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STIMULATION OF VASCULAR PROSTACYCLIN SYNTHESIS BY EXTRACELLULAR ADP AND ATP

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SUMMARY: Extracellular ADP and ATP stimulated the synthesis of prostacyclin - as reflected by the release of 6-keto-$PGF_{1\alpha}$ - in the rabbit aorta, the rabbit pulmonary artery and the rat aorta. A doubling of 6-keto-$PGF_{1\alpha}$ output was produced by 3 μM ADP. Adenosine had no effect and the stimulation by ADP was blocked by quinidine, but not by theophylline. This stimulation was abolished by indomethacin and lost after mechanical removal of the endothelium. Stimulation of vascular prostacyclin synthesis by ADP released from aggregating platelets could help localize thrombus formation to areas of vascular damage.

Prostacyclin is a potent inhibitor of platelet aggregation produced by vascular endothelial and smooth muscle cells. Stimulation of vascular prostacyclin synthesis by mediators generated or released during the hemostatic response could constitute a potentially useful feedback mechanism, limiting the number of platelets involved and helping to localize thrombus formation. So far three such mediators have been identified : thrombin (1, 2), platelet-derived growth factor (3) and serotonin (4). In this paper, we show that the platelet secretory product ADP is a potent stimulator of prostacyclin synthesis by the rabbit aorta and pulmonary artery. This action is shared by ATP and might involve a P_2-purinergic receptor (5).

MATERIALS AND METHODS

White male rabbits weighing around 3 kgs and white male rats (Sprague-Dawley) weighing around 250 g were sacrificed by a blow on the neck. The aorta was quickly dissected from the iliac bifurcation up to the arch. In some experiments, the extrapulmonary portion of the pulmonary artery was also

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removed. The vessels were trimmed free of fat and connective tissue and cut into rings (± 3 mm large). The incubation of the rings was performed at 37°C, under constant shaking (80 rpm), in a medium of the following composition : NaCl, 124 mM; KCl, 5 mM; $MgSO_4$, 1.25 mM; $CaCl_2$, 1.45 mM; KH_2PO_4, 1.25 mM; Hepes buffer pH 7.4, 25 mM; glucose, 8 mM. Usually the rings were incubated for 5 periods of 30 min : the medium was renewed at the end of each period. Since the basal output of 6-K-$PGF_{1\alpha}$ was very high initially and reached a steady-state after 120 min, stimulating agents were added during the last period. Inside an experiment performed with one animal, each condition was tested in duplicate or triplicate.

The production of prostacyclin was assessed by the radioimmunoassay (RIA) of 6-keto-$PGF_{1\alpha}$ (6-K-$PGF_{1\alpha}$), which was performed directly in the incubation medium, without extraction and chromatography. The following reagents were added in polypropylene tubes :
- 0.1 ml of [^{3}H]-6-K-$PGF_{1\alpha}$ (22,000 dpm) in Tris buffer 50 mM, pH 7.4;
- 0.1 ml of a 1 g/dl solution of bovine gamma globulin in Tris buffer 50 mM, pH 7.4;
- 0.1 ml of incubation medium sample diluted 5-fold with Tris buffer 50 mM, pH 7.4 or 0.1 ml of 6-K-$PGF_{1\alpha}$ standard prepared in the same buffer;
- 0.1 ml of an antiserum solution in Tris buffer 50 mM, pH 7.4, binding 30 % of the tracer.

This reaction mixture was left for 1 hour at room temperature. Then 0.4 ml of a cold 25 % (w/w) solution of polyethylene glycol 6000 was added. After vigorous shaking, the tubes were centrifuged for 20 min at 3,000 g. The supernatants were removed by aspiration and the pellets were dissolved in 0.1 ml NaOH 0.1 M. 2 ml of Optifluor scintillation liquid were then added and the tubes were counted in a liquid scintillation counter. A similar procedure was used to assay PGE_2 and $PGF_{2\alpha}$, except that the samples were not diluted before the assay.

