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EFFECT OF DIPYRIDAMOLE ON THE FORMATION OF 6-OXO-PROSTAGLANDIN $F_{1\alpha}$ BY THE RAT ISOLATED AORTA AND RAM SEMINAL VESICLE MICROSOMES

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1 Dipyridamole (1 and 10 μM) enhanced the production of 6-oxo-prostaglandin $F_{1\alpha}$ by rat aortic tissue.

2 Dipyridamole (5 to 40 μM) did not influence the PGI_2-synthetase activity in ram seminal vesicle microsomes whereas, in concentrations ranging from 100 to 200 μM, it reduced the metabolism of exogenously added arachidonic acid. The latter effect may be due to an inhibition of the cyclo-oxygenase.

Introduction

The antiplatelet agent dipyridamole, is known to inhibit phosphodiesterase in platelets (Mills & Smith, 1971) and its anti-aggregating activity has been ascribed to an increase in platelet adenosine cyclic monophosphate (cyclic AMP). Moreover, it has been suggested that this effect depends on the presence of circulating prostacyclin (PGI_2) (Moncada & Korbut 1978). In accordance with these observations a synergistic effect of dipyridamole and low doses of the cyclo-oxygenase inhibitor acetylsalicylic acid, on the inhibition of platelet functions, has been shown (Rajah, Penny, Crow, Pepper & Watson, 1979). More recently, dipyridamole has been suggested to act more directly on the arachidonic acid (AA) metabolic pathway, i.e. by inhibiting thromboxane A_2(TXA_2) production (Greenwald, Wong, Rao, Bianchine & Panganamala, 1978) as well as by stimulating PGI_2 formation (Neri Serneri, Masotti, Abbate, Poggesi, Gensini, Favilla, Galanti & Laureano, 1979; Blass, Block, Förster & Pönicke, 1980). Since it is still unclear which of these effects contribute to the antiaggregating properties of dipyridamole, its effects on the PGI_2 production were investigated in two different systems, known to possess cyclo-oxygenase and PGI_2-synthetase activity, i.e. ram seminal vesicle microsomes and the isolated aortic tissue of the rat.

Methods

Materials

Ram seminal vesicles were collected in a local slaughter-house. A particulate fraction, containing cyclo-oxygenase activity was prepared as described by Takeguchi, Kohno & Sih (1971) and stored as a lyophilized powder at − 20°C.

Radioactive [1-^{14}C]-arachidonic acid (56.5 mCi/mmol) and [5,8,9,11,12,14,15-^{3}H] 6-oxoprostaglandin $F_{1\alpha}$ (100.0 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabelled arachidonic acid (AA) was obtained from Sigma Chemical Co. (St. Louis, MO). Dipyridamole was a gift of Dr A. Goossens, Boehringer Ingelheim, Belgium. All prostaglandin standards ($PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2 and 6-oxo-$PGF_{1\alpha}$) were a gift of Dr J. Pike (Upjohn, Kalamazoo, MI, U.S.A.). Thin layer chromatography (t.l.c.) was performed on silica plates $60F_{214}$ (Merck, Darmstadt, F.R.G.). All solvents used were of analytical grade (Merck).

Incubations of ram seminal vesicle microsomes with low doses of dipyridamole

Enzymatic reactions were carried out at room temperature, in a final volume of 1 ml Tris-HCl buffer (100 mM, pH 7.5 at 25°C) for 20 min. Each tube contained 10 mg lyophilized enzyme preparation, 0.8 nmol [1-^{14}C]-AA and various concentrations of unlabelled AA (6.5, 16 and 33 μM) and of dipyridamole (5, 10, 20 and 40 μM). Dipyridamole was added in 15 μl dimethylsulphoxide (DMSO). Control incubations, in the presence of 15 μl DMSO were performed simultaneously. The reactions were stopped by acidification with HCl (0.5 N) to pH 3. Reaction products were extracted twice with 2 ml ethylacetate and the combined organic phases were evaporated under nitrogen. The residues were redissolved in 50 μl chloroform:methanol (2:1 v/v) together with authentic standards (6-oxo-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2 and PGA_2: 2 μg of each)

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and developed by t.l.c., using the organic phase of 2,2,4-trimethylpentane:ethylacetate:acetic acid: water (5:11:2:10). The different metabolites were located by radiochromatogram scanning, visualized by spraying with phosphomolybdic acid, and scraped off. The radioactivity in each zone was quantified by liquid scintillation counting.

