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MICONAZOLE INHIBITION OF PLATELET AGGREGATION BY INHIBITING CYCLOOXYGENASE

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Abstract—Platelet dysfunction was found in rabbits to which a dose of miconazole nitrate (1.6 mg/kg body wt) therapeutic for human subjects had been given intravenously. The present experiments were conducted to elucidate the mechanism of inhibitory effects of miconazole on platelet function. After administration of a single dose of miconazole, rabbit platelet aggregation induced by collagen and sodium arachidonate was inhibited significantly for approximately 24 hr. On the other hand, hypertriglycemia, one of the major side effects of this drug, was not seen during 2 days of observations, nor were any other outstanding manifestations observed. In *in vitro* experiments, miconazole nitrate (10 μM) also significantly inhibited rabbit and human platelet aggregation ($P < 0.01$). Biochemical analyses revealed that the stimulant-induced formation of prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2), metabolites via cyclooxygenase, was inhibited by miconazole nitrate in both human and rabbit platelets *in vitro*. PGE_2 production was decreased dose-dependently with the increase of micronazole concentration (10 to 100 μM), and the decrease was in parallel with a decrease of TXB_2 production. In addition, malondialdehyde (MDA) production of human and rabbit platelets induced by exogenous arachidonate and collagen was also inhibited significantly by miconazole. Chromatographic studies showed that the amount of 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), a metabolite via lipoxygenase, was increased markedly in accordance with the miconazole-induced decrease of TXB_2 and 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) formation in both human and rabbit platelets. These results indicate that miconazole nitrate inhibits platelet cyclooxygenase, without affecting the stimulant-induced release of arachidonic acid from platelet phospholipids. Use of this drug in the treatment of sytemic fungal infection appears to be increasing. Careful attention should be paid to the inhibitory effects of miconazole on platelet function, especially in the case of intravenous treatment.

Miconazole, a synthetic imidazole derivative, is used as an antifungal agent [1, 2]. In the case of systemic fungal infection, intravenous administration of miconazole has been recognized to produce good results [3, 4]. Thus, recently, use of this drug in the treatment of systemic candidiasis as well as cryptococcosis appears to be increasing [5]. It has been reported that adverse reactions of this drug consist of anaphylaxis, gastrointestinal complaints, cardiorespiratory toxicity (cardiac arrest and respiratory arrest), thrombophlebitis, anemia, thrombocytopenia, and hyperlipidemia [3–6]. However, little attention has been paid to the inhibitory effects of miconazole on platelet function. Recently, we found inhibitory effects of miconazole on platelet aggregation in rabbits intravenously injected with miconazole. The purpose of the present study was to clarify the inhibitory effects of miconazole on animal and human platelets. The results of this study indicate that miconazole is a potent cyclooxygenase inhibitor, but not a selective thromboxane (TX†) synthetase inhibitor, and that the inhibitory effect of this drug on platelets is not long-lasting *in vivo* as compared with aspirin [7].

MATERIALS AND METHODS

Blood collection and preparation of platelet rich plasma. Venous blood was collected from male Japan White rabbits (2.3 to 2.6 kg body wt) and from six healthy male volunteers [mean age 29.3 ± 4.8 (S.D.) years]. Both animals and human subjects were starved for 12 hr before blood collection. Administration of drugs and chemical agents that might affect platelet function was carefully avoided for a month. Blood (9 vol.) was collected in 1 vol. of 3.8% trisodium citrate as an anticoagulant. The citrated blood was gently mixed and centrifuged at 180 *g* for 20 min at 20°, and the supernatant (platelet rich plasma; PRP) fraction was carefully transferred to a plastic tube by plastic pipette. The platelet concentration of PRP was adjusted to approximately 300,000/μl by addition of an appropriate amount of platelet poor plasma (PPP) prepared by centrifugation of the remainder of the blood at 3000 *g* for 20 min at 4°. Platelet concentration of PRP was measured with a platelet counter (Towa model PL-100, Tokyo, Japan).