Reversed-phase-high-performance liquid chromatography (RP-HPLC) was performed on a u Bondapak C_{18} column (3.9 x 300 mm, 10 u particles, Waters Associates). The injector (U6K) and the pump (6000A) were from Waters Associates. Elution was performed with a mixture of acetonitrile-water-acetic acid (26:74:0.1, v/v) at a flow rate of 2 ml/min. 2 ml fractions were collected and lyophilised before the RIA of 6-K-$PGF_{1\alpha}$.

ATP, ADP, AMP, AMP-PNP, GTP and adenosine were purchased from either Sigma Chem. Co. or Boehringer-Mannheim. 6-K-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ were purchased from Upjohn Diagnostics. ^{3}H-labeled tracers of PGE_2, $PGF_{2\alpha}$ and 6-K-$PGF_{1\alpha}$ were obtained from Amersham. Antisera against PGE_2 and $PGF_{2\alpha}$ were purchased respectively from Institut Pasteur de Paris and Clinical Assays. The antiserum against 6-K-$PGF_{1\alpha}$ was a generous gift of Dr A. Herman (Universitaire Instelling Antwerpen, Antwerp). Optifluor scintillation fluid was obtained from Packard Instrument.

RESULTS

The addition of ADP rapidly increased the release of 6-K-$PGF_{1\alpha}$ from rings of rabbit aorta (fig. 1). In order to confirm that the product formed in response to ADP was authentic 6-K-$PGF_{1\alpha}$, RIA was repeated after RP-HPLC purification of the samples : all the immunoreactivity was indeed recovered in the fractions where a standard of 6-K-$PGF_{1\alpha}$ is eluted (fig. 1, inset). A dose-response curve of the stimulatory effect of ADP is shown in figure 2.

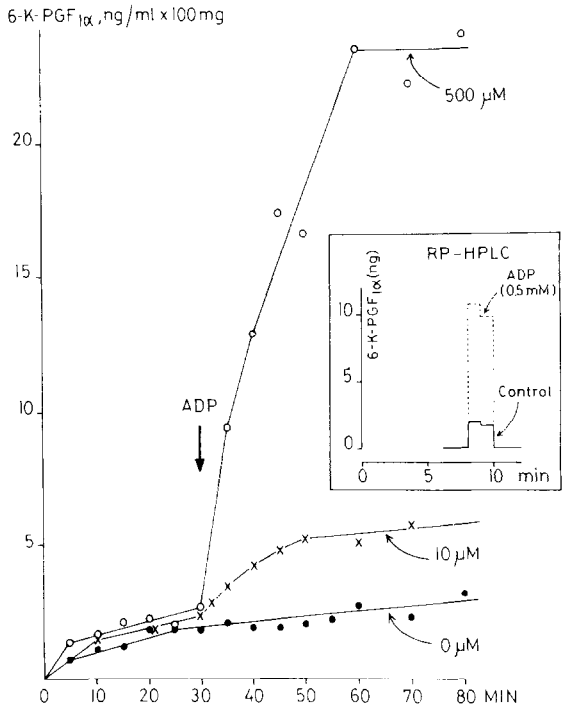

Figure 1. Kinetics of the ADP effect on 6-K-$PGF_{1\alpha}$ release by rings of rabbit aorta. Rings of rabbit aorta were incubated in separate flasks : after 30 min, ADP was added at a 10 μM or a 0.5 mM final concentration. Aliquots of the incubation medium were removed every 5 min and assayed for 6-K-$PGF_{1\alpha}$. Inset : 1 ml of incubation medium was removed at time 90 min from the control and the 0.5 mM ADP-stimulated flasks and injected on a C_{18} u Bondapak column; RIA of 6-K-$PGF_{1\alpha}$ was performed in the various fractions collected; [^{3}H]-6-K-$PGF_{1\alpha}$ used as standard was recovered in fractions 9 and 10.