Incubations of ram seminal vesicle microsomes with higher doses of dipyridamole

Incubations were carried out as described above, with some modifications. Dipyridamole (100, 150 and 200 μM) was added in 100 μl of a mixture of polyethylene glycol (50 g/l) and tartaric acid (2 g/l) in distilled water. The reactions were carried out in isotonic HEPES buffer solution (mM): NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 5, HEPES 10) and compared to controls containing 100 μl of the solvent mixture.

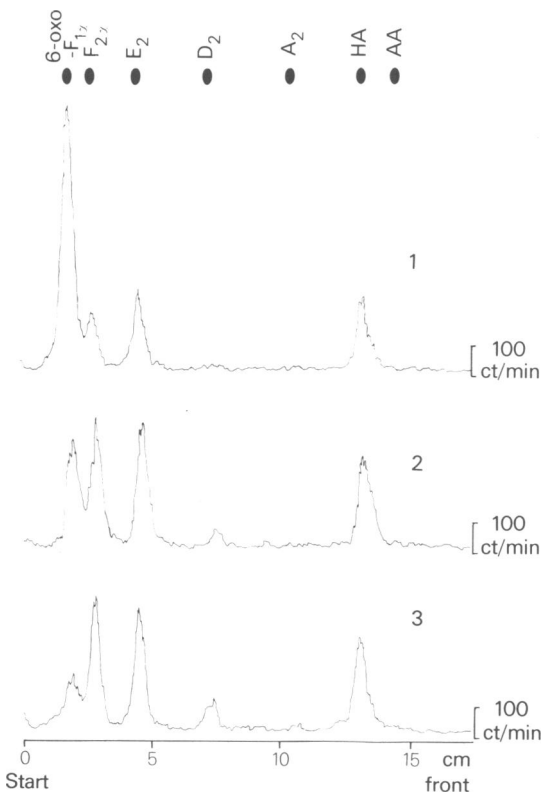

Figure 1 Radiochromatograms of incubations of ram seminal vesicle microsomes with 0.8 nmol [1-^{14}C]-arachidonic acid ([1-^{14}C]-AA) and 6.5 (1), 16 (2) and 33 (3) nmol AA in the absence of dipyridamole. 6-oxo-$F_{1\alpha}$ = 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$); $F_{2\alpha}$ = $PGF_{2\alpha}$; E_2 = PGE_2; D_2 = PGD_2; A_2 = PGA_2; HA = hydroxy-fatty acids; AA = arachidonic acid.

Measurement of endogenous 6-oxo-prostaglandin $F_{1\alpha}$ formation by rat aortic tissue

Rats (Wistar, males, 200–300 g) were killed by a blow on the head and thoracic aortae were immediately removed and placed in ice-cold Tris buffer (50 mM, pH 8.5 at 4°C). The aortae were carefully freed from adjacent tissue, opened longitudinally and cut in 3 pieces of 1 cm. The 3 pieces of each aorta were randomly placed in tubes containing 1 ml Tris buffer with 0.02% DMSO and 0, 1 or 10 μM dipyridamole.

The tissues were then preincubated in a shaking water bath at 37°C for 15 min, and, thereafter transferred to 1 ml fresh identical buffer (Tris buffer with 0.02% DMSO and 0,1 or 10 μM dipyridamole) and incubated for 15 min at 37°C. The reactions were stopped by cooling to 0°C, acidification with citric acid (2M) to pH 3 and removal of the tissues. The wet weight of the tissue pieces was determined. After addition of [^{3}H]-6-oxo-$PGF_{1\alpha}$ (1000 ct/min) the incubation fluid was extracted twice with 2 ml ethylacetate. The combined ethylacetate phase was taken to dryness under a stream of nitrogen and the residue was dissolved in 1 ml Tris buffer (50 mM, pH 7.5 at 25°C, containing 1% gelatine) and used for determination of the recovery and radioimmunoassay of 6-oxo-$PGF_{1\alpha}$. Anti 6-oxo-$PGF_{1\alpha}$ antiserum was raised in New Zealand white rabbits and the assay was run according to Salmon (1978). Cross reactivities with classical prostaglandins were 1% ($PGF_{2\alpha}$) and less than 0.1% (PGE_2,15-oxo-PGE_2).

