^{2+}-free and BSA-free Tyrode solution containing 75 μCi mL^{-1} [^{3}H]inositol and 1 mM EDTA. After incubation for 2 h at 37°C, the platelets were collected by centrifugation (500 *g*, 4 min) and suspended in Ca^{2+}-free Tyrode solution. The reaction was carried out at 37°C for 6 min with 1 mL platelet suspension in a 3·5-mL cuvette with a stirring bar driven at 900 rev min^{-1}. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 mL supernatant was pooled and trichloroacetic acid was removed by extracting with 5 × 2 vol diethyl ether. The aqueous phase, containing the inositol phosphate, was adjusted to pH 7–8 and diluted to 4 mL with distilled water before its application to a Dowex-1 ion-exchange column for separation of the inositol phosphates, as described previously by Neylon & Summers (1987). All the experiments were carried out in the presence of 5 mM LiCl to inhibit inositol phosphate phosphatase. Because the levels of inositol bisphosphate and inositol trisphosphate were very low, we measured the inositol monophosphate as an index of the total inositol phosphate formation.

Materials

Gingerol (Fig. 1) was isolated from *Zingiber officinale*. Collagen (type 1, bovine achilles tendon), obtained from Sigma Chemical Co., St Louis, MO, was homogenized in 25 mM acetic acid and stored at −70°C at a concentration of 1 mg mL^{-1}. Thrombin (bovine) was purchased from Parke Davis & Co., Detroit, MI, and dissolved in 50% glycerol to give a stock solution of 100 NIH units mL^{-1}. Platelet-activating factor (PAF) was purchased from Sigma Chemical Co. and dissolved in chloroform. ADP, arachidonic acid, EDTA (disodium salt), luciferin-luciferase, dimethylsulphoxide (DMSO), Dowex-1 (100–200 mesh: X8, chloride) resin, myoinositol, indomethacin, U46619 (9,11-dideoxy-9α,11α-methano-epoxy-PGF$_{2\alpha}$) and trichloroacetic acid were purchased from Sigma Chemical Co. Thromboxane B_2 RIA kits, prostaglandin D_2 RIA kits and myo[2-^{3}H]inositol were obtained from New England Nuclear Co., Boston, MA.

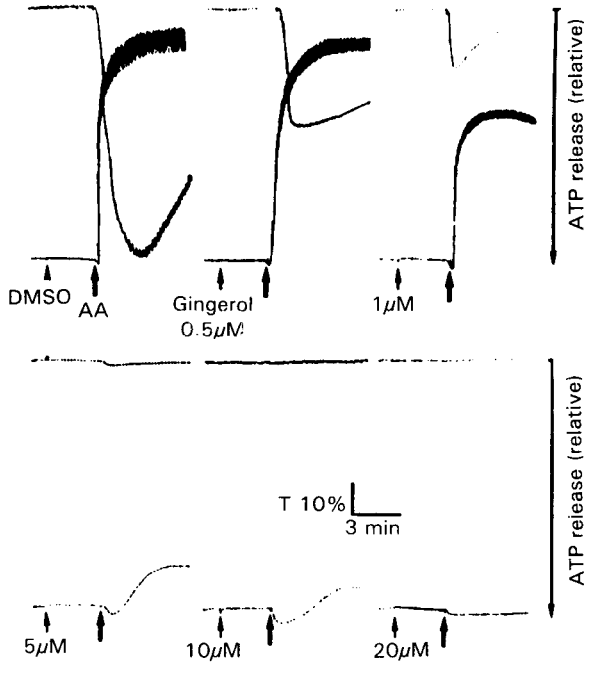

FIG. 2. Inhibitory effect of gingerol on platelet aggregation and ATP release induced by arachidonic acid. Rabbit washed platelets were incubated with DMSO (0·5%) or various concentrations of gingerol for 3 min, then arachidonic acid (AA, 100 μM) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings). T is the change in light transmission.

