



DIFFERENTIAL EFFECTS OF THE PYRIMIDO-PYRIMIDINE DERIVATIVES, DIPYRIDAMOLE AND MOPIDAMOL, ON PLATELET AND VASCULAR CYCLOOXYGENASE ACTIVITY

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Abstract—The chronic administration of 10 mg/kg/day of dipyridamole to rats produced 33.7% inhibition of platelet aggregation induced with ADP and a 93% increase in 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) in vascular samples, versus saline-treated rats. Mopidamol, 8.3 mg/kg/day, caused 50.6% inhibition of ADP-induced platelet aggregation, 37.6% inhibition of aggregation induced with arachidonic acid, a 47.6% decrease in serum levels of thromboxane B_2 and a 23.7% increase in the vascular production of 6-keto-PGF $_{1\alpha}$, versus saline-treated rats. Dipyridamole showed a higher *in vitro* anti-aggregating effect in whole blood (IC_{50} 6.6 μ M) than in platelet-rich plasma (PRP) (IC_{50} 210 μ M), when ADP was used as inducer, and had no effect in the presence of arachidonic acid. Mopidamol exerted a similar effect in whole blood (IC_{50} 3.7–20 μ M, depending on the inducer) and PRP (IC_{50} 11–17.3 μ M), and showed a dose-dependent inhibition of platelet aggregation and thromboxane B_2 synthesis induced with arachidonic acid (IC_{50} 16.8–22.3 μ M). Mopidamol also inhibited enzymatically induced lipid peroxidation (IC_{50} 89 \pm 5.9 μ mol/L) and had no effect on free radical-induced lipid peroxidation. The dose-dependent increase in 6-keto-PGF $_{1\alpha}$ in vascular samples after incubation with dipyridamole showed a negative linear correlation with inhibition of lipid peroxidation ($r^2 = 0.77$). It is concluded that the phosphodiesterase inhibitors, dipyridamole and mopidamol, interfere in a different manner with platelet function. It seems that mopidamol may also exert a selective effect on platelet thromboxane synthesis.

Dipyridamole, the most representative compound amongst pyrimido-pyrimidine derivatives (Fig. 1), acts as a coronary vasodilator and platelet aggregation inhibitor [1–3]. A chemical congener, mopidamol (Fig. 1), is endowed with *in vivo* and *ex vivo* antiplatelet effects [2, 4–8] and has shown antimitogenic properties by inhibiting the adherence of cancer cells to platelets or endothelial cells [9–11]. Although both compounds cause inhibition of cAMP phosphodiesterase (K_i values of 12 ± 2 μ mol/L for dipyridamole and 0.55 ± 0.15 μ mol/L for mopidamol) [12], mopidamol is 10 times more potent than dipyridamole. It has been reported that the antiplatelet activity of dipyridamole and the related compound, RA-642, is not dependent on platelet cyclooxygenase [4, 6, 13], but we have found no references to the effects of mopidamol on the cyclooxygenase activity of platelets. We reported that mopidamol inhibited lipid peroxidation induced by arachidonic acid, although it did not prevent platelet activation induced by oxygen-derived free radicals [13–16]. This finding would suggest a possible effect of mopidamol on platelet cyclooxygenase. In this study, we evaluate the effects of mopidamol

on platelet TxA_2 † and/or vascular prostacyclin biosynthesis in comparison with dipyridamole.

MATERIALS AND METHODS

Three different types of experiments were carried out consecutively: *ex vivo* assays in rats, *in vitro* platelet aggregometry and *in vitro* biochemical tests.

Ex vivo assays in rats. A total of 60 male Wistar rats weighing 200–250 g were housed in plastic cages with unlimited access to food and water. The animals were divided at random into three experimental groups. In group I, 20 animals received daily oral (endogastric cannula) doses of 1 mL/kg of normal saline; in group II, 20 animals received oral doses of 10 mg/kg/day of dipyridamole (Boehringer Ingelheim España, S.A., Barcelona, Spain) in a volume of 1 mL/kg; and in group III, 20 animals were given oral doses of 8.3 mg/kg/day of mopidamol (Dr Karl Thomae, Biberach an der Riss, Germany) in a volume of 1 mL/kg (equimolar concentration to that of dipyridamole). The duration of treatment was 15 days. One hour after the last dose, animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) (Nembutal®, Abbott) and a blood sample from the abdominal aorta at the level of its bifurcation into the common iliac arteries was obtained, and two 5-mm sections of the thoracic aorta (in the midpoint between the left subclavian artery and the diaphragm) were removed.

