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In vivo interaction of prostacyclin with an inhibitor of cyclic nucleotide phosphodiesterase, HL 725

Harald Darius, Allan M. Lefer, István Leprán[1] & J. Bryan Smith*

Department of Physiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and Specialized Center for Thrombosis Research*, Temple University, Philadelphia, Pennsylvania 19140

1 Prostacyclin (PGI_2) inactivates platelets by stimulation of adenylate cyclase, and its effect can be potentiated *in vitro* by simultaneous inhibition of cyclic AMP phosphodiesterase.

2 The interaction of synthetic PGI_2 and the potent phosphodiesterase inhibitor HL 725 was studied in a model of systemic platelet activation by intravenous injection of collagen. Platelet aggregate formation was evaluated by continuous on-line measurement of the circulating platelet count.

3 Collagen injection in rabbits receiving vehicle caused a 30 ± 3% decrease in the circulating platelet count. Infusion of PGI_2 (0.05, 0.1 and 0.75 $\mu g\,kg^{-1}\,min^{-1}$) dose-dependently inhibited this decrease. HL 725 (0.5, 1 and 3 $\mu g\,kg^{-1}\,min^{-1}$) caused a slight but significant effect.

4 Combinations of PGI_2 and HL 725, respectively, at 0.25 + 1.0 and 0.1 + 0.5 $\mu g\,kg^{-1}\,min^{-1}$ inhibited platelet aggregate formation to a greater extent than when either substance was used alone and produced a comparable inhibition to PGI_2 at 0.75 $\mu g\,kg^{-1}\,min^{-1}$.

5 Collagen induced an acute fall in the mean arterial blood pressure (MABP) which also was inhibited by PGI_2, HL 725 and their combinations.

6 The infusion of a combination of PGI_2 and HL 725 before collagen produced a decrease in the MABP which was greater than when either compound was used on its own.

7 Thus, PGI_2 and the phosphodiesterase inhibitor HL 725 interact *in vivo* to inhibit platelet aggregation and lower MABP.

Introduction

Prostacyclin (PGI_2) and its synthetic derivatives are under experimental and clinical investigation as antithrombotic and platelet preserving compounds. In particular, Ubatuba *et al.* (1979) demonstrated the effective inhibition of arterial thrombosis by PGI_2 and Longmore *et al.* (1981) showed that PGI_2 enhances platelet survival in extracorporeal circulatory devices in man. Additionally, therapeutic administration of PGI_2 is being tried in various diseases where PGI_2 deficiency and platelet activation may play a role (Lewis & O'Grady, 1981). A major problem with this approach is the vasodilator activity of PGI_2 which can lead to a marked decrease in arterial blood pressure. In human trials, the necessary blood concentrations of PGI_2 are in a range where only mild blood pressure effects occur, but its therapeutic usefulness is limited by facial flushing, restlessness, nausea and vomiting (Data *et al.*, 1981).

It is currently postulated that the primary mechanism of platelet inactivation is by intracellular adenosine 3′:5′ cyclic monophosphate (cyclic AMP) accumulation (Mills & Smith, 1971), and that PGI_2 increases platelet cyclic AMP by adenylate cyclase activation (Gorman *et al.*, 1977). In addition to adenylate cyclase, platelet cyclic AMP is also dependent on the activity of the cyclic AMP degradatory enzyme phosphodiesterase (Vigdahl *et al.*, 1971). PGI_2 and phosphodiesterase inhibitors act synergistically to increase platelet cyclic AMP (Gorman *et al.*, 1977) and to inhibit platelet aggregation *in vitro* (Jorgensen *et al.*, 1979).

Moncada & Korbut (1978) suggested that the platelet inactivating properties of phosphodiesterase inhibitors (e.g., dipyridamole and theophylline) depend on the activation of adenylate cyclase by endogenous circulating PGI_2 (Moncada *et al.*, 1978). However, more recent results could not confirm PGI_2 as a circulating hormone in concentrations sufficient to affect platelets systemically (Smith *et al.*, 1978;

[1]Present address: Department of Pharmacology, Medical University of Szeged, Szeged, Hungary.