Table 1. Effect of gingerol on the aggregation of rabbit washed platelets induced by arachidonic acid, collagen, PAF, U46619 and thrombin.

	Aggregation (%)	
	Control (DMSO, 0·5%)	Gingerol (300 μM)
Arachidonic acid (100 μM)	93·5 ± 1·4	0 ± 0***
Collagen (10 μg mL^{-1})	92·8 ± 1·0	44·3 ± 13·5*
PAF (2 ng mL^{-1})	94·8 ± 0·7	92·6 ± 0·9
U46619 (1 μM)	90·7 ± 1·8	91·6 ± 1·1
Thrombin (0·1 units mL^{-1})	94·8 ± 1·2	93·9 ± 1·3

Rabbit washed platelets were preincubated with gingerol (300 μM) or DMSO (0·5%) at 37°C for 3 min, then the inducer was added. Values are presented as means ± s.e.m. (n = 6). *P < 0·05, ***P < 0·001 compared with the respective control.

Data analysis

The experimental results are expressed as the means ± s.e.m. Statistical significance was assessed by Student's t-test and P values less than 0·05 were considered significant.

Results

Effects of gingerol on platelet aggregation and ATP-release reaction

Gingerol concentration-dependently inhibited the platelet aggregation and ATP release induced by arachidonic acid (100 μM) and reached complete inhibition at 20 mM (Fig. 2).

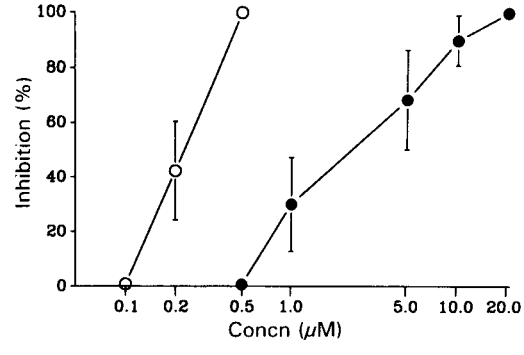

FIG. 3. Concentration-response curves of indomethacin and gingerol on the platelet aggregation induced by arachidonic acid. Rabbit washed platelets were incubated with various concentrations of indomethacin (○) or gingerol (●) for 3 min, then arachidonic acid (100 μM) was added. Percent inhibition is presented as means ± s.e.m. (n = 6).

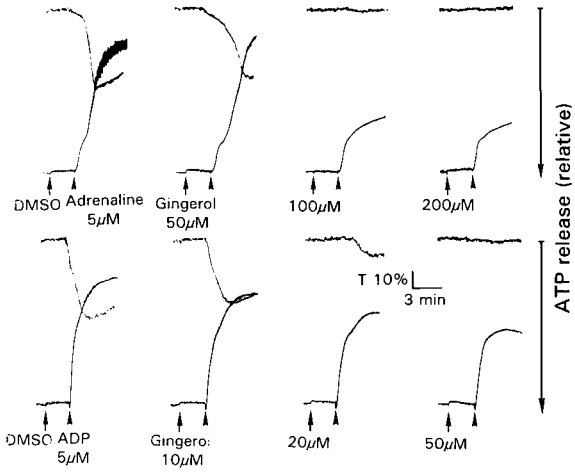

FIG. 4. Effects of gingerol on the aggregation and ATP release of human platelet-rich plasma induced by adrenaline and ADP. Platelet-rich plasma was incubated with gingerol of various concentrations or DMSO (0·5%) for 3 min, then adrenaline (5 μM) or ADP (5 μM) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings). T is the change in light transmission.