Blood samples were divided into two parts, one

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† Abbreviations: TxA_2 , thromboxane A_2 ; TxB_2 , thromboxane B_2 ; MDA, malondialdehyde; FeAs, ferrousulphate and ascorbic acid; 6-keto-PGF $_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PRP, platelet-rich plasma.

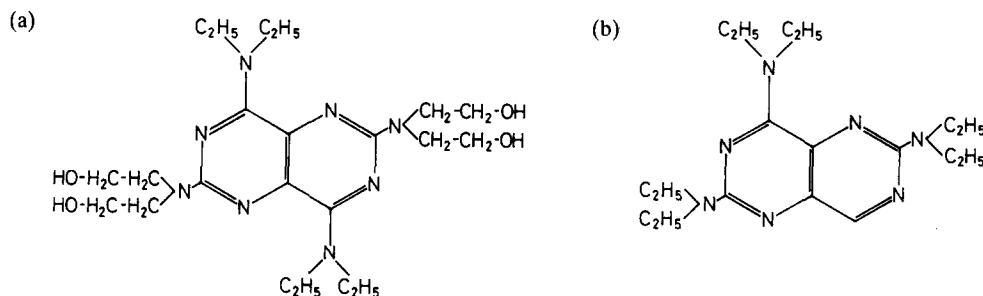


Fig. 1. Chemical structures of dipyridamole (a) and mopidamol (b).

of which was anticoagulated with a solution of 3.8% trisodium citrate, 1:10; the other was incubated at 37° for 30 min, centrifuged at 2500 g for 10 min at 4°, and serum samples were stored frozen at -70° before analysis. Aortic sections were transferred to a buffer solution containing (in g/L) 6.21 NaCl, 0.29 KCl, 1.68 NaHCO₃, 0.28 Na₂SO₄, 5.58 trisodium citrate, 0.5 glucose and 0.6 Tris (pH 8.2) and incubated at 37° for 10 min. They were subsequently incubated at 37° for 5 min in the buffer mentioned above with 100 µmol/L of arachidonic acid (Bio Data Corp., U.S.A.); vascular samples were weighed and supernatants were kept frozen at -70°.

The concentrations of TxB₂, a stable metabolite of TXA₂, in serum samples were determined by a commercial radioimmunoassay (Amersham International plc, U.S.A.). The production of 6-keto-PGF_{1α}, a stable metabolite of prostacyclin, in vascular samples was measured by a commercial radioimmunoassay (Amersham International plc, U.S.A.).

Platelet aggregation in whole blood was measured by the electric impedance method described by Cardinal and Flower [17] as the maximum change in impedance (ohms) 10 min after the addition of the aggregating agent. Samples were diluted in physiological saline (pH 7.4, 1:1 v/v) and aggregometry was performed at 37° with continuous stirring at 1000 r.p.m. ADP (10 µmol/L) (Diagnostica Stago, Asnières, France) and arachidonic acid (0.8 mmol/L) were used as inducers.

In vitro platelet aggregometry. Venous blood was obtained from healthy male volunteers aged between 25 and 40 years, who had not taken any drug for at least 30 days prior to blood collection. Blood was anticoagulated with a solution of 3.8% trisodium citrate, 1:10. Platelet aggregometry was carried out in whole blood and PRP. PRP was obtained from whole blood by centrifugation (180 g for 10 min at 20°) and diluted up to 300 platelets × 10⁹/L with autologous platelet-poor plasma (1800 g for 15 min at 20°). Platelet aggregation was measured by the electronic impedance method [17] using ADP (2.5 µmol/L), collagen (1 µg/mL) (Diagnostica Stago, Asnières, France) and arachidonic acid (0.8 mmol/L) as inducers. In PRP samples, different concentrations of arachidonic acid (0.05–1.6 mmol/L) were used as aggregating agents. Samples were

incubated at 37° for 5 min in increasing concentrations of dipyridamole or mopidamol prior to the addition of inducers. Dose-response curves were constructed and the concentrations of dipyridamole and mopidamol that produced 50% inhibition of platelet aggregation (IC₅₀) were calculated. The influence of different concentrations of dipyridamole and mopidamol on the concentration-response curve of arachidonic acid in PRP was also assessed.