Results

The influence of low doses of dipyridamole on the conversion of exogenous arachidonic acid by ram vesicle microsomes

An example of the reaction products formed upon incubation of ram seminal vesicle microsomes with 6.5, 16 and 33 μM AA is shown in Figure 1. Under all conditions AA was completely converted by the cyclo-oxygenase. At 6.5 μM AA the resulting prostaglandin-endoperoxide, PGH_2, was almost completely transformed by prostacyclin-synthetase and 6-oxo-$PGF_{1\alpha}$ was the major metabolite, whereas some hydroxy-fatty acids, mainly HHT, were also present. With increasing concentrations of AA, there is a relative increase of non-enzymatic PGH_2 metabolites, namely (in order of importance) $PGF_{2\alpha}$, PGE_2, HHT and PGD_2. This results in an apparent decrease in 6-oxo-$PGF_{1\alpha}$ biosynthesis with increasing AA concentrations.

In Figure 2, the quantitative formation of PGI_2 measured as 6-oxo-$PGF_{1\alpha}$ is shown. It is clear that

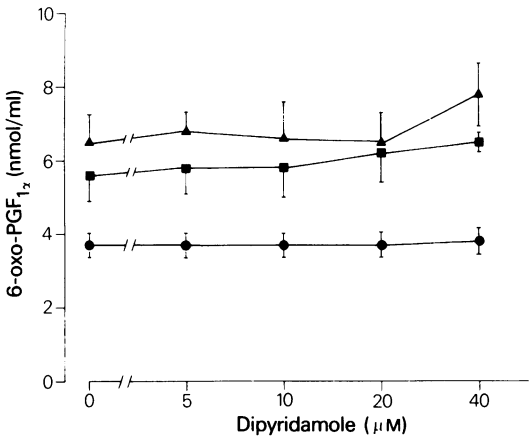

Figure 2 Effects of dipyridamole on the formation of 6-oxo-prostaglandin F$_{1\alpha}$ (6-oxo-PGF$_{1\alpha}$) by ram seminal vesicle microsomes in the presence of 0.8 nmol [1-^{14}C]-arachidonic acid ([1-^{14}C]-AA) and 6.5 (●), 16 (■) and 33 (▲) nmol AA. Data are given as mean values ($n = 6$); vertical lines show s.e.mean.

PGI_2 biosynthesis is still increasing in the substrate range used (6.5, 16 and 33 μM AA), in contrast to the impression suggested by the data from Figure 1. Dipyridamole (5 to 40 μM) did not change the 6-oxo-PGF$_{1\alpha}$ production (Figure 2) and had no influence on the qualitative nor on the quantitative pattern of the other cyclo-oxygenase metabolites at any of the AA concentrations used (results not shown).

The effect of high doses of dipyridamole on the conversion of exogenous arachidonic acid by ram seminal vesicle microsomes

In these experiments the presence of increasing concentrations of dipyridamole caused a significant, dose-dependent reduction of the conversion of AA (Table 1). This reduced conversion coincided with a decreased formation of the various AA metabolites but this was not always statistically significant. The effects of dipyridamole on the amount of unconverted AA and on the formation of 6-oxo-PGF$_{1\alpha}$ and hydroxy acids are shown in Tables 1, 2 and 3.

Measurement of the endogenous 6-oxo-prostaglandin F$_{1\alpha}$ formation by rat aortic tissue

As shown in Figure 3, 1 and 10 μM dipyridamole enhanced the absolute amounts of the 6-oxo-PGF$_{1\alpha}$ released; this was statistically significant for the higher concentration of dipyridamole. When the increase was compared with the paired control (percentage increase) statistical significance was found for both concentrations.

Figure 4 shows that a statistically significant negative correlation existed between the basal release of 6-oxo-PGF$_{1\alpha}$ and the absolute stimulation due to 1 μM dipyridamole.

Table 1 Amounts of unconverted arachidonic acid (AA) in the presence of various concentrations of dipyridamole

	Dipyridamole (μM)			
AA (nmol/ml)	0	100	150	200
6.5	0.6±0.2	1.2±0.2**	1.6±0.4**	1.9±0.3**
16	1.3±0.6	2.3±0.6*	3.3±0.8**	3.8±0.8**
33	2.6±0.7	4.1±0.2*	5.3±0.7**	5.9±0.4**

Each value is the mean ± s.e.mean (nmol/ml, $n = 4$).
Significantly different from control (0 μM dipyridamole): *$P < 0.05$; **$P < 0.01$, Student's t test.

Table 2 Effect of various concentrations of dipyridamole on the transformation of arachidonic acid (AA) to 6-oxo-prostaglandin F$_{1\alpha}$

	Dipyridamole (μM)			
AA (nmol/ml)	0	100	150	200
6.5	3.9±0.3	3.5±0.4	3.3±0.3*	3.2±0.4*
16	8.3±1.7	8.3±1.4	7.7±1.1	7.5±1.0
33	16.8±2.1	15.4±3.2	15.9±2.0	15.6±1.4

Each value is the mean ± s.e.mean (nmol/ml, $n = 4$).
Significantly different from control (0 μM dipyridamole): *$P < 0.05$, Student's t test.

