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Inhibition of Platelet Thromboxane Formation and Phosphoinositides Breakdown by Diisoeugenol

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Abstract—Diisoeugenol inhibited the platelet aggregation and ATP release of rabbit platelets caused by ADP, arachidonic acid, platelet-activating factor (PAF), collagen and thrombin. Prolongation of the incubation time of platelets with diisoeugenol did not cause further inhibition and the aggregability of platelets could not be restored after washing. In human platelet-rich plasma, diisoeugenol inhibited the biphasic aggregation and ATP release induced by adrenaline and ADP in a concentration-dependent manner. Thromboxane B_2 formation caused by arachidonic acid, collagen and thrombin was markedly inhibited by diisoeugenol in a concentration-dependent manner. Diisoeugenol also inhibited the formation of inositol monophosphate caused by collagen, PAF and thrombin. The cAMP level of washed platelets was not changed by diisoeugenol. It is concluded that the antiplatelet effect of diisoeugenol is due to the inhibition of thromboxane formation and phosphoinositides breakdown.

In platelets, thromboxane A_2 is mainly produced from prostaglandin (PG) endoperoxide, which is derived from arachidonic acid liberated in the cell membrane (Hamberg et al 1975). Thromboxane A_2 plays an important role in platelet activation and is involved in the development of thrombosis (Ogletree 1987). It has been reported that phosphoinositides breakdown is another important pathway, and it may be a primary event in agonist-induced platelet activation (Berridge 1984). Mural thrombus formation can restrict the flow of blood to vital tissues or organs leading to peripheral, cerebral or coronary ischaemia. Thus, inhibition of platelet function may be a promising approach for the prevention of thrombosis. It has been reported that eugenol and its analogues can inhibit prostaglandin E biosynthesis in ram seminal vesicles and have antiplatelet actions in rabbits in-vitro and in-vivo (Laekeman et al 1986).

In this paper, we have investigated the antiplatelet action of diisoeugenol.

Materials and Methods

Materials

Diisoeugenol (Fig. 1) was prepared from isoeugenol by refluxing in 90% HCOOH (Ching & Li 1978). Thrombin (bovine) was purchased from Park Davis Co (Detroit, MI). and dissolved in 50% glycerol to give a stock solution of 100 NIH units mL^{-1}. Collagen (Type I, bovine achilles tendon), obtained from Sigma Chemical Co. (St Louis, MO) was homogenized in 25 mM acetic acid and stored at $-70°C$. Platelet-activating factor (PAF), ADP, arachidonic acid, ethylene diaminetetraacetic acid (EDTA), sodium citrate, bovine serum albumin, indomethacin, luciferase-luciferin, Dowex-1 (100–200 mesh: ×8, chloride) and myo-inositol were purchased from Sigma Chemical Co. Myo-[2-^{3}H]inositol was purchased from Amersham, UK. Thromboxane B_2 RIA kits were obtained from New England

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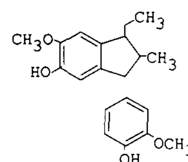

FIG. 1. Chemical structure of diisoeugenol.

Nuclear Co. (MA, USA). Cyclic AMP (cAMP) enzyme immunoassay kit was purchased from Cayman Chemical Co. (MI, USA).