The mean increase above the control was 100 % ± 24 % at 3 μM ADP (n=5), 190 % ± 47 % at 10 μM (n=5), 460 % ± 57 % at 20 μM (n=9) and 710 % ± 25 % at 0.5 mM (mean ± SE, n = number of experiments). ADP also stimulated the release of PGE and $PGF_{2\alpha}$, but the amounts produced were much lower than those of 6-K-$PGF_{1\alpha}$ (fig. 2). ATP and its non-hydrolysable analog adenylyl-imido-diphosphate (AMP-PNP) produced the same maximal effect as ADP : 0.5 mM ATP increased 6-K-$PGF_{1\alpha}$ release to 670 % ± 60 % of the control (mean ± SE, 13 experiments). On the contrary, 5'-AMP, GTP and adenosine were completely inactive (fig. 3). The increased release of 6-K-$PGF_{1\alpha}$ triggered by ADP was abolished without delay by indomethacin (fig. 4, right part). The stimulatory effect of ADP was inhibited by quinidine (fig. 4, left part) and was unaffected by theophylline 1 mM (not shown). Stimulation of 6-K-$PGF_{1\alpha}$ output by

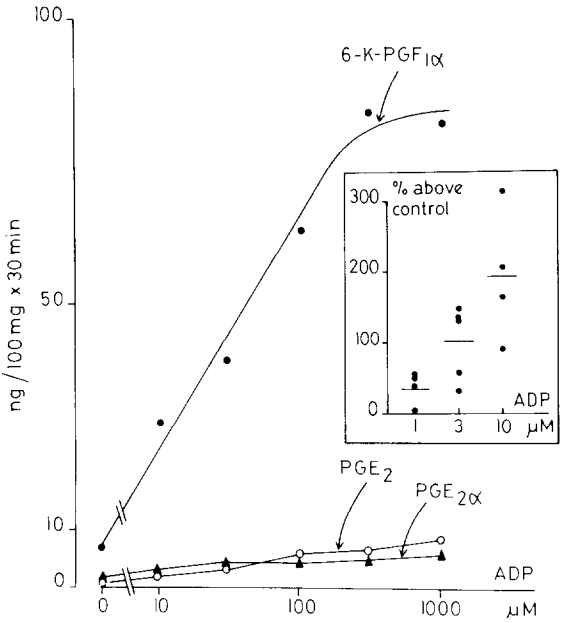

Figure 2. Stimulation by ADP of 6-K-$PGF_{1\alpha}$ release from rings of rabbit aorta : dose-response curve. Results are the mean of duplicate determinations, from 1 representative experiment out of 5. Inset : the effect of low concentrations of ADP was tested in triplicate in 5 experiments; the mean 6-K-$PGF_{1\alpha}$ release in each experiment is represented as % above the control value.

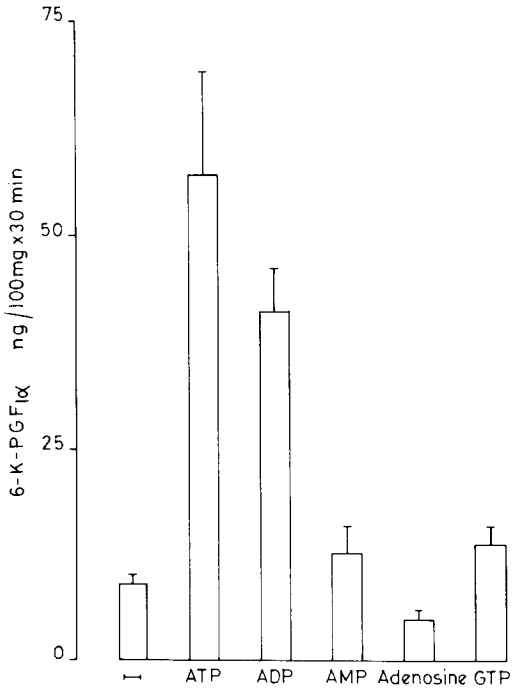

Figure 3. Specificity of the purinergic stimulation of 6-K-$PGF_{1\alpha}$ production by rings of rabbit aorta. All the compounds tested were present at a 0.5 mM final concentration. Results are expressed as mean ± SD of 6 determinations from 3 distinct experiments.