Preparation of gel-filtrated platelets. Gel filtration of platelets was carried out as reported by Tangen *et al.* [8] on siliconized glass columns (2.5 by 45 cm) (Pharmacia, Sweden). The columns were equipped with gel-supporting nets having pore diameters of 40 μm and were packed to a height of 25 cm with Sepharose 2B (Pharmacia, Sweden). The columns were previously equilibrated with Ca^{2+}-free Tyrode's

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† Abbreviations: TX, thromboxane; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; MDA, malondialdehyde; and PG, prostaglandin.

solution containing 1.6% human serum albumin (Sigma). The PRP was gently layered on top of the gel and eluted with the same solutions with which the columns were equilibrated. The most concentrated fractions of gel-filtrated platelets appearing just after the void volumes were collected. The collected fractions were diluted with Ca^{2+}-free Tyrode's solution containing 1.6% human serum albumin and 5 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma), pH 7.35, to make a final concentration of 300,000 cells/μl. After the addition of $CaCl_2$ (final concentration 4 mM), platelet aggregation was measured.

Platelet aggregation. Platelet aggregation was examined between 60 and 100 min after blood sampling [9] at 37° in a Lumi-Aggregometer (Chrono-Log Corp., Havertown, PA) according to the method of Feinman *et al.* [10]. Aggregation was measured as percent light transmission, using the light transmission of PRP as 0% and that of PPP as 100%; the values shown are the maximum % light transmission within 5 min after stimulant was added. Added chemicals consisted of 10% of the final volume of the assay mixture. Sodium arachidonate (Sigma) was prepared and kept under nitrogen as described by Silver *et al.* [11]. Collagen reagent (HORM) was obtained from Hormon-Chemie (München, West Germany) and diluted in the HORM buffer. Disodium ADP (10^{-2} M) was prepared in veronal buffer (pH 6.8) and was further diluted in saline prior to the aggregation assay.

Preparation of solutions of miconazole nitrate and aspirin for in vitro *experiments.* Miconazole nitrate was obtained from the Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Aspirin (acetylsalicylic acid) was from the Shionogi Pharmaceutical Co., Ltd. (Osaka, Japan). *N,N*-Dimethylformamide was obtained from Wako Pure Industries, Ltd. (Osaka, Japan). The stock solutions of miconazole nitrate and aspirin, containing 10^{-1} moles/l, were prepared by dissolving them in dimethylformamide. Each solution was diluted with saline just before aggregation measurements.

Measurements of MDA formation induced by sodium arachidonate and collagen. Malondialdehyde (MDA) formation was measured by the spectrometric assay of Smith *et al.* [12] as modified by Villa *et al.* [13]. PRP was cooled to 4°, and EDTA was added to give a final concentration of 1 mM. The PRP was centrifuged at 2000 *g* for 20 min at 4°. The supernatant fraction was discarded, and the platelet pellet was resuspended in an equal volume of buffered saline containing EDTA (1 mM EDTA, 0.15 M phosphate buffer, pH 7.4). The platelets were centrifuged at 2000 *g* for 15 min at 4° and finally resuspended in an appropriate volume of the above solution without EDTA. A 0.9-ml sample of the platelet suspension, containing 6.0×10^5 platelets/μl, was preincubated with 0.1 ml of either dimethylformamide (1%) or drug at 37° for 15 min before addition of the stimulating agent. The reaction was stopped by adding 0.5 ml of 100% trichloroacetic acid after 5 min of incubation with the stimulating agent. Readings at 532 nm were taken with a Hitachi 124 dual-beam spectrometer, and the results were expressed as nmoles/1.0×10^9 platelets. Preliminary experiments showed a linear relationship between the platelet count in PRP (150,000–1,500,000/μl) and the amount of MDA formed in our system by each stimulus. In addition, miconazole nitrate did not influence the measurements of MDA.