Platelet aggregation and ATP release reaction

Platelet-rich plasma (PRP) was obtained from human blood, anticoagulated with sodium citrate (3·8%, 1:14) and centrifuged for 10 min, 90 *g*, room temperature (21°C). Rabbit platelet suspension was obtained from EDTA (1:14, v/v)-anticoagulated PRP according to the washing procedures described previously (Teng et al 1987). Platelet numbers were counted by Coulter Counter (Model ZM) and adjusted to $4·5 \times 10^{8}$ platelets mL^{-1}, and suspended in Tyrode solution of the following composition (mM): NaCl 136·8, KCl 2·8, $NaHCO_3$ 11·9, $MgCl_2$ 2·1, NaH_2PO_4 0·33, $CaCl_2$ 1·0 and glucose 11·2 with bovine serum albumin (0·35%). Aggregation was measured by the turbidimetric method (O'Brien 1962) and assigned the absorbance of PRP or platelet suspension as 0% aggregation and the absorbance of platelet-poor plasma or platelet-free Tyrode solution as 100% aggregation. ATP release from platelets was detected by the bioluminescence method as described by DeLuca & McElory (1978). Both the aggregation and release of ATP were measured simultaneously by Lumi-aggregometer (Chrono-Log Co., USA) connected to two dual channel recorders. The platelet suspension was stirred at 1200 rev min^{-1}. To eliminate the effect of the drug solvent, dimethylsulphoxide (DMSO), on the aggregation, the final concentration of DMSO was fixed at 0·5%.

Table 1. Effect of diisoeugenol on the platelet aggregation induced by some aggregation inducers.

	Aggregation (%)				
	ADP (20 μM)	Arachidonic acid (100 μM)	PAF (2 ng mL^{-1})	Collagen (10 μg mL^{-1})	Thrombin (0·1 units mL^{-1})
Control (0·5% DMSO)	82±3	89±1	91±1	88±2	96±2
Diisoeugenol 20 μg mL^{-1}	51±8**	72±7*	85±2	78±2**	95±2
50 μg mL^{-1}	1±1***	1±1***	7±3***	1±1***	35±12***

Rabbit washed platelets were preincubated with DMSO (0·5%, control) or diisoeugenol at 37°C for 3 min, then the inducer ADP, arachidonic acid, PAF, collagen or thrombin was added. Values are presented as means±s.e.m. (n = 3–5). *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ as compared with the respective control.

Thromboxane B_2 assay

After activating platelets with the aggregation inducer for 6 min, 2 mM EDTA and 50 μM indomethacin were added. Thromboxane B_2 was assayed from the supernatant after centrifugation of the platelet suspension in an Eppendorf centrifuge (Model 5414) for 2 min, using the radioimmunoassay kits according to the procedure described by the manufacturer.

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

The 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} myo-[2-^{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 Tyrode solution. The reaction was carried out at 37°C for 6 min with 1 mL of 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 100 *g* for 10 min, 1 mL of the supernatant was pooled and trichloroacetic acid was removed by washing with 5×2 vol diethyl ether. The aqueous phase containing the inositol phosphates was adjusted to pH 7–8 and diluted to 4 mL with distilled water before application to a Dowex-1 ion-exchange column for separation of the inositol phosphates, as described by Neylon & Summers (1987). All the experiments were carried out in the presence of 5 mM LiCl to inhibit inositol monophosphate phosphatase. Because the levels of inositol bisphosphate and inositol trisphosphate were very low, we measured the inositol monophosphate as an index of inositol phosphate formation.

Estimation of platelet cAMP

The method of Karniguian et al (1982) was followed. Platelet suspension was warmed at 37°C for 1 min, then PGE_1 or diisoeugenol was added and incubated for 1 min. The incubation was stopped by adding 10 mM EDTA and immediate boiling for 5 min. After cooling to 4°C, the precipitated protein was sedimented by centrifugation in an Eppendorf centrifuge (Model 5414). The supernatant was used to determine the cAMP content by a cAMP enzyme immunoassay kit as described by the manufacturer.

