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DIPYRIDAMOLE AND VASCULAR PROSTACYCLIN PRODUCTION

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Abstract—The action of dipyridamole on the vascular production of prostacyclin (PGI_2) has been investigated. Dipyridamole (1–100 μM) did not induce a significant stimulation of PGI_2 release in any of the following experimental models: rings of rabbit aorta, cultured endothelial cells from bovine aorta or human umbilical vein, cultured explants of bovine aortic smooth muscle. The activity of known stimuli of PGI_2 release (ADP, suloctidil, serotonin) and the capacity of dipyridamole to inhibit adenosine uptake into endothelial cells were carefully checked. Pretreatment of the rabbit aorta with dipyridamole (10–100 μM) prolonged the transient stimulation of PGI_2 release induced by mechanical deendothelialization: this effect was probably due to a partial protection of the cyclooxygenase against oxidative self-inactivation. Our largely negative results are consistent with the current theory that the antiplatelet action of dipyridamole is mediated by adenosine and not by PGI_2.

Dipyridamole exerts an antiplatelet and antithrombotic action *in vivo*, in experimental animals [1] and in man [2]. It has been difficult to reproduce *in vitro* this inhibition of platelet aggregation by dipyridamole in the range of therapeutic concentrations [3]. One possible explanation for this discrepancy might be the involvement of an unstable platelet inhibitor, the effect of which would vanish *ex vivo*. Moncada and Korbut proposed indeed that dipyridamole acts by potentiating the antiplatelet effect of prostacyclin (PGI_2) [4]. On the other hand, several authors, using different experimental models and assay methods, reported that dipyridamole enhanced vascular PGI_2* biosynthesis [5–10]: the mechanism and the cellular localization of this effect were not characterized. We have re-examined this question and studied the effects of dipyridamole on PGI_2 release from the whole aorta *in vitro* (intact or deendothelialized) and from cultured vascular endothelial and smooth muscle cells.

MATERIALS AND METHODS

Preparation and incubation of aortic tissue. Rabbits weighing ±3 kg were killed by a blow on the neck, and the aorta was quickly dissected from the iliac bifurcation up to the arch, trimmed free of fat and connective tissue and cut into rings (±3 mm diameter). The rings were incubated at 37°, under constant shaking (80 rpm), in a medium of the following composition: 124 mM NaCl; 5 mM KCl; 1.25 mM $MgSO_4$; 1.45 mM $CaCl_2$; 1.25 mM KH_2PO_4; 25 mM Hepes buffer, pH 7.4; 8 mM glucose. The ratio of tissue weight to medium volume was roughly 25 mg/2 ml. In some experiments, the rings were opened by a longitudinal incision and the intimal surface of the resulting strips was scraped with a scalpel. Rings and strips were incubated for several 30-min or 60-min periods: the medium was collected and replaced at the end of each period. Within an experiment (performed with one aorta), each experimental condition was tested in duplicate or triplicate.

Preparation and culture of endothelial cells. Bovine aortic endothelial cells were obtained by mild collagenase digestion of aorta excised from freshly slaughtered cows, as previously described [11, 12]. These cells were cultured on 94 mm Petri dishes in a medium of the following composition: Dulbecco's modification Eagle's medium (DMEM: 60%), Ham F_{12} medium (20%), fetal calf serum (20%), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml). At confluency, they were detached by a 5-min incubation in a Ca- and Mg- free Hanks buffer containing trypsin (10 mg/dl) and EDTA (1 mM) and subcultured in 35 mm Petri dishes. Endothelial cells from human umbilical vein were obtained and cultured as described [13]. With both types of cells, the experiments were performed using confluent monolayers (±10^6 cells/dish) between passage 2 and 5. The culture medium was removed and, after rinsing, the cells were incubated in 1 ml Dulbecco's phosphate-buffered saline, for 20 or 30 min, or in 1 ml DMEM for several periods of 60 min.

Preparation and culture of bovine aortic media explants. After removal of the intima and the adventitia from an aorta excised from a freshly slaughtered cow, the media was cut into small fragments (1–2 mm^2). Four to five such explants (20–50 mg) were put in 60 mm Petri dishes and cultured in 2.5 ml of the same medium which was used for culturing endothelial cells (see above). After 24 hr, the medium was removed, the explants were rinsed and incubated for 3 periods of 60 min in 2.5 ml DMEM: the medium was collected and changed at the end of each period.