Determination of TXB_2 and PGE_2 production induced by sodium arachidonate and collagen by using radioimmunoassay. Platelets suspended in Tris/NaCl buffer, counting 6.0×10^5 platelets/μl, were preincubated with either miconazole nitrate or 0.1% dimethylformamide (control) for 15 min at 37°. After the preincubation, arachidonic acid or collagen was added to each platelet suspension to give a final concentration of 0.5 mM or 20 μg/ml, respectively, and the mixture was incubated for 5 min at 37°. TXB_2 and PBE_2 were measured according to an established radioimmunoassay (RIA) [9, 14, 15] using a [^{3}H]-RIA kit (New England Nuclear, Boston, MA, U.S.A.).

Chromatographic separation of arachidonic acid and its metabolites. Samples of 50 ml of PRP were incubated at 37° for 2 hr with 2 μCi of [^{14}C]arachidonic acid (52.1 mCi/mmole) (Amersham Corp.). At the end of the incubation period, EDTA was added to give a final concentration of 2 mM, and the platelets were pelleted by centrifugation at 3000 *g* for 20 min at 4°. The pellet was resuspended gently in 50 ml NaCl/Tris–HCl (120 mM/30 mM) buffer, pH 7.4 (Tris/NaCl buffer).

One-milliliter samples of the labeled platelets (1×10^9 cells/ml) were preincubated with either 0.1% dimethylformamide (control) or miconazole nitrate for 15 min at 37°. Collagen was added to give a final concentration of 20 μg/ml, and the mixture was incubated at 37° for 5 min. Lipid extraction of the whole incubation mixture was done according to

Table 1. Effects of miconazole nitrate on rabbit platelet aggregation induced by collagen, sodium arachidonate, and ADP *in vitro*

Stimulants (final concn)	Light transmission (%)					
	Miconazole nitrate (μM)					
	1.0	10	25	50	100	Control
ADP (10 μM)	73 ± 3.5	70 ± 5.9	70 ± 4.7	72 ± 5.8	70 ± 4.5	73 ± 3.6
Collagen (10 μg/ml)	75 ± 3.6	54 ± 4.1*	40 ± 6.9*	20 ± 6.8*	1.4 ± 0.7*	78 ± 4.5
Sodium arachidonate (0.5 mM)	72 ± 4.1	55 ± 4.5*	41 ± 4.1*	29 ± 6.1*	16 ± 3.3*	72 ± 5.7

Values are mean ± S.D. of four experiments performed in PRP preincubated for 15 min with miconazole.
* $P < 0.01$, as compared to control.

Table 2. Effects of miconazole nitrate on human platelet aggregation induced by various stimulants

Stimulants (final concn)	Light transmission (%)					
	Miconazole (μM)				Aspirin (μM)	
	1.0	10	25	100	100	Control
ADP (10 μM)	72 ± 2.1	70 ± 3.3	70 ± 4.1	70 ± 4.3	70 ± 5.7	75 ± 2.9
Collagen (2 μg/ml)	61 ± 9.9	37 ± 9.7*	30 ± 10*	4.0 ± 2.9*	18 ± 5.1*	76 ± 4.6
Sodium arachidonate (1.7 mM)	69 ± 0.5	57 ± 4.0*	41 ± 2.0*	1.8 ± 1.8*	5.0 ± 2.0*	74 ± 5.0

Values are mean ± S.D. of five experiments performed in PRP preincubated for 15 min with miconazole.
* $P < 0.01$, as compared to control.

Billah *et al.* [16]. Dried lipids were dissolved in chloroform and spotted on silica gel G plates (E. M. Merck, Darmstadt, West Germany).

The solvent used for separation was the upper phase from a mixture of ethylacetate–2,2,4-trimethylpentane–acetic acid–water (90:50:20:100, by vol.), which gives a good separation of cyclooxygenase and lipoxygenase products (Fig. 1). Radioactive spots were visualized by autoradiography, and radioactivity in each spot was determined by liquid scintillation counting. Standard HETE, HHT and TXB_2 were made available by members of the Experimental Chemistry Unit of the Ono Pharmaceutical Co., Ltd. (Osaka, Japan).