Results

Diisoeugenol (20 μg mL^{-1}) partly inhibited the aggregation of rabbit platelets induced by ADP (20 μM), arachidonic acid (100 μM) and collagen (10 μg mL^{-1}). A higher concentration (50 μg mL^{-1}) of diisoeugenol suppressed completely the platelet aggregation induced by ADP, arachidonic acid and collagen, and markedly those by PAF (2 ng mL^{-1}) and thrombin (0·1 units mL^{-1}) (Table 1). Indomethacin (10 μM), a cyclo-oxygenase inhibitor, partially inhibited the aggregation of rabbit platelets induced by collagen (10 μg mL^{-1}) (% aggregation 86·1±1·2% (n = 5) for the control and 48·1±5·2% (n = 5) for indomethacin), while it did not affect the aggregation induced by ADP (20 μM) (% aggregation 80·7±1·6% (n = 5) for the control and 77·9±1·8% (n = 5) for indomethacin). In the presence of 10 μM indomethacin, 50 μg mL^{-1} diisoeugenol completely inhibited collagen- and ADP-induced platelet aggregation.

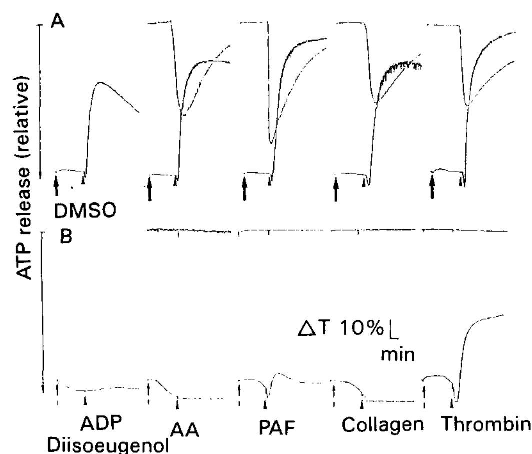

FIG. 2. Effect of diisoeugenol on the platelet aggregation and ATP release induced by ADP, arachidonic acid, PAF, collagen and thrombin. Rabbit washed platelets were preincubated with DMSO (0·5%, control, panel A) or diisoeugenol (50 μg mL^{-1}, panel B) for 3 min, then ADP (20 μM), arachidonic acid (AA, 100 μM), PAF (2 ng mL^{-1}), collagen (10 μg mL^{-1}) or thrombin (0·1 units mL^{-1}) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

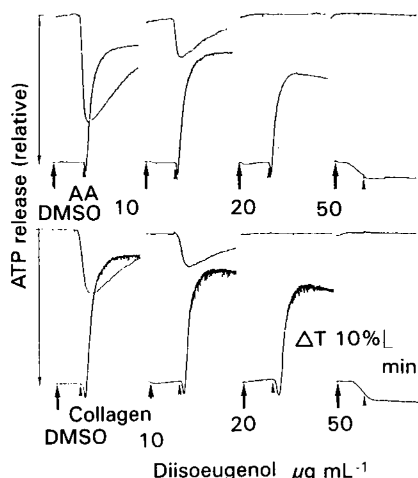

FIG. 3. Inhibitory effect of diisoeugenol on the platelet aggregation and ATP release induced by arachidonic acid and collagen. Rabbit washed platelets were preincubated with various concentrations of diisoeugenol or DMSO (0·5%) for 3 min, then arachidonic acid (AA, 100 μM) or collagen (10 μg mL^{-1}) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

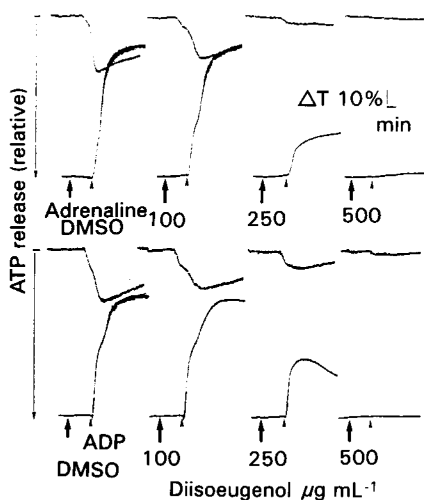

FIG. 4. Effect of diisoeugenol on the aggregation and ATP release reaction of human platelet-rich plasma induced by adrenaline and ADP. Platelet-rich plasma was preincubated with various concentrations of diisoeugenol or DMSO (0·5%) for 3 min, then adrenaline (5 μM) or ADP (5 μM) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