* Abbreviations used: PGI_2 prostacyclin; 6-K-$PGF_{1\alpha}$, prostaglandin 6-K-$F_{1\alpha}$; RIA, radioimmunoassay.

Prostaglandin radioimmunoassay (RIA). The production of PGI_2 was measured by the RIA of its stable degradation product, prostaglandin 6-keto-$F_{1\alpha}$ (6-K-$PGF_{1\alpha}$), performed directly in the incubation medium, without extraction and chromatography. A rabbit antiserum was raised against 6-K-PGF_{1a} coupled to BSA, as described [14]: the limit of detection was 16 pg and the cross-reactions were 1.2% with $PGF_{2\alpha}$, 0.3% with PGE_2 and <0.1% with thromboxane B_2. 100 μl aliquots of incubation media, [^{3}H]6-K-$PGF_{1\alpha}$ (11,000 dpm), anti 6-K-$PGF_{1\alpha}$ antiserum (final dilution: 10^{-4}) and bovine gamma globulins (0.25 g/dl) in Tris buffer (50 mM, pH 7.4) were incubated in a total volume of 0.4 ml for 60 min at room temperature. Then 0.4 ml of a cold 25% (wt/wt) solution of polyethylene glycol was added to separate bound and free antigen.

Measurement of adenosine uptake. Confluent monolayers of bovine aortic endothelial cells were incubated in Dulbecco's phosphate-buffered saline in the presence of dipyridamole or of the ethanol vehicle (0.5%) alone. After 5 min [^{3}H] adenosine (2 μCi/ml; 2 μM) was added. Aliquots of the incubation medium were removed at various times thereafter for liquid scintillation counting.

Statistical analysis. The statistical significance of observed differences was established using the analysis of variance for repeated measurements with two within factors (time period and treatment) and one grouping factor (animal). Computerizations were performed with the use of the P_2V program of the BMDP statistical software [15].

Materials. Dipyridamole was received from Boehringer-Ingelheim and suloctidil from Continental Pharma. The two drugs were added in ethanol, at a final concentration of 0.5% (v/v): the same concentration was added in the controls and it was checked that it did not interfere with PGI_2 release. ADP, serotonin and adenosine were purchased from Sigma Chem. Co. [^{3}H] adenosine and [^{3}H] 6-K-$PGF_{1\alpha}$ were obtained from Amersham. DMEM, Ham F_{12}, glutamine, penicillin, streptomycin, amphotericin B and collagenase type II were purchased from Flow Laboratories. Fetal calf serum was obtained from Gibco.

RESULTS

During a 30 min incubation with rings of rabbit aorta, dipyridamole (1–100 μM) did not produce a significant increase of PGI_2 output (Fig. 1, lower panel). Preexposure of the rings to dipyridamole did not potentiate the PGI_2 stimulation induced by ADP [16] (Fig. 1, upper panel). Repeated additions of dipyridamole during prolonged periods of incubation were also unable to elicit an increase of PGI_2 production, whereas a tremendous effect of suloctidil was observed in these experimental conditions (Boeynaems *et al.*, submitted for publication) (Fig. 2).

Dipyridamole (2–20 μM) did not stimulate the release of PGI_2 from cultured bovine aortic endothelial cells, whereas ADP was highly effective inside the same experiments [12] (Fig. 3A). Additional experiments showed that dipyridamole remained inactive at concentrations as high as 100 μM (not shown). In the same cells, dipyridamole produced its well-known inhibitory effect on adenosine uptake [17, 18] (Fig. 3B). PGI_2 release from human umbilical vein endothelial cells was not increased during a prolonged (3 hr) incubation with dipyridamole (1–100 μM), whereas suloctidil again produced a tremendous stimulation (not shown). Dipyridamole had no effect on the production of PGI_2 by cultured explants from bovine aortic media, a preparation of aortic smooth muscle, which is responsive to serotonin and serum (Demolle and Boeynaems, submitted for publication). Dipyridamole did not increase PGI_2 release *per se* (Fig. 4) nor did it potentiate the stimulatory effect of fetal calf serum (Fig. 5).