In vitro biochemical tests. The concentrations of TxB₂ were determined by radioimmunoassay (as described in the *ex vivo* experiments) in PRP samples. After platelet aggregation assay, indomethacine (100 µmol/L) (Sigma Chemical Co., St Louis, MO, U.S.A.) was added, samples were centrifuged at 10,000 g for 2 min and the supernatant was stored frozen at -70°.

The formation of MDA by platelets was taken to be an indicator of lipid peroxidation [18]. Briefly, 1 mL of trichloroacetic acid was added to PRP samples used in the platelet aggregation assay with 0.4 mmol/L arachidonic acid. The product was centrifuged at 10,000 g for 2 min. Subsequently, 0.5% thiobarbituric acid (1:5 v/v) was added and samples were incubated at 100° for 15 min. The amount of MDA (enzymatic MDA) produced was measured by the spectrophotometric absorbance of the supernatant at 532 nm using a Perkin-Elmer spectrophotometer (C-53200001). The absorbances obtained were compared to that of a standard curve using MDA-bis-diethyl-acetol (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). The same experiments were carried out using 100 µmol/L ferrous sulphate and 100 µmol/L ascorbic acid (FeAs) to induce lipid peroxidation via the formation of hydroxyl anions (FeAs-induced MDA).

The method described by Roos and Pleger [19] was used to measure the uptake of adenosine into erythrocytes. The pellet obtained in the above-mentioned process of PRP preparation was removed and the red cells were washed twice with a buffer solution containing (in g/L) 9 NaCl, 0.9 glucose and 0.6 Tris (pH 7.4), by centrifugation at 1000 g for 20 min. The final count of red blood cells was 0.2 × 10¹²/L. To each tube 1 µmol/L of [8-¹⁴C]-adenosine (sp. act. 55 µCi/mmol) (Amersham International plc) was added in assays without drugs and in those with increasing concentrations of dipyridamole and mopidamol. Samples were incu-

Table 1. Effects of oral administration of dipyridamole and mopidamol for 15 days on platelet aggregation and TxB_2 synthesis, and vascular 6-keto-PGF $_{1\alpha}$ production

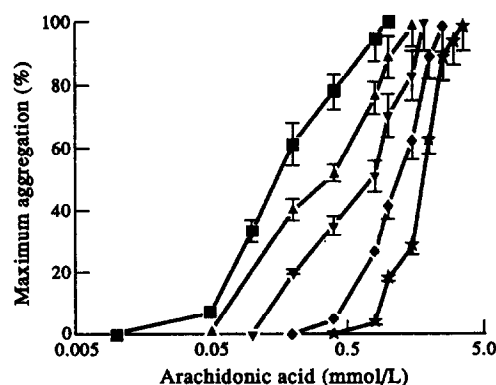
	Controls (saline)	Dipyridamole (10 mg/kg/day)	Mopidamol (8.3 mg/kg/day)
No. of animals	20	20	20
Maximum platelet aggregation (ohms)			
ADP (10 $\mu\text{mol/L}$)	8.3 \pm 0.7	5.5 \pm 0.4*	4.1 \pm 0.2†
AA (0.8 mmol/L)	11.7 \pm 0.4	11.9 \pm 1.3	7.3 \pm 1.0†
TxB_2 (ng/mL)	235 \pm 34	222 \pm 10	123 \pm 10‡
6-keto-PGF $_{1\alpha}$ (ng/mg aorta)	12.2 \pm 0.3	23.6 \pm 4.0‡	15.1 \pm 1.1*

AA, arachidonic acid.

Data are means \pm SEM.* $P < 0.007$, † $P < 0.0005$, ‡ $P < 0.002$ vs controls.Table 2. IC_{50} values for dipyridamole and mopidamol for human platelet aggregation in whole blood and PRP

Platelet aggregometry	IC_{50} ($\mu\text{mol/L}$)	
	Dipyridamole	Mopidamol
Whole blood		
ADP (2.5 $\mu\text{mol/L}$)	6.6 \pm 0.2	3.7 \pm 0.7
Collagen (1 $\mu\text{g/mL}$)	65.8 \pm 13.0	18.3 \pm 2.1*
AA (0.8 mmol/L)	>1000	20.6 \pm 1.2
PRP		
ADP (2.5 $\mu\text{mol/L}$)	210 \pm 20	17.3 \pm 1.4†
Collagen (1 $\mu\text{g/mL}$)	378 \pm 6.9	15.9 \pm 1.1†
AA (0.8 mmol/L)	>1000	11.0 \pm 0.8

AA, arachidonic acid.