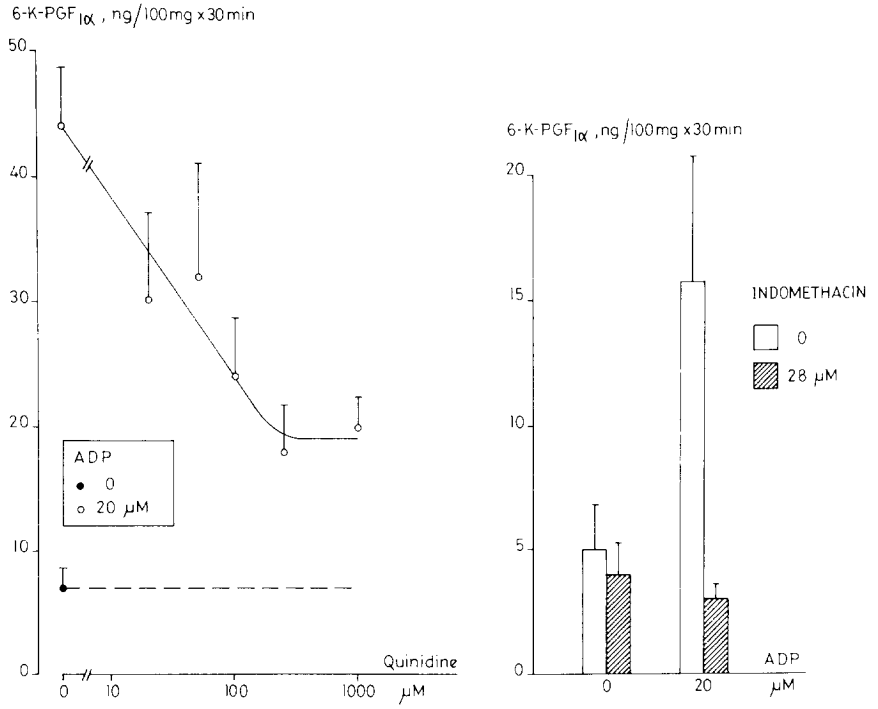

Figure 4. Left part. Dose-dependent inhibition by quinidine of the stimulatory effect of ADP on 6-K-$PGF_{1\alpha}$ release by the rabbit aorta. Results are expressed as the mean ± SD of 8 determinations in 4 distinct experiments.

Right part : Suppression by indomethacin of the increased output of 6-K-$PGF_{1\alpha}$ induced by ADP. Rings of rabbit aorta were incubated for 5 periods of 30 min and the medium was renewed at the end of each period. Indomethacin was added at the beginning of the last incubation period and ADP was added 5 min later. Results express the release of 6-K-$PGF_{1\alpha}$ during this period (mean ± SD of 6 determinations in 2 experiments).

ADP and ATP was completely lost in a calcium-free medium and after mechanical removal of the endothelium (rubbing of the intimal surface against filter paper) (not shown). ADP (0.5 mM) produced a 5-fold increase of 6-K-$PGF_{1\alpha}$ release from the extrapulmonary part of the rabbit pulmonary artery (mean of 3 experiments). In the rat aorta, the production of 6-K-$PGF_{1\alpha}$ was increased to 270 % of the control by ADP (0.5 mM) and to 900 % of the control by ATP (0.5 mM) (mean of 4 experiments).