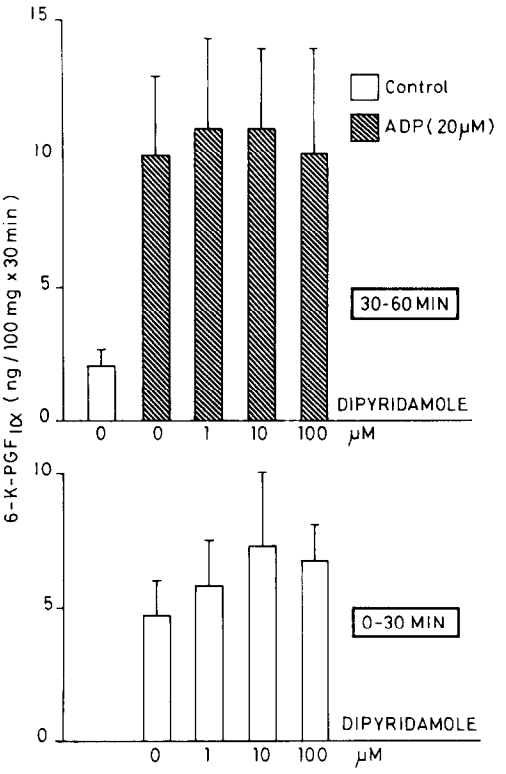

Fig. 1. Lack of dipyridamole effect on the basal and ADP-stimulated release of PGI_2 from the rabbit aorta *in vitro*. Rings of rabbit aorta were incubated for 5 periods of 30 min: the medium was changed at the end of each period. Dipyridamole was added at the beginning of the 4th and 5th periods, whereas ADP was only present during the 5th period. Results represent the amounts of 6-K-$PGF_{1\alpha}$ accumulated in the incubation medium at the end of the 4th period (lower panel: 0–30 min) and the 5th period (upper panel: 30–60 min). They are expressed as mean ± SD of 6 measurements (triplicate determinations in 2 separate experiments).

We have previously shown that the *in vitro* removal of the endothelium from the rabbit aorta induces an immediate and transient stimulation of PGI_2 release: it is believed that the rapid decline of this stimulation is due to the oxidative self-inactivation of cyclooxygenase [19]. *In vitro* pretreatment of the rabbit aorta with dipyridamole had no effect on the immediate stimulation of PGI_2 release following scraping of the endothelial cells (Fig. 6).

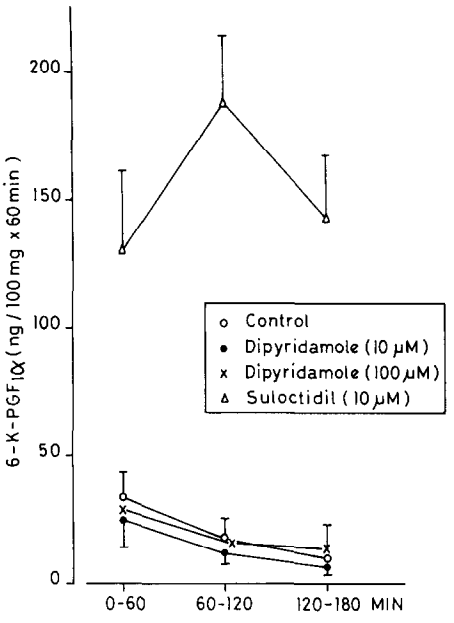

Fig. 2. Lack of dipyridamole effect on PGI_2 release from rings of rabbit aorta, during prolonged incubations. The rings were incubated for 3 periods of 60 min: the medium was changed after each period. When added, dipyridamole and suloctidil were present throughout the experiment. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the incubation medium at the end of each period (mean ± SD of 6 measurements: triplicate determinations in 2 separate experiments).