Each value represents the mean \pm SEM of six to eight independent experiments.* $P < 0.005$ and † $P < 0.0001$ as compared with dipyridamole.Fig. 2. Dose-response curves of platelet aggregation induced by increasing concentrations of arachidonic acid in PRP assays (■); inhibitory effect of 5 (▲), 10 (▼), 15 (◆) and 20 (★) $\mu\text{mol/L}$ of mopidamol. Each point represents the mean \pm SEM of six to eight independent experiments.

bated at 37° for 5 min and centrifuged at 10,000 g for 3 min. The uptake of adenosine into erythrocytes was measured by the percentage of radioactivity in the pellet using a liquid-scintillation spectrophotometer (Betamatic, Kontron, Switzerland). Finally, the production of 6-keto-PGF $_{1\alpha}$ and enzymatic MDA (not induced with FeAs) was determined in vascular samples from untreated rats (controls).

The Student's t -test for unpaired variables and least-squares linear regression analysis were applied for statistical analysis using the Statgraphics computer program (STSC Inc., MD, U.S.A.). Statistical significance was set at $P < 0.05$. All values are presented as means \pm SEM.

RESULTS

Ex vivo assays in rats

The chronic administration of oral dipyridamole (10 mg/kg/day) caused a statistically significant increase in the vascular production of 6-keto-PGF $_{1\alpha}$ (93% increment vs controls) and a decrease in ADP-induced platelet aggregation (33.7% inhibition vs controls). When mopidamol was given, there was 50.6% inhibition of ADP-induced platelet

aggregation, 37.6% inhibition of aggregation induced by arachidonic acid, a 47.6% decrease in serum levels of TxB_2 and a 23.7% increase in the vascular production of 6-keto-PGF $_{1\alpha}$ (Table 1).

In vitro human platelet aggregometry

Dipyridamole showed a dose-related inhibitory effect on platelet aggregation both in whole blood and in PRP when ADP (range 0.5–20 $\mu\text{mol/L}$) and collagen (range 5–100 $\mu\text{mol/L}$) were used as inducers. However, dipyridamole at <1000 $\mu\text{mol/L}$ had no effect on platelet aggregation induced with arachidonic acid.

Mopidamol (range 0.5–50 $\mu\text{mol/L}$) also showed a dose-related inhibitory effect on platelet aggregation both in whole blood and PRP when ADP, collagen or arachidonic acid was used as inducer (Table 2). Figure 2 shows the dose-response curves of platelet aggregation induced by arachidonic acid (EC_{50} 0.2 \pm 0.01 mmol/L) in PRP assays. In the presence of 5, 10, 15 and 20 $\mu\text{mol/L}$ of mopidamol, the EC_{50} values of arachidonic acid were 0.34 \pm 0.02,

Table 3. IC₅₀ values for dipyridamole and mopidamol for different *in vitro* human platelet tests (lipid peroxidation and TxB₂ synthesis), and human erythrocyte adenosine uptake

	IC ₅₀ (μmol/L)	
	Dipyridamole	Mopidamol
Adenosine uptake into erythrocytes	0.76 ± 0.06	125 ± 10*
MDA induced with		
AA (0.8 mmol/L)	140 ± 7.5	89.0 ± 5.9†
FeAs (100 μmol/L)	15.5 ± 1.7	>1000
TxB ₂ induced with		
AA (0.1 mmol/L)	>1000	16.8 ± 0.9
AA (0.2 mmol/L)	>1000	17.0 ± 1.1
AA (0.4 mmol/L)	>1000	19.3 ± 2.4
AA (0.8 mmol/L)	>1000	22.3 ± 2.6

AA, arachidonic acid.
Each value represents the mean ± SEM of six to eight independent experiments.
* P < 0.0001 and †P < 0.01 as compared with dipyridamole.

Table 4. Effects of dipyridamole and mopidamol on the production of 6-keto-PGF_{1α} and enzymatic MDA in vascular samples

	Dipyridamole	Mopidamol
	Concentration (μmol/L) that increases 2-fold the baseline values	
6-keto-PGF _{1α} induced with		
AA (0.1 mmol/L)	45.3 ± 5.8	>1000
AA (0.2 mmol/L)	68.4 ± 5.1	>1000
AA (0.4 mmol/L)	73.0 ± 6.9	>1000
AA (0.8 mmol/L)	75.6 ± 7.9	>1000
NDA induced with	IC ₅₀ (μmol/L)	
AA (0.1 mmol/L)	73.0 ± 6.5	70.1 ± 6.9
AA (0.2 mmol/L)	81.5 ± 7.3	83.4 ± 8.8
AA (0.4 mmol/L)	93.7 ± 8.6	88.7 ± 7.6
AA (0.8 mmol/L)	90.0 ± 8.8	92.6 ± 9.7

AA, arachidonic acid.
Each value represents the mean ± SEM of six to eight independent experiments.