Effects of miconazole nitrate administration on platelet function and plasma triglyceride levels in rabbits. Miconazole nitrate was dissolved in 0.9% saline and filtered through a Millipore filter (0.22 μm). Miconazole nitrate (1.6 mg/kg body wt) was infused intravenously in rabbits within 5 min. Blood was withdrawn before and at 1, 2, 6, 12, 24 and 48 hr after the start of the miconazole nitrate administration for the determination of plasma triglyceride levels and for the measurement of platelet aggregation. Triglyceride levels in plasma were determined by the method of enzymatic analysis [17].

Statistics. Data were analyzed by Student's *t*-test for significance of differences.

RESULTS

Effects of miconazole nitrate on rabbit platelet aggregation induced by collagen and sodium arachidonate in vitro. As can be seen in Table 1, miconazole nitrate inhibited collagen (10 μg/ml)- and sodium arachidonate (0.5 mM)-induced platelet aggregation in a dose-dependent manner. Miconazole nitrate at a concentration of 10 μM showed significant inhibitory effects on arachidonate- and collagen-induced platelet aggregation. Miconazole nitrate at a concentration of 100 μM completely inhibited both collagen- and arachidonate-induced aggregation, although the first wave of ADP-induced aggregation was not inhibited by miconazole nitrate at this high concentration (100 μM). As seen in the miconazole-treated platelets, aspirin did not inhibit the first wave of ADP-induced aggregation, although it did inhibit the second wave.

Effects of miconazole nitrate on human platelet aggregation induced by collagen and sodium arachidonate in vitro. Miconazole nitrate, at a concentration of 100 μM, completely inhibited human platelet aggregation induced by collagen (2.0 μg/ml) and sodium arachidonate (1.7 mM), as observed with aspirin (100 μM) (Table 2). The degree of miconazole-induced inhibition on platelet aggregation found by using PRP was almost the same as that obtained by using gel-filtrated human platelets. However, the second wave of ADP-induced aggregation of human platelets was inhibited, as seen in miconazole-treated rabbit platelets.

Effects of pretreatment of human platelets with miconazole nitrate on collagen- and arachidonate-induced MDA formation. Table 3 shows that both miconazole nitrate and aspirin inhibited stimuli-induced MDA formation. The results indicate that endogenous and exogenous arachidonic acid was not normally metabolized in platelets pretreated with miconazole nitrate or aspirin. In addition, miconazole nitrate also inhibited MDA formation of rabbit platelets induced by collagen or arachidonate (data not shown).

Effects of miconazole nitrate on the production of TXB_2 and PGE_2 induced by sodium arachidonate or collagen. As shown in Fig. 2, when stimulated at an appropriate concentration of sodium arachidonate

Table 3. Effects of miconazole nitrate on platelet MDA (malondialdehyde) formation induced by collagen and sodium arachidonic acid

Stimulants	MDA formed (nmoles/10^9 platelets)		
	Control	Miconazole (100 μM)	Aspirin (100 μM)
Collagen (20 μg/ml)	2.9 ± 0.20	1.2 ± 0.20*	0.12 ± 0.05*
Sodium arachidonic acid (0.5 mM)	8.0 ± 0.46	4.7 ± 0.14*	1.25 ± 0.10*

Values are means ± S.D. of three experiments.
* $P < 0.01$, as compared to control.