Christ-Hazelhof & Nugteren, 1981; Blair *et al.*, 1982).

In this investigation, the *in vivo* interactions of synthetic PGI_2 and the new and the potent phosphodiesterase inhibitor 9,10-bimethoxy-3-methyl-2-mesityl-imino-3,4,6,7-tetrahydro-2-H-pyrimido(6,1-A)-isochinoline-4-on-hydrochloride (HL 725; Hoechst, Frankfurt, W. Germany) (Ruppert & Weithmann, 1982) were studied. The experiments were performed in anaesthetized rabbits which were challenged by intravenous injections of collagen. Drug effects on platelet aggregate formation were measured by continuous on-line platelet counting using a previously described technique (Smith & Freuler, 1973).

Methods

Male New Zealand white rabbits weighing 2.5–3.2 kg were used in this study. After induction of anaesthesia by intravenous pentobarbitone (30 mg kg^{-1}), a tracheotomy was performed and animals were allowed to breathe spontaneously. The left femoral artery was prepared for measurement of mean arterial blood pressure (MABP) by a Statham P-23 Db pressure transducer. A double lumen cannula was placed in the right femoral vein which allowed permanent blood drawing at a constant rate of 0.1 ml min^{-1}. Sodium citrate (3.8%) at a rate of 0.015 ml min^{-1} was delivered to the tip of the cannula through a second channel, so that the blood was anticoagulated immediately after leaving the animal (Smith & Freuler, 1973). The citrated blood was diluted with a mixture of ammonium oxalate (1%) and saponin (0.002%) to lyse the red cells. The number of platelets was counted by light refraction in a continuous flow cell and recorded on a precalibrated strip chart recorder. The apparatus used was an Autocounter (Technicon, Tarrytown, NY, U.S.A.) and was calibrated by Quanticel Platelet Reference (Cooper Biomedical, Malvern, PA, U.S.A.).

After a stabilization period of 20–30 min, platelet count recorded in the control experiments remained unaltered for at least 3 h. Platelets were stimulated *in vivo* by three consecutive intravenous bolus injections of collagen (100 $\mu g\,kg^{-1}$) (Type I, Chronolog Corp., Havertown, PA, U.S.A.) every 40 min. Continuous infusion of HL 725 (0.2, 0.5, 1 or 3 $\mu g\,kg^{-1}\,min^{-1}$) with or without PGI_2 (0.05, 0.1, 0.25 or 0.75 $\mu g\,kg^{-1}\,min^{-1}$) via the ear veins started 30 and 10 min before the first collagen challenge, respectively. Both infusions were terminated 10 min after the first collagen injection. In control experiments, the respective solvents (0.9% w/v NaCl solution for HL 725 or 1 mM Na_2CO_2 for PGI_2) were infused at the appropriate rate (i.e., 8 $\mu l\,min^{-1}$).

The data were calculated as means ± standard errors of the mean (s.e.means) for n observations. After validation of standard normal distribution by F test, further statistical analysis was performed by two-tailed Student's t test for independent means unless otherwise stated. P values of less than 0.05 were regarded as statistically significant.

Results

Three consecutive injections of collagen (100 $\mu g\,kg^{-1}$) led to a 30 ± 3, 29 ± 2 and 25 ± 2% decrease in circulating platelet count ($n = 9$). During the 40 min period after each injection, the platelet count recovered to a mean of 94.4 ± 1.4% ($n = 27$) of the preinjection value. Infusion of PGI_2 reduced the decrease in platelet count in a dose-dependent manner. PGI_2 at 0.75 $\mu g\,kg^{-1}\,min^{-1}$ reduced the decrease in platelet count during the first challenge to 9 ± 2% ($P < 0.01$). Also, the responses to the second and third collagen injections were significantly diminished although PGI_2 infusions had been terminated before that time. Infusion of PGI_2 at a rate of 0.1 $\mu g\,kg^{-1}\,min^{-1}$ caused significant inhibition of the decrease of the platelet count only with the first collagen injection ($P < 0.05$), whereas an infusion rate of 0.05 $\mu g\,kg^{-1}\,min^{-1}$ was without significant effect (Figure 1).