In contrast, gingerol had no inhibitory effect on platelet aggregation induced by PAF (2 ng mL^{-1}), U46619 (1 μM) and thrombin (0·1 units mL^{-1}) even at the high concentration of 300 μM, but had partial and significant inhibition on that caused by collagen (10 μg mL^{-1}) (Table 1). Since gingerol specifically inhibited the effect caused by arachidonic acid, comparison of the concentration-response curves of indomethacin with gingerol was made (Fig. 3). Indomethacin, as well as gingerol, in a concentration-dependent manner, inhibited the platelet aggregation induced by arachidonic acid, with IC50 values of 0·22 and 2·59 μM, respectively. In human platelet-rich plasma, adrenaline (5 μM) and ADP (5 μM) caused biphasic aggregation. Secondary aggregation and ATP release were concentration-dependently suppressed by gingerol, while the primary aggregation was little affected (Fig. 4).

Effect of gingerol on formation of thromboxane B_2 and prostaglandin D_2

Thromboxane B_2 and prostaglandin D_2 formation in rabbit washed platelets were measured at 6 min after arachidonic acid was added. Table 2 shows that gingerol concentration-dependently inhibited the thromboxane B_2 and prostaglandin D_2 formation evoked by arachidonic acid. In contrast, indomethacin (0·5 μM) completely abolished both events caused by arachidonic acid. Whereas, imidazole (1 mM) inhibited the thromboxane B_2 formation but markedly increased the prostaglandin D_2 formation.

Table 2. Effects of gingerol on thromboxane B_2 and prostaglandin D_2 formation evoked by arachidonic acid in rabbit washed platelets.

		Thromboxane B_2 (ng/4·5 × 10^8 platelets)	Prostaglandin D_2 (ng/4·5 × 10^8 platelets)
Control		639 ± 60	1·68 ± 0·33
Gingerol	0·5 μM	209 ± 24***	
	1	121 ± 67***	
	2	81 ± 29***	
	5	29 ± 10***	0·36 ± 0·14*
	10	13 ± 3***	0·22 ± 0·01**
Indomethacin	0·5 μM	5 ± 3***	0·05 ± 0·01***
Imidazole	1 mM	30 ± 2***	32·06 ± 4·82***

Values are presented as means ± s.e.m. (n = 6). Thromboxane B_2 and prostaglandin D_2 levels of resting platelets were 0·8 ± 0·2 and 0·010 ± 0·003 ng/4·5 × 10^8 platelets, respectively. *P < 0·05, **P < 0·01, ***P < 0·001 compared with the respective control (arachidonic acid, 100 μM).

Effect of gingerol on breakdown of phosphoinositide

Phosphoinositide breakdown is the major activating pathway of platelet aggregation. In this study, arachidonic acid, collagen, PAF and thrombin induced inositol monophosphate formation 2·8 ± 0·4, 3·0 ± 0·6, 2·6 ± 0·5 and 4·5 ± 1·1 times, respectively, as compared with that of resting platelets. Indomethacin and gingerol decreased the formation of inositol monophosphate evoked by arachidonic acid to 0·9 ± 0·2- and 1·2 ± 0·4-fold, respectively, but had no inhibitory effects on those caused by collagen, PAF or thrombin (Table 3).

Effect of incubation time on gingerol inhibitory activity

The inhibitory effect of gingerol on platelet aggregation induced by arachidonic acid was proportional to the incubation time. When the incubation time was more than 30 min, the inhibitory effect reached the maximum (93·4 ± 3·3%, n = 6). In addition, after treatment of platelets with gingerol (20 μM) for 3 min (100% inhibition of platelet aggregation) at room temperature (21°C) and then washing the platelets with the suspending solution, the inhibitory effect of gingerol was decreased (71·3 ± 19·1, 31·8 ± 17·5 and 7·3 ± 2·4% inhibition of platelet aggregation after one, two and three washings, respectively) and arachidonic acid-induced platelet aggregation was recovered.