Table 3 Effect of various concentrations of dipyridamole on the transformation of arachidonic acid (AA) to HHT

	Dipyridamole (μM)			
AA (nmol/ml)	0	100	150	200
6.5	1.0±0.1	0.9±0.1	0.8±0.1*	0.8±0.1*
16	2.6±0.6	2.3±0.4	2.0±0.1	1.9±0.3
33	4.9±0.5	4.2±0.4	3.8±0.5	3.2±0.8*

Each value is the mean ± s.e.mean (nmol/ml, $n = 4$).
Significantly different from control (0 μM dipyridamole): $^*P < 0.05$, Student's t test.

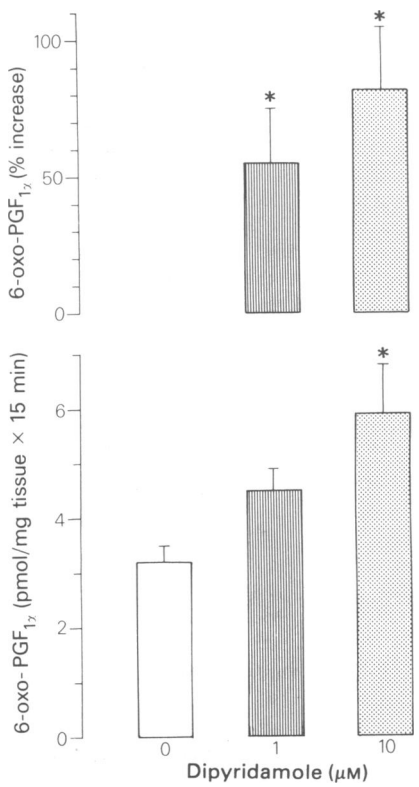

Figure 3 Effects of dipyridamole (1 and 10 μM) on the production of 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) by rat aortic tissue. Lower half, absolute amounts of 6-oxo-$PGF_{1\alpha}$ (pmol/mg) formed in 15 min. Upper half, increase in 6-oxo-$PGF_{1\alpha}$ production in the presence of dipyridamole, expressed as percentage of the paired control. Data are given as mean values ($n = 10$); vertical lines show s.e.mean; $^*P < 0.05$, Wilcoxon test for paired samples, two sided, dipyridamole-treated versus control.

Discussion

By measuring the endogenous release of 6-oxo-$PGF_{1\alpha}$, as an indication of the production of PGI_2 by rat aortic tissue, it was found that dipyridamole stimulated the release of 6-oxo-$PGF_{1\alpha}$. This effect of dipyridamole was demonstrated in a concentration range (1 to 10 μM) which could be of clinical interest. It is in agreement with the stimulation of the release of PGI_2-like material, assessed by bioassay, by rabbit aortic tissue in the presence of similar concentrations of dipyridamole (Neri Serneri *et al.*, 1979).

The negative correlation between the basal release of 6-oxo-$PGF_{1\alpha}$, and the enhancement of its formation in the presence of 1 μM dipyridamole suggests that the effect of dipyridamole on PGI_2 biosynthesis is greater when the basal PGI_2 release is rather low. The increased 6-oxo-$PGF_{1\alpha}$ levels may be caused by a stimulation of PGI_2 production or an inhibition of its metabolic degradation.

In order to investigate whether dipyridamole

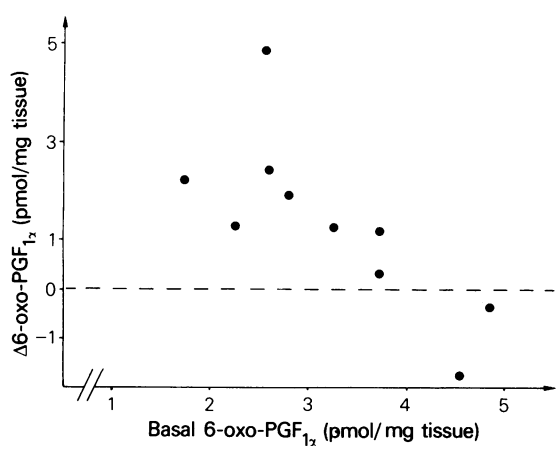

Figure 4 Relationship between the basal release of 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) in the control aorta and the increase due to 1 μM dipyridamole. A statistically significant negative correlation was present ($P < 0.05$, Spearman test).