0.56 ± 0.03, 1.3 ± 0.06 and 2.3 ± 0.4 mmol/L, respectively.

In vitro biochemical tests

Mopidamol (range 10–500 μmol/L) showed a dose-dependent inhibition of adenosine uptake by human erythrocytes (IC₅₀ values 165 times greater than those of dipyridamole) (Table 3). Mopidamol also showed a dose-related inhibitory effect on the production of enzymatic human platelet MDA (IC₅₀ values 1.5 times lower than those of dipyridamole). In contrast to dipyridamole, however, mopidamol had no effect on the production of FeAs-induced human platelet MDA (IC₅₀ > 1000 μmol/L) (Table 3). Mopidamol (range 1–100 μmol/L) showed a dose-dependent inhibitory effect on human platelet TxB₂ after induction with arachidonic acid. This effect was not observed in assays in which dipyridamole was used (IC₅₀ > 1000 μmol/L) (Table 3).

The production of 6-keto-PGF_{1α} in rat vascular samples showed a dose-dependent increase after incubation with dipyridamole (Table 4). A slight

effect was only observed after incubation with mopidamol, i.e. 500 μmol/L mopidamol produced a maximal increase of 38.6 ± 5.6% when 0.1 mmol/L arachidonic acid was used as inducer. Both dipyridamole and mopidamol showed a dose-related inhibitory effect on the production of enzymatic MDA in rat vascular samples (Table 4).

There was a statistically significant positive correlation between the human platelet production of TxB₂ and maximal aggregation of human platelets induced with arachidonic acid in the presence of different concentrations of mopidamol (Fig. 3); in the case of dipyridamole, no significant correlations were found (data not shown). Likewise, there was a statistically significant negative correlation between the production of 6-keto-PGF_{1α} and MDA in rat vascular samples in the presence of different concentrations of dipyridamole (Fig. 3); in the case of mopidamol, no significant correlations were observed (data not shown).

DISCUSSION

The results obtained in *ex vivo* experiments in

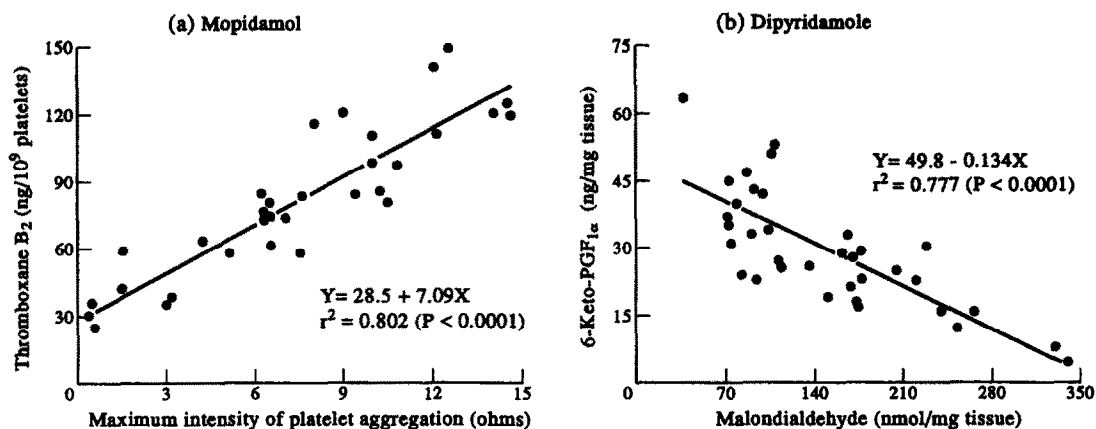


Fig. 3. Linear correlations between the production of TxB_2 and maximal aggregation of human platelets (left panel) and the production of 6-keto- $\text{PGF}_{1\alpha}$ and MDA in rat vascular samples (right panel), in the presence or absence of different concentrations of mopidamol and dipyridamole (assays with 0.4 mmol/L arachidonic acid).