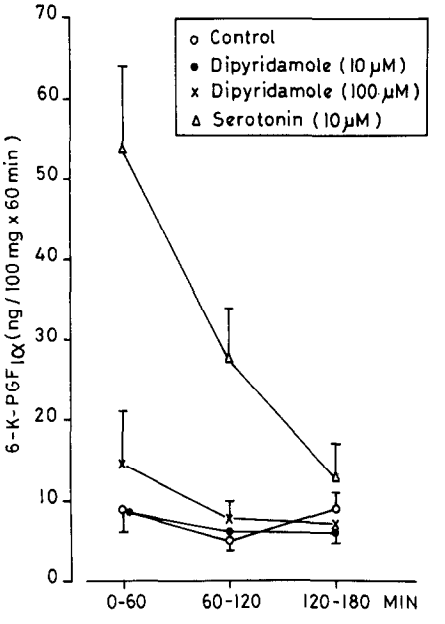

Fig. 4. Lack of dipyridamole effect on PGI_2 release from cultured explants of bovine aortic media. The explants were incubated for 3 periods of 60 min: after each incubation, the media were collected and replaced and the tested agents were readded. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of each period (mean ± SD of 6 measurements: triplicate dishes in 2 separate experiments).

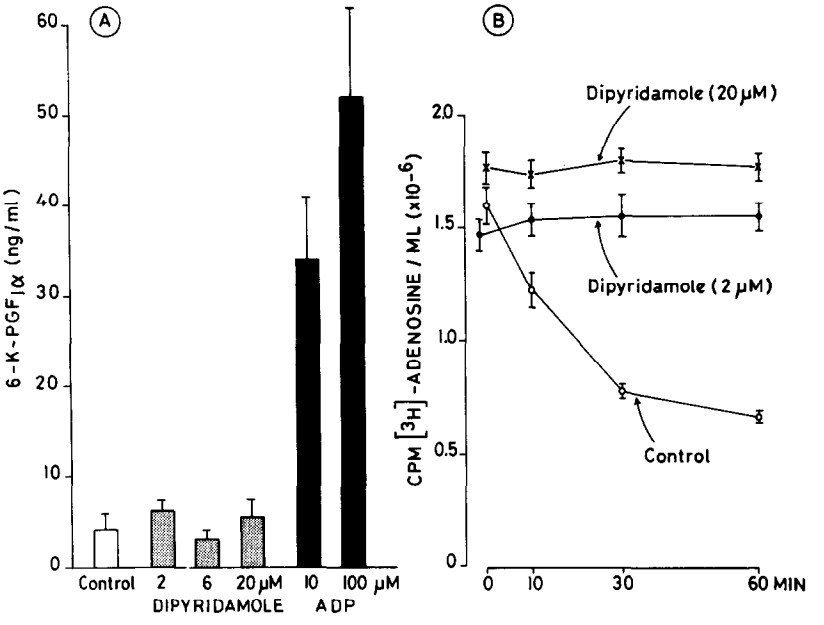

Fig. 3. (A) Lack of dipyridamole effect on PGI_2 release from bovine aorta endothelial cells. The cells were incubated for 20 min, in the presence of dipyridamole or ADP. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the incubation medium: mean ± SD of triplicate determinations in separate cell dishes, for 1 representative experiment out of 7. (B) Inhibition by dipyridamole of adenosine uptake into bovine aortic endothelial cells. Cells were incubated with [^{3}H] adenosine, as indicated under Methods. Aliquots were withdrawn at various times to measure the residual radioactivity in the incubation medium. Results represent the mean ± SD of 4 measurements (duplicate determinations in duplicate cell dishes) in 1 representative experiment out of 3. The results depicted in panels A and B of the figure were obtained on the same day, with the same preparation of cells.