stimulated PGI_2 synthetase directly, its effects on the conversion of AA to PGI_2 by microsomes from ram seminal vesicles were studied. This particulate fraction contains both cyclo-oxygenase and PGI_2-synthetase activities, and in this system the latter enzyme is the rate-limiting step in the overall conversion of AA to PGI_2 (Beetens, Claeys & Herman, 1981). In the range of AA concentrations used (6.5–33 μM), all the substrate was converted to prostaglandin endoperoxides; at 6.5 μM AA, the resulting PGH_2 was subsequently almost completely transformed to PGI_2, but with 16 and 33 μM AA, PGI_2-synthetase was unable to cope with all the PGH_2 supplied by the cyclo-oxygenase activity. Thus, the formation of 6-oxo-PGF$_{1\alpha}$ still increased with the rise in AA concentrations (Figure 2), but the proportion of non enzymatic PGH_2 metabolites became more and more important (Figure 1). If dipyridamole had raised the turnover rate (maximum velocity) of PGI_2-synthetase or prevented the inactivation of the enzyme, an increased biosynthesis of PGI_2 was to be expected at the highest concentrations of AA. Under similar conditions, vitamin C promotes PGI_2 biosynthesis by a reduction of the inactivation of PGI_2-synthetase by active oxygen species derived from PGG_2 (Beetens *et al.*, 1981). However, dipyridamole (5 to 40 μM) failed to stimulate PGI_2-synthetase in this subcellular system. This result is at variance with the small stimulation (21 ± 7%) of the conversion of a relatively low concentration of exogenous AA to prostacyclin by homogenates of the rat stomach fundus in the presence of 10 μM dipyridamole (Blass *et al.*, 1980). However, the enzyme activities in the latter preparation are less well characterized, and apart from cyclo-oxygenase and PGI_2-synthetase, various other enzymes, such as acylhydrolases (eg. phospholipase A_2), fatty acyl CoA transferases, and prostaglandin-metabolizing enzymes are likely to be present. Furthermore, significant amounts of endogenous AA could interfere with the conversion of the exogenously added substrate. Therefore, it is impossible to distinguish between stimulation of PGI_2-synthetase activity (Blass *et al.*, 1980) and one of the numerous other mechanisms that could explain an increased formation of 6-oxo-PGF$_{1\alpha}$ in the presence of dipyridamole.

Higher concentrations of dipyridamole (100 μM and more) are reported to stimulate the formation of 6-oxo-PGF$_{1\alpha}$ in rat stomach homogenates more substantially, and 100 μM dipyridamole enhanced the conversion of PGH_2 to prostacyclin by pig aortic microsomes (Blass *et al.*, 1980). Therefore, the effects of high concentrations of dipyridamole (100–200 μM) on the conversion of AA to PGI_2 by ram seminal vesicles have also been studied, although it is clear that these concentrations are at least one order of magnitude higher than those found in human plasma after ingestion of 100 mg dipyridamole, even in the form of a sustained release preparation (Rajah *et al.*, 1979, Chevolet, Van de Velde, Weisenberger, Herman & Dresse, 1981).

The experiments had to be carried out in HEPES buffer, since 100 μM dipyridamole neither dissolved in 50 μM Tris buffer, nor in the 25 mM phosphate buffer used for experiments with pig aortic microsomes (Blass *et al.*, 1980). In HEPES buffer about 8% of the AA was not converted to prostaglandin endoperoxides, and with increasing dipyridamole doses (from 100 μM) the amount of unconverted AA increased up to about 26% (see Table 1), indicating that these high concentrations did not stimulate, but inhibited the cyclo-oxygenase reaction to some extent. As a result, the formation of 6-oxo-PGF$_{1\alpha}$, as well as the non-enzymatic PGH_2 metabolites decreased proportionally, but again a shift in favour of PGI_2 was not observed. A similar action could explain the decreased TXA_2 formation by platelets in the presence of high dipyridamole concentrations (Greenwald *et al.*, 1980).

Thus, dipyridamole does not promote the activity of PGI_2-synthetase, and it is unlikely that it enhances cyclo-oxygenase activity (cf. Table 1). Recently, it has been shown that dipyridamole interferes with the uptake of PGE_2 by hamster isolated perfused lungs, but does not inhibit 15-hydroxyprostaglandin dehydrogenase (Uotila & Männistö, 1981). In conclusion, enhanced availability of endogenous AA probably explains the increased release of 6-oxo-PGF$_{1\alpha}$ by rat aortic tissue, although the possibility that dipyridamole interferes with the uptake of PGI_2 by vascular tissue has still to be investigated.

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