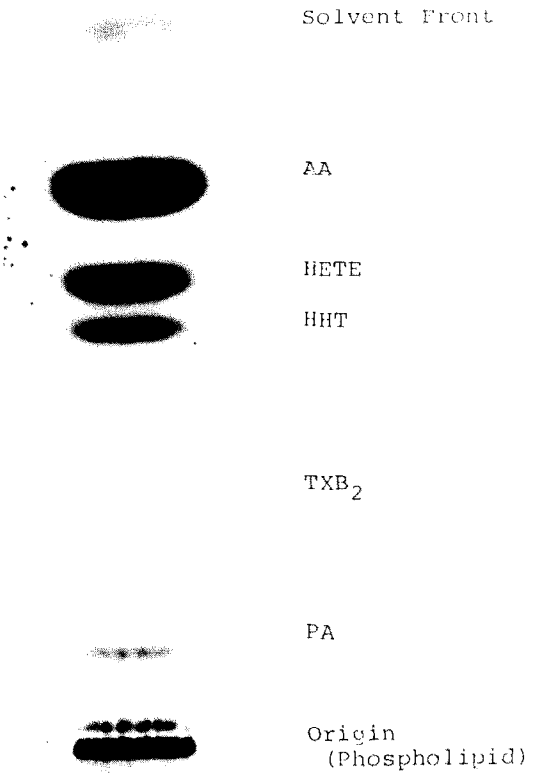

Fig. 1. Thin-layer chromatographic separation of radioactive arachidonate and its metabolites. Human platelets were prelabeled with [^{14}C]arachidonic acid and incubated with 0.1% dimethylformamide for 15 min at 37° and then exposed to 20 μg/ml collagen. Products were extracted and analyzed by thin-layer chromatography using a silica gel G plate. Radioactive spots were visualized by autoradiography. Abbreviations: TXB_2, thromboxane B_2; PA, phosphatidic acid; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; and AA, arachidonic acid.

and collagen, the amount of TXB_2 production induced by exogenous arachidonic acid was much more than that induced by collagen. Miconazole nitrate (100 μM) almost completely inhibited TXB_2 production induced by arachidonic acid and collagen. These results are consistent with the results shown in Table 2. PGE_2 production was decreased with the increased of miconazole concentration (10 to 100 μM), and the decrease of PGE_2 production was in parallel with a decrease of TXB_2 production. There was no discrepancy between the decrease of TXB_2 production and that of PGE_2 production. Similar inhibitory effects on the stimulus-induced production of TXB_2 and PGE_2 were observed using rabbit platelets (data not shown).

Effects of miconazole nitrate on collagen-induced production of arachidonate metabolites (Fig. 3). The formation of the radioactive arachidonate metabolites via cyclooxygenase (TXB_2 and HHT) following collagen stimulation was apparently inhibited

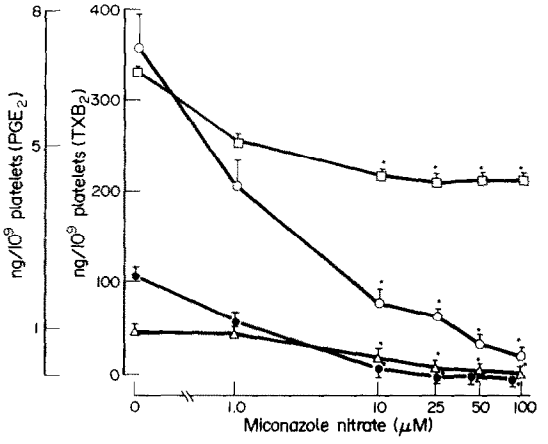

Fig. 2. Effect of miconazole nitrate on arachidonate metabolite formation induced by arachidonic acid and collagen. These results were obtained by radioimmunoassay methods. Each point represents the mean ± S.D. of three experiments. Key: (○—○) TXB_2 formation induced by arachidonic acid; (△—△) TXB_2 formation induced by collagen; (□—□) PGE_2 formation induced by arachidonic acid; (●—●) PGE_2 formation induced by collagen; and (*) $P < 0.01$, as compared to control.

by miconazole nitrate. In contrast, HETE, an arachidonate metabolite via lipoxygenase, was increased markedly. An increase of HETE was apparently mirrored by a decrease in TXB_2 formation. Similar inhibitory effects on the collagen-induced production of radioactive arachidonate metabolites were observed using rabbit platelets (data not shown).