In preliminary experiments, it was confirmed that HL 725, like other phosphodiesterase inhibitors, acted synergistically with PGI_2 to inhibit collagen-induced platelet aggregation *in vitro*.

Treatment of rabbits with HL 725 alone at 0.2, 0.5 and 3 $\mu g\,kg^{-1}\,min^{-1}$ was followed by a dose-dependent diminution of collagen effects which was statistically significant only for the highest dose used. In Figure 2, the effect of PGI_2 and HL 725 infusions during the first collagen challenge are compared to that of the vehicle.

Combined infusion of HL 725 and PGI_2 during the first collagen injection resulted in a significant attenuation of the decrease in platelet count. The combinations of PGI_2 0.25 and HL 725 1.0 $\mu g\,kg^{-1}\,min^{-1}$ and of PGI_2 0.1 and HL 725 0.5 $\mu g\,kg^{-1}\,min^{-1}$ reduced the collagen induced decrease in platelet count to 9 ± 3 and 8 ± 2% of initial value, respectively ($P < 0.01$ *versus* vehicle). Further reduction of the PGI_2 infusion rate to 0.05 $\mu g\,kg^{-1}\,min^{-1}$ in combination with HL 725, 0.5 $\mu g\,kg^{-1}\,min^{-1}$, showed a diminished protective effect, although inhibition was still detectable when compared to vehicle controls ($P < 0.05$) (Figure 3).

Figure 4 shows typical recordings of alterations in circulating platelet count during the first collagen injection. Both the groups of animals receiving only the vehicle or PGI_2 (0.1 $\mu g\,kg^{-1}\,min^{-1}$) alone, exhibited a marked fall in the number of circulating platelets. In contrast, when the combination of PGI_2 (0.1 $\mu g\,kg^{-1}\,min^{-1}$) with HL 725 (0.5 $\mu g\,kg^{-1}\,min^{-1}$)

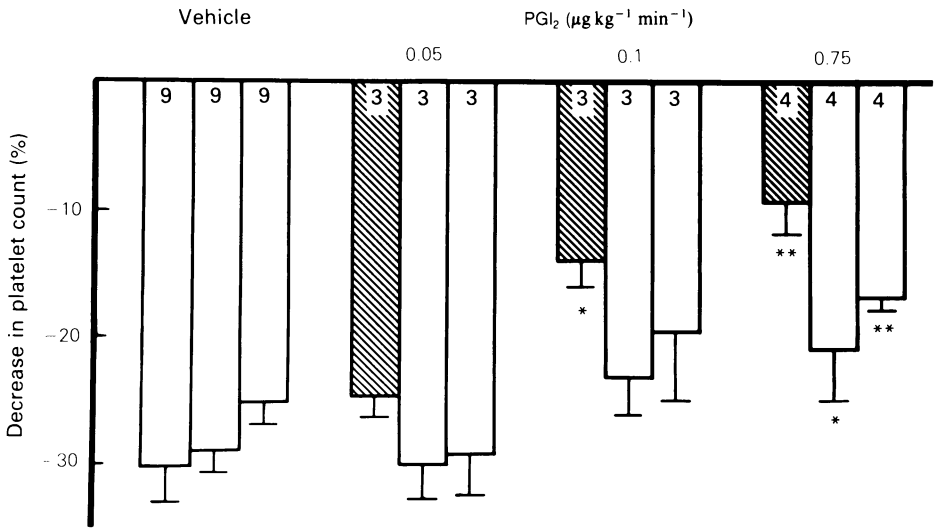

Figure 1 Effects of prostacyclin (PGI_2; 0.05, 0.1 and 0.75 $\mu g\,kg^{-1}\,min^{-1}$) infusion (hatched columns) on the decreases in circulating platelet count due to three consecutive injections of collagen (100 $\mu g\,kg^{-1}$). The numbers inside the columns represent the number of experiments. $*P<0.05$ and $**P<0.01$, *versus* vehicle.

was used, the platelets were markedly protected. The effect of these combinations of agents was similar to that of PGI_2 alone at the highest dose investigated (0.75 $\mu g\,kg^{-1}\,min^{-1}$).