Discussion

Exogenous addition of arachidonic acid is converted by platelet cyclo-oxygenase to the prostaglandin endoperoxides (prostaglandins G_2 and H_2), which in turn are converted by thromboxane synthase to thromboxane A_2, a potent aggregating agent, and then causes platelet aggregation (Hamberg et al 1974, 1975; Parise et al 1984). According to the previous reports, the rabbit platelet aggregation induced by PAF as well as thrombin was not mediated by thromboxane A_2 synthesis, while platelet aggregation induced by U46619, the PGH_2/TxA_2-receptor agonist, was not through the cyclo-oxygenase pathway. Thus, the effects induced by the above inducers were not affected by aspirin and indomethacin (Teng et al 1987; Lapetina et al 1978). In the present study, gingerol (20 μM) completely inhibited the platelet aggregation and ATP release induced by arachidonic acid. However, at the high concentration of 300 μM, gingerol had no inhibitory effect on those induced by PAF, U46619 and thrombin but had partial blockade on those induced by collagen. In contrast, indomethacin (0·5 μM) completely abolished the platelet aggregation caused by arachidonic acid and had partial inhibition on that induced by collagen (21·2 ± 5·2%, n = 4, P < 0·001), but had no influence on those induced by PAF, U46619 and thrombin

Table 3. Inhibitory effect of gingerol on the formation of inositol monophosphate in rabbit washed platelets.

	Inositol monophosphate (folds)			
	Arachidonic acid	Collagen	PAF	Thrombin
Control (DMSO, 0·5%)	2·8 ± 0·4	3·0 ± 0·6	2·6 ± 0·5	4·5 ± 1·1
Gingerol 20 μM	1·2 ± 0·4**			
300 μM		2·9 ± 0·6	2·2 ± 0·6	5·3 ± 1·4
Indomethacin 2 μM	0·9 ± 0·2***			

[^{3}H]Inositol-labelled platelets were incubated with arachidonic acid (100 μM), collagen (10 μg mL^{-1}), PAF (2 ng mL^{-1}) or thrombin (0·1 units mL^{-1}) in the presence of calcium for 6 min. Values are presented as means ± s.e.m. (n = 4). **$P < 0·01$, ***$P < 0·001$ compared with the respective control.

(data not shown). This indicates that the anti-aggregatory effect on gingerol has high selectivity to arachidonic acid and reveals the same effect as indomethacin. Gingerol and indomethacin markedly inhibited thromboxane B_2 and prostaglandin D_2 formation evoked by arachidonic acid. Imidazole, a thromboxane-synthase inhibitor, abolished the thromboxane B_2 formation but potentiated the prostaglandin D_2 formation. These results indicate that gingerol is not a thromboxane-synthase inhibitor. In adrenaline- and ADP-stimulated human platelet-rich plasma, gingerol inhibited the secondary aggregation and ATP release. This second phase was known to be due to the formation of thromboxane A_2 and release of ADP (Weiss 1983), and was affected by aspirin (Zucker & Peterson 1968). In the present study, gingerol concentration-dependently inhibited the secondary aggregation and ATP release induced by adrenaline and ADP.

It has been reported that the phosphoinositide breakdown in platelets may provide a source of free arachidonic acid via the diglyceride lipase pathway (Bell et al 1979) leading to thromboxane A_2 formation. Arachidonic acid also can be liberated by phospholipase A_2 from membrane phospholipids (McKean et al 1981), then the subsequent thromboxane A_2 activates further phosphoinositide breakdown (MacIntyre et al 1985). In the presence of indomethacin, to abolish the further formation of thromboxane A_2, the phosphoinositide breakdown caused by collagen, PAF and thrombin were not affected by the addition of gingerol (300 μM). In contrast, the phosphoinositide breakdown induced by arachidonic acid was markedly inhibited by indomethacin (2 μM) or gingerol (20 μM). The inhibition of arachidonate metabolism and thromboxane A_2 formation rather than the direct inhibition of phosphoinositide breakdown, was responsible for the failure of blockade on phosphoinositide breakdown caused by collagen, PAF and thrombin. In addition, the anti-aggregatory effect of gingerol was decreased when the gingerol-treated platelets were washed with Tyrode solution, indicating that the anti-aggregatory effect of gingerol was reversible.

We conclude that the antiplatelet action of gingerol is due to the inhibition of arachidonate metabolism.

Acknowledgements

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