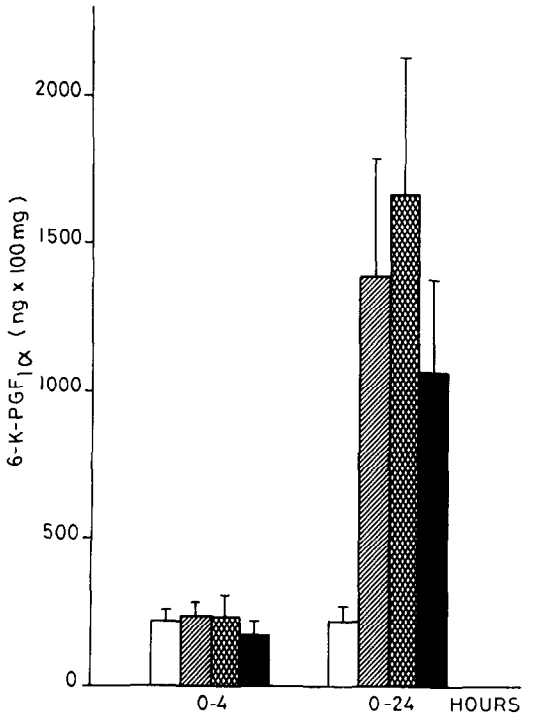

Fig. 5. Lack of dipyridamole effect on the release of PGI_2 from cultured explants of bovine aortic media stimulated by fetal calf serum (FCS). The explants were incubated for 4 hours or 24 hours in the following conditions: □, no FCS, no dipyridamole; ▨, FCS (20%), no dipyridamole; ▩, FCS (20%), dipyridamole (6 μM); ■, FCS (20%), dipyridamole (60 μM). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of the indicated period (mean ± SD of 6 measurements: triplicate dishes in 2 separate experiments).

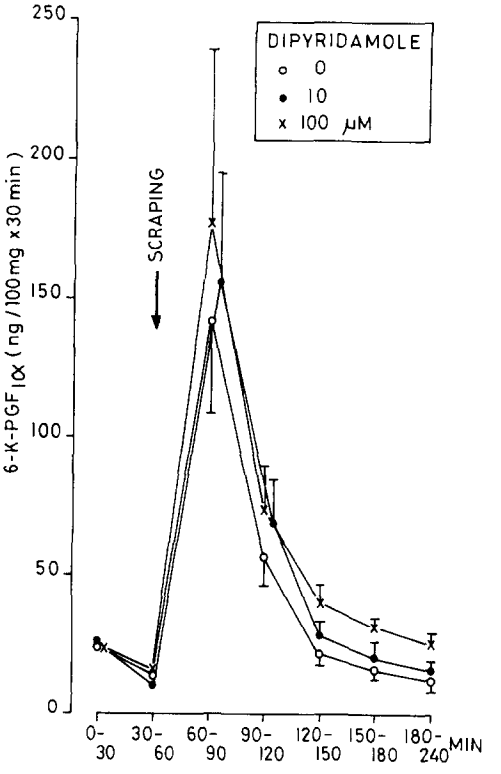

Fig. 6. Prolongation by dipyridamole of the PGI_2 release induced by endothelium removal from the rabbit aorta *in vitro*. Rings of rabbit aorta were incubated for 6 periods of 30 min: the medium was collected and replaced at the end of each period and the same concentration of dipyridamole (0, 10 or 100 μM) was readded. At the end of the 2nd period, the rings were opened by a longitudinal incision and the intimal surface of the resulting strips was scraped with a scalpel. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of each period (mean ± SD of 8 measurements: duplicate determinations in 4 separate experiments).

However, a difference between control- and dipyridamole- treated tissue became apparent later, when the production of PGI_2 had almost returned to its pre-stimulation level: starting at 120 min for at least 90 min, the aortic strips pretreated with dipyridamole (100 μM) released twice as much PGI_2 as the control strips (Fig. 6). This difference was highly significant: $P = 0.001$.

DISCUSSION

Most of our experiments with dipyridamole provided negative results. Dipyridamole, either in the range of therapeutic concentrations (2–6 μM: [3, 20]) or at much higher concentrations (up to 100 μM), was tested during periods ranging from 20 min to 3 hr. It did not stimulate the release of PGI_2 in the 4 experimental models studied: rings of rabbit aorta, cultured endothelial cells from bovine aorta, cultured endothelial cells from human umbilical vein, cultured explants of bovine aortic smooth muscle. Previously identified stimuli of PGI_2 production were used as controls in these experiments: ADP active in the rabbit aorta [16] and in bovine aortic endothelial cells [12], suloctidil active in the rabbit aorta and human umbilical vein endothelial cells (Boeynaems *et al.*, submitted for publication), serotonin active on the aortic smooth muscle (Demolle and Boeynaems, submitted for publication). Furthermore, we checked that dipyridamole produced its well-known inhibitory effect on adenosine uptake into endothelial cells [17, 18]: in the same experiments where dipyridamole completely blocked that uptake (at a concentration as low as 2 μM), no increase of PGI_2 release could be detected in response to the drug. Neri Serneri *et al.* [5, 7] reported that dipyridamole (1–10 μM) stimulated the release of PGI_2 from rings of rabbit aorta: the discrepancy between their results and ours might be due to the methodology, since these authors used a superfusion cascade technique to measure PGI_2-like activity. Bult *et al.* [9, 10] observed that dipyridamole stimulated the release of PGI_2 from rat aortic strips: although significant, this effect was of small magnitude (75% above the control at 10 μM dipyridamole). The discrepancy with our negative results might be due to a species difference or to a difference in the incubation conditions: they worked with an hypotonic buffer at alkaline pH instead of the isotonic medium buffered at pH 7.4 used in our study. Alternatively intra- and inter-animal variability might have masked a small effect