DISCUSSION

We have shown that, in the aorta and the pulmonary artery of the rabbit and the aorta of the rat, extracellular ADP and ATP stimulate the release of

6-K-$PGF_{1\alpha}$. The rapid and complete inhibition of this increased release by indomethacin suggests that it results from an enhanced synthesis of prostacyclin. This effect does not involve a phosphorylation reaction, since AMP-PNP, the non-hydrolysable analog of ATP, is also active. The stimulation of prostacyclin synthesis by ADP might be classified as a P_2-purinergic response, since adenosine is inactive and the stimulation is unaffected by theophylline (5) : the inhibition of the response by quinidine is consistent with that hypothesis, although this drug cannot be considered as a specific antagonist of purinergic receptors (5). A purinergic stimulation of prostaglandin synthesis has indeed been observed in several organs (6, 7). The stimulatory effect of adenine nucleotides on 6-K-$PGF_{1\alpha}$ release is lost after removal of the endothelium, suggesting that the purinergic response occurs in endothelial cells. It has been shown that the relaxing effect of ATP and ADP on isolated arteries involves an inhibitory signal generated by endothelial cells (8-10). These two actions of adenine nucleotides-increased release of 6-K-$PGF_{1\alpha}$ and endothelium - dependent relaxation - are characterized by similar dose-response curves, by the same agonist specificity (inactivity of adenosine) and both are insensitive to theophylline (9). However, prostacyclin cannot be the mediator of the purinergic relaxation, since this relaxation is maintained in the presence of indomethacin (10).

ADP is contained in the dense granules of the platelets, from which it is secreted during the release reaction. It appears now that this released ADP can exert two opposite actions on platelet aggregation : a direct stimulation and an inhibition mediated by prostacyclin released from the vascular wall. ADP might thus be part of a negative feedback loop limiting the extent of platelet aggregation and helping to localize the thrombus to areas of vascular damage. Two other platelet secretory products - platelet-derived growth factor and serotonin - and thrombin can also increase the vascular production of prostacyclin. Moreover, in some experimental conditions, vascular endothelial cells can convert prostaglandin endoperoxides released from platelets into prostacyclin (11). The redundancy of these control

mechanisms might be only apparent, since they would not all operate at the same sites : serotonin is active on smooth muscle cells and not on endothelial cells (4), whereas thrombin stimulates prostacyclin synthesis in endothelial cells from umbilical veins, but not from aorta (12).

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REFERENCES

1. Weksler, B.B., Ley, C.W. and Jaffe, E.A. (1978) J. Clin. Invest. 62,923.
2. Czervionke, R.L., Smith, J.B., Hoak, J.C., Fry, G.L. and Haycraft, D.L. (1979) Thromb. Res. 14, 781.
3. Coughlin, S.R., Moskowitz, M.A., Zetter, B.R., Antoniades, H.N. and Levine, L. (1980) Nature 288, 600.
4. Coughlin, S.R., Moskowitz, M.A., Antoniades, H.N. and Levine, L. (1981) Proc. Natl. Acad. Sci. USA 78, 7134.
5. Burnstock, G. (1981) J. Physiol. 313, 1.
6. Needleman, Ph., Minkes, M.S. and Douglas, J.R. (1974) Circ. Res. 34, 455.
7. Anderson, G.F. (1982) J. Pharmacol. Exp. Ther. 220, 347.
8. Furchgott, R.F. (1981) TIPS , 173.
9. De Mey, J.G. and Vanhoutte, P.M. (1981) J. Physiol. 316, 347.
10. De Mey, J.G., Claeys, M. and Vanhoutte, P.M. J. Pharmacol. Exp. Ther. in press.
11. Marcus, A.J., Weksler, B.B., Jaffe, E.A. and Broekman, M.J. (1980) J. Clin. Invest. 66, 979.
12. Goldsmith, J.C. and Kisker, C.T. (1982) Thromb. Res. 25, 131.