Time-courses for the collagen-induced platelet aggregation after intravenous administration of miconazole nitrate in rabbit (Fig. 4). When miconazole nitrate was given intravenously to three rabbits in a 1.6 mg/kg dose, this drug distinctly inhibited collagen-induced platelet aggregation. The inhibitory effects of miconazole nitrate on platelet aggregation persisted for approximately 24 hr. On the

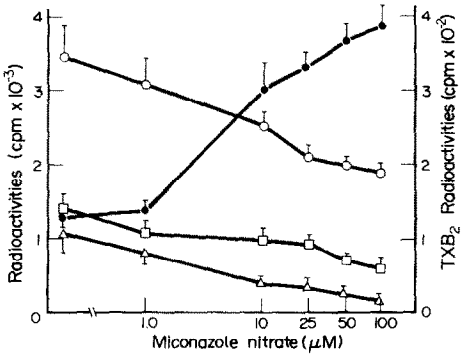

Fig. 3. Effect of miconazole nitrate on arachidonate metabolites induced by collagen. Human platelets prelabeled with [^{14}C]arachidonic acid were incubated with various concentrations of miconazole nitrate for 15 min at 37° and then exposed to collagen (20 μg/ml). Products were extracted and analyzed by thin-layer chromatography. Radioactive spots were counted by a liquid scintillation counter. Key: (●) HETE; (○) TXB_2; (△) HHT; and (□) AA.

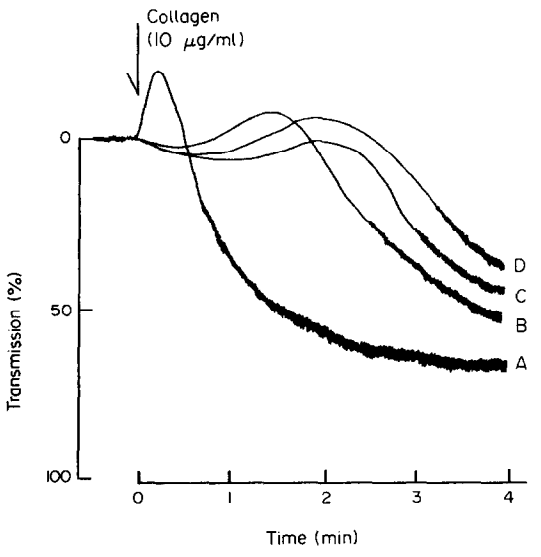

Fig. 4. Time-courses for the collagen-induced platelet aggregation after intravenous administration of miconazole nitrate (1.6 mg/kg body wt) in rabbits. Curve A, before administration and 48 hr after administration; curve B, 24 hr; curve C, 6 hr; and curve D, 1 hr.

other hand, the level of venous triglyceride was 50 ± 13 mg/dl before treatment of miconazole, and hypertriglycemia was not observed in the treated animals during 2 days of observation. Furthermore, the rabbits did not show any apparent adverse reactions. In addition, arachidonate-induced platelet aggregation was also inhibited in rabbits treated with miconazole nitrate. These results are consistent with the results obtained *in vitro* (Tables 1 and 2).

DISCUSSION

The results of this study clearly demonstrate that miconazole nitrate inhibits platelet cyclooxygenase. Preincubation with miconazole nitrate (10–100 μM) inhibited rabbit and human platelet aggregation induced by arachidonic acid and collagen (Tables 1 and 2). The extent of inhibition on platelet aggregation was dose dependent with the increase of miconazole nitrate concentration. To elucidate the mechanism of the inhibition of miconazole nitrate on platelet aggregation, we investigated the effects of miconazole nitrate on exogenous arachidonate metabolism and stimulus-induced membrane arachidonate metabolism. The formation of PGE_2 and TXB_2 was decreased in accordance with the increase of miconazole nitrate concentration (10 to 100 μM). A decrease of PGE_2 production and a decrease of TXB_2 formation, as measured by radioimmunoassay, started at the same concentration on of miconazole nitrate (Fig. 2). MDA production of human platelets induced by sodium arachidonate and collagen was also inhibited by miconazole (Table 3). These results seem to be consistent with the observed inhibition of stimuli-induced platelet aggregation *in vitro* as mentioned above (Tables 1 and 2). Chromatographic studies, furthermore, showed that the collagen-induced production of arachidonate metabolites via cyclooxygenase (HHT and TXB_2) from [^{14}C]arachidonic acid-labeled platelets was inhibited significantly by miconazole nitrate, in contrast to a marked increase of HETE, an arachidonate metabolite via lipoxygenase (Fig. 3). These observations coincide with the reported effects of aspirin [18], a representative cyclooxygenase inhibitor. The data in this study thus strongly indicate that miconazole nitrate inhibits platelet cyclooxygenase, without affecting the stimulus-induced release of arachidonic acid from platelet phospholipids.