in our study: anyway the biological significance of such a small effect would be questionable, as compared to the large stimulations of PGI_2 release induced by either the physiological agonist ADP or the drug suloctidil and to the clearcut effect of dipyridamole on adenosine uptake. Finally, we can exclude neither an action of dipyridamole in other blood vessels than the aorta or the umbilical vein, nor the possibility of a delayed effect, obtained only after a prolonged exposure to the drug.

We have previously shown that the removal of the endothelium from the rabbit aorta *in vitro* induces an immediate mobilization of free arachidonic acid, resulting in a large stimulation of PGI_2 release [19]: whereas arachidonate mobilization is sustained, the increased release of PGI_2 is a transient phenomenon, probably because of the oxidative self-inactivation of cyclooxygenase, which has been observed also in other instances [19, 21–23]. The only effect of dipyridamole that we have detected is a prolongation of this increased release of PGI_2 from the deendothelialized rabbit aorta, which might result from a partial protection of the cyclooxygenase. This hypothesis is supported by the data of Marnett *et al.* [24], who showed that dipyridamole, an easily oxidized substrate, can increase PGI_2 biosynthesis in ram seminal vesicle microsomes by two mechanisms: as a peroxidase reducing substrate, it enhances the reduction of hydroperoxide inhibitors of PGI_2 synthase; as a radical scavenger, it may trap the oxidized species, formed during the reduction of prostaglandin endoperoxide G_2 into prostaglandin endoperoxide H_2, which is responsible for cyclooxygenase inactivation [21]. The relevance of this *in vitro* effect of dipyridamole to the clinical efficacy of the drug is questionable, since it was obtained in the 10–100 μM range, whereas therapeutic concentrations range from 2 to 6 μM [3, 20]. During the completion of our work, Deckmyn *et al.* reported that dipyridamole did not increase PGI_2 production by fresh rat aortic rings, but delayed the exhaustion of the PGI_2-forming capacity during repetitive incubations in Tris buffer [25]. Although it is not clear whether the PGI_2 measured in that study came from endothelial or smooth muscle cells, the kinetics and dose-dependency of the dipyridamole effect were quite similar to our data, depicted in Fig. 6.

Recently, Gresele *et al.* [3] demonstrated that dipyridamole, in the range of therapeutic concentrations, inhibits platelet aggregation measured in whole blood, but not in platelet-rich plasma. This discrepancy suggests the following mechanism: by inhibiting its uptake into erythrocytes (and also into endothelial cells, *in vivo*), dipyridamole would increase the plasma concentration of adenosine, which is a known inhibitor of platelet aggregation [26], acting via the stimulation of platelet adenylate cyclase [27]. The action of adenosine might be potentiated by a second effect of dipyridamole, the selective inhibition of two forms of cyclic nucleotide phosphodiesterase in platelets [28, 29], which might explain the synergism between dipyridamole and PGI_2 [4]. That the antiplatelet effect of dipyridamole is mediated by adenosine, and not by PGI_2, is perfectly compatible with our failure to detect a stimulation of vascular PGI_2 by the drug. The lack of a predominant involvement of PGI_2 in dipyridamole action is consistent with the known antithrombotic efficacy of the dipyridamole-aspirin combination, which has been demonstrated in experimental models [30, 31] and in human patients [32–36].

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