In addition to aggregation, ATP release from washed rabbit platelets induced by the agonists was also inhibited by diisoeugenol (50 μg mL^{-1}) (Fig.2). The inhibition of ATP release was concentration-dependent and more sensitive than inhibition of the platelet aggregation caused by the agonists (arachidonic acid and collagen, Fig. 3). After treating the platelets with diisoeugenol (50 μg mL^{-1}) for 3 min at 37°C and then washing them twice with the suspending solution, collagen (10 μg mL^{-1})-induced platelet aggregation was not restored. Incubation of diisoeugenol (20 μg mL^{-1}) with platelets for 60 min at 37°C did not cause more marked inhibition than that incubated for 3 min (data not shown). In human platelet-rich plasma, 5 μM adrenaline and 5 μM ADP caused biphasic aggregation. The aggregation and ATP release were suppressed by diisoeugenol in a concentration-dependent manner (Fig. 4). Diisoeugenol showed a less inhibitory effect on the aggregation of human PRP compared with rabbit platelet suspension.

Thromboxane B_2 formation in platelet suspension was measured at 6 min after the aggregation inducer was added. Diisoeugenol (10–50 μg mL^{-1}) markedly inhibited the thromboxane B_2 formation induced by 100 μM arachidonic acid, 10 μg mL^{-1} collagen or 0·1 units mL^{-1} thrombin in a concentration-dependent manner (Table 2).

As shown in Table 3, 2 ng mL^{-1} PAF, 10 μg mL^{-1} collagen and 0·1 units mL^{-1} thrombin caused 3·5 ± 0·2-, 3·2 ± 0·2- and 8·0 ± 0·8-fold, respectively, increases of inositol monophosphate formation compared with the resting level. The formation of inositol monophosphate caused by these three agonists was decreased to 2·2 ± 0·2-, 1·5 ± 0·1- and 3·8 ± 1·0-fold, respectively, by 50 μg mL^{-1} diisoeugenol.

The level of cAMP in unstimulated platelets was 4·8 ± 0·5 pmol mL^{-1} (n = 5); 1 μM PGE_1 caused the cAMP level to increase to 93·5 ± 15·6 pmol mL^{-1}. However, 50 μg mL^{-1}

Table 2. Effect of diisoeugenol on the thromboxane B_2 formation of rabbit washed platelets.

	Thromboxane B_2 (ng mL^{-1})		
	Arachidonic acid (100 μM)	Collagen (10 μg mL^{-1})	Thrombin (0·1 units mL^{-1})
Control	410·9 ± 47·6 (6)	460·3 ± 103·7 (6)	204·6 ± 47·4 (4)
Diisoeugenol (μg mL^{-1})			
10	199·4 ± 46·5 (5)*	318·5 ± 77·4 (4)	172·4 ± 56·1 (4)
20	130·3 ± 22·0 (5)**	248·9 ± 107·9 (5)	140·9 ± 79·1 (5)
50	19·7 ± 4·1 (5)**	2·2 ± 1·3 (5)**	14·5 ± 5·8 (5)**

Platelets (4·5 × 10^8 mL^{-1}) were preincubated with various concentrations of diisoeugenol or DMSO (0·5%, control) at 37°C for 3 min, then arachidonic acid, collagen or thrombin was added. Aggregation and thromboxane B_2 formation were terminated by EDTA (2 mM) and indomethacin (50 μM), 6 min after the addition of the aggregation inducer. Values are presented as means ± s.e.m. (n). *$P < 0·01$, **$P < 0·001$ compared with the respective control.

Table 3. Inhibition of diisoeugenol on the formation of inositol monophosphate in rabbit washed platelets caused by PAF, collagen and thrombin.