animals suggest that dipyridamole and mopidamol may interfere with different aspects of platelet function. The anti-aggregatory activity of dipyridamole is exerted presumably via stimulation of adenylyl cyclase (ADP-induced aggregometry) and prostacyclin formation, while platelet cyclooxygenase (arachidonic acid-induced aggregometry and production of TxB_2) appears to be involved in the mode of action of mopidamol. Other studies of the effects of dipyridamole on platelet activity have shown similar results [6–8]. We have found no references to the effect of mopidamol on thromboxane biosynthesis. It should be noted that mopidamol caused a 47.6% decrease in serum levels of TxB_2 with a concomitant 23.7% increase in the vascular production of 6-keto- $\text{PGF}_{1\alpha}$, a finding reported for thromboxane synthetase inhibitors.

In the human *in vitro* experiments, dipyridamole showed a greater inhibitory effect on aggregation induced with ADP as compared with collagen, had no effect on platelet aggregation induced with arachidonic acid and exerted a greater antiplatelet activity in whole blood than in PRP [13, 20, 21], which is consistent with its inhibitory effect on adenosine uptake by erythrocytes [19, 21]. In contrast, mopidamol showed inhibitory effects in the presence of the three aggregating agents, exerted a similar antiplatelet activity in whole blood and PRP, and had a poor effect on the inhibition of adenosine uptake into erythrocytes (IC_{50} values 165 times greater than those of dipyridamole).

In addition to the reported increase in cAMP by mopidamol [22, 23], the present study provides evidence which suggests that this drug inhibits the metabolic pathway of arachidonic acid. Mopidamol inhibited *in vitro* platelet aggregation induced with arachidonic acid and showed a dose-dependent inhibitory effect on the production of TxB_2 after induction with arachidonic acid. This effect was not observed in assays in which dipyridamole was used [13, 20].

On the other hand, mopidamol showed a higher

inhibitory effect on the production of enzymatic MDA as compared with dipyridamole and had no effect on FeAs-induced lipid peroxidation (as opposed to dipyridamole). These findings coincide with previous studies made by our group in different animal and human tissues [13–16], which showed that the antiperoxidative effect of dipyridamole may be explained by a scavenging action of oxygen-derived free radicals [15, 24], whereas mopidamol would inhibit lipid peroxidation by interfering in the metabolic pathway of arachidonic acid [13, 25].

It may be postulated that the inhibitory effect of mopidamol on thromboxane may be exerted through an increase in the negative regulators of thromboxane biosynthesis, cAMP and cGMP [22]. However, the dose-response curves of platelet aggregation induced by increasing concentrations of arachidonic acid in the absence and presence of mopidamol agree with antagonism of the cyclooxygenase pathway, for three reasons: (a) the presence of a linear correlation between the inhibition of arachidonic acid-induced platelet aggregation and the inhibition of the TxB_2 synthesis; (b) the arachidonic acid maximal rate of aggregation remains unchanged in the presence of mopidamol, but there is a right-parallel displacement of the dose-response curves, which would not have been observed if cyclooxygenase inhibition was exerted through an indirect mechanism via stimulation of cAMP or cGMP; (c) when arachidonic acid is used as a platelet aggregating agent in PRP, the observed rate of aggregation depends on TxA_2 synthesis [26]. Therefore, direct inhibition of some enzyme in the cyclooxygenase pathway appears to be involved in the mode of action of mopidamol.

It has been suggested that phosphodiesterase inhibitors could have an antithrombotic activity by potentiating prostacyclin synthesis [27–31]. In the present study, dipyridamole showed a potent enhancing effect on 6-keto- $\text{PGF}_{1\alpha}$ in vascular samples which, in turn, correlated with the production of MDA (inhibition of lipid peroxidation) [32–34]. In contrast, the lack of effects of mopidamol on the

production of free radicals and the smaller vascular antiperoxidative effect as compared with the concentration range that inhibited platelet aggregation or thromboxane synthesis support a mechanism of action on the pathway of arachidonic acid neither directed towards inhibition of cyclooxygenase nor to stimulation of prostacyclin biosynthesis. Therefore, it is postulated that mopidamol may exert its inhibitory effect on the synthesis of platelet thromboxane through a negative interaction with the enzyme, thromboxane synthetase, because mopidamol is not a cyclooxygenase inhibitor and vascular 6-keto-PGF_{1α} is not modified using concentrations that exert the other effects, described and observed in the present study, of mopidamol.

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