Little attention has been paid to the effects of miconazole nitrate on platelet function. Recently, miconazole nitrate has been reported to be a selective TX synthetase inhibitor, based on the observations that miconazole decreases the TX production of platelets with a coincident increase in the PGE_2 production [19]. In the report, however, the formation of TXB_2, PGD_2 and PGE_2 induced by exogenous arachidonate was investigated by only one method, radioimmunoassay, *in vitro*. In addition, the effect of this drug on the stimulus-reaction system of platelets has not been studied. In the present work, a decrease of PGE_2 production was observed using platelets stimulated by two different stimuli when an inhibition of TXB_2 formation by miconazole nitrate took place. The increase of PGE_2 formation and the decrease of TXB_2 production, as determined by radioimmunoassay, occurred at the same time and dose-dependently with the concentration of miconazole nitrate (Fig. 2). Furthermore, as demonstrated by chromatographic studies, HETE, a lipoxygenase product, increased markedly, mirroring the decrease of TXB_2 and HHT formations in platelets pretreated with miconazole (Fig. 3). Such a tremendous increase of HETE has not been reported in platelets treated with established TX synthetase inhibitors, including OKY-046 [20], CGS-13080 [21], and UK-38485 [22]. These results indicate that miconazole nitrate inhibited cyclooxygenase, but not TX synthetase.

On the other hand, TX synthetase inhibitors have been shown to fail to inhibit arachidonate-induced platelet aggregation in PRP from some donors, designated "non-responders" [23, 24]. Recently, Bertelé *et al.* [24] reported that inhibition of thromboxane synthetase does not *per se* prevent platelet aggregation. The present study shows that miconazole nitrate strongly and dose-dependently inhibited both arachidonic acid- and collagen-induced platelet aggregation in PRP from six volunteers. Although only six subjects were studied in the present study, our results further support the conclusion that miconazole nitrate is not a TX synthetase inhibitor but a cyclooxygenase inhibitor.

When miconazole nitrate was given intravenously to rabbits, at a dose therapeutic for human subjects (1.6 mg/kg), it distinctly inhibited platelet aggregation *in vivo*. However, hypertriglycemia, one of the major side effects of this drug, was not seen when platelet function was apparently inhibited by miconazole. This finding indicates that platelet dysfunction is one of the first side effects of this drug to appear. Miconazole nitrate has been recognized as an effective antifungal agent, and the application of high dose intravenous miconazole treatment to the

treatment of systemic fungal infections appears to be increasing [5]. Careful attention should be paid to inhibitory effects of miconazole nitrate on platelet aggregation in the case of intravenous treatment. It is interesting to note, however, that inhibitory effects of miconazole nitrate on platelet aggregation persisted for only approximately 24 hr; that is, in contrast to aspirin, the action of miconazole is short-acting and reversible.

It is generally accepted that stimulus-induced arachidonate metabolites are essential mediators in stimulus–response reactions of various cells [25]. It may be possible that the therapeutic dose of miconazole nitrate inhibits cyclooxygenase in these cells. While it is beyond the scope of this paper to discuss the other toxic effects of miconazole nitrate, the fact that miconazole nitrate has inhibitory effects on cyclooxygenase, as shown in this paper, may open new vistas about the mechanism of biological action and the toxicity of this drug.

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