	Fold increase in inositol monophosphate		
	PAF (2 ng mL^{-1})	Collagen (10 μg mL^{-1})	Thrombin (0·1 units mL^{-1})
Resting	1·0	1·0	1·0
Control	3·5 ± 0·2	3·2 ± 0·2	8·0 ± 0·8
With diisoeugenol (50 μg mL^{-1})	2·2 ± 0·2**	1·5 ± 0·1**	3·8 ± 1·0*

[^{3}H]Inositol-labelled platelets were preincubated with PAF, collagen or thrombin in the presence of calcium (1 mM) for 6 min. Resting values of inositol monophosphate were 785 ± counts min^{-1}/ 4·5 × 10^8 platelets (n = 3). Fold increase of inositol monophosphate from resting levels is presented as means ± s.e.m. (n = 3). *$P < 0·05$, **$P < 0·001$ as compared with the respective control (without diisoeugenol).

diisoeugenol had no significant effect on platelet cAMP level ($5{\cdot}8 \pm 0{\cdot}6$ pmol mL^{-1}).

Discussion

Diisoeugenol inhibited the platelet aggregation and ATP release reaction caused by ADP, arachidonic acid, collagen, PAF and thrombin in rabbit washed platelets. Thromboxane A_2 is an important mediator of aggregation and release reaction in platelets (Ogletree 1987). The formation of thromboxane B_2 caused by arachidonic acid, collagen or thrombin was inhibited by diisoeugenol. The result indicates that the antiplatelet effect of diisoeugenol was due, at least partly, to the inhibition of thromboxane A_2 formation. Diisoeugenol inhibited the aggregation caused by ADP and collagen even in the presence of indomethacin. The results also indicate that diisoeugenol possesses an additional inhibitory effect which is not related to the inhibition of thromboxane A_2 formation.

Phosphoinositides breakdown is observed in platelets activated by many agonists (Lazenave et al 1979; Broekman et al 1980), and this process may be a primary event in the agonist-induced activation (Berridge 1984). The products of phosphoinositides breakdown may have roles as secondary messengers. Diacylglycerol activates protein kinase C, causing protein phosphorylation and release reaction. Inositol trisphosphate triggers calcium mobilization from intracellular calcium stores, resulting in phosphorylation of proteins and release reaction (Mayerus et al 1985). The inositol monophosphate production due to PAF, collagen and thrombin was suppressed by diisoeugenol. This result indicates that diisoeugenol may suppress platelet activation and ATP release, partly by inhibiting the breakdown of phosphoinositides.

The elevation of cAMP levels, either by stimulation of adenylate cyclase or by inhibition of cAMP phosphodiesterase, is one of the most potent mechanisms of inhibition of platelet functions (Packham & Mustard 1977). In our experimental data, diisoeugenol did not elevate the cAMP content of platelets; this indicates that the antiplatelet effect of diisoeugenol is not mediated by elevation of platelet cAMP levels.

Although phosphoinositide breakdown is believed to be an earlier step than thromboxane synthesis, the inhibition of thromboxane formation caused by diisoeugenol was not entirely from the inhibition of phosphoinositides breakdown. For example, thromboxane B_2 formation caused by exogenous arachidonic acid was inhibited by diisoeugenol. Although thromboxane A_2 also can activate the phosphoinositide breakdown, the decrease of phosphoinositide breakdown by diisoeugenol was a direct action, but not due to the inhibition of thromboxane A_2 formation. PAF-stimulated phosphoinositides breakdown in platelets is independent of thromboxane A_2 formation and ADP release (MacIntyre & Pollack 1983); therefore, diisoeugenol may exert its inhibitory action primarily on both thromboxane and inositol monophosphate formation. These actions are not necessarily directly linked.

Less inhibition of platelet aggregation was obtained by diisoeugenol in human PRP than in rabbit washed platelet suspension. This could be due to the binding capacity of plasma for diisoeugenol or to species differences.

In summary, the inhibitory effect of diisoeugenol on rabbit platelets may be due to the direct inhibition of arachidonic acid metabolism and phosphoinositide breakdown.

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