Blood pressure effects

The initial mean arterial blood pressure (MABP) in unchallenged anaesthetized rabbits varied from 87 ± 6 to 102 ± 4 mmHg for the different groups studied.

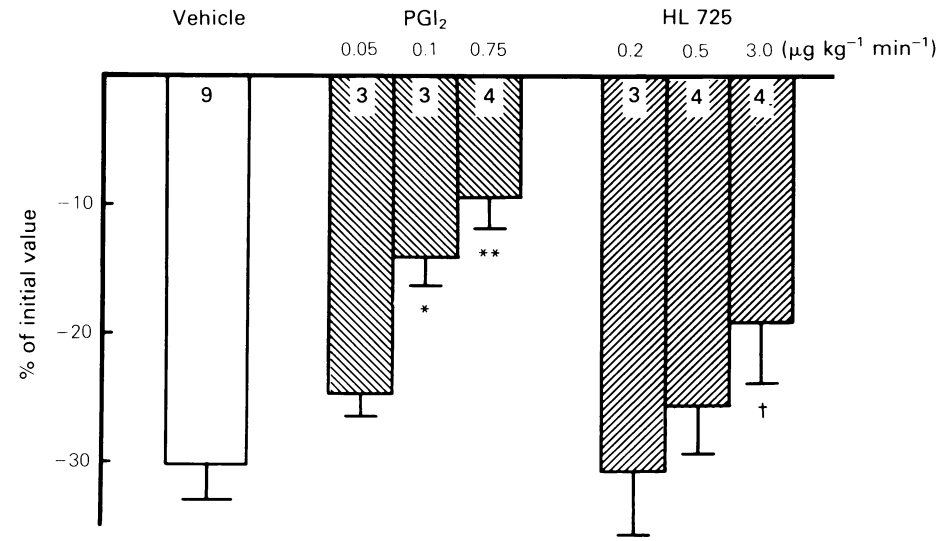

Figure 2 Effects of prostacyclin (PGI_2; 0.05, 0.1 and 0.75 $\mu g\,kg^{-1}\,min^{-1}$) and HL 725 (0.2, 0.5 and 3.0 $\mu g\,kg^{-1}\,min^{-1}$ infusion (hatched columns) on the decrease in circulating platelet count due to one injection of collagen (100 $\mu g\,kg^{-1}$). The numbers inside the columns represent the number of experiments. $*P<0.05$ and $**P<0.01$, *versus* vehicle. $\dagger P<0.05$ versus vehicle in one-tailed t test.

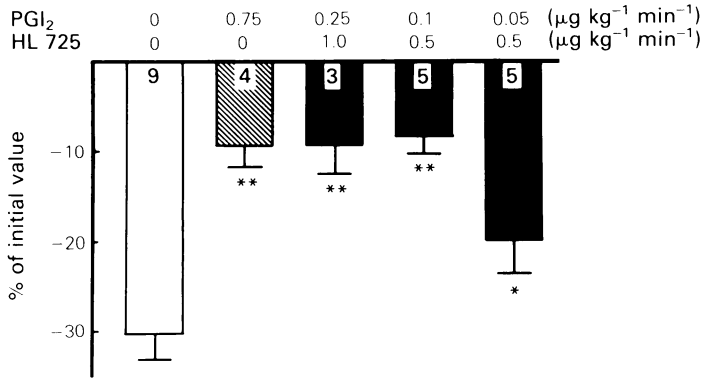

Figure 3 Inhibition of collagen ($100\,\mu g\,kg^{-1}$) induced decrease in peripheral platelet count by prostacyclin, PGI_2 $0.75\,\mu g\,kg^{-1}\,min^{-1}$) and by combinations of PGI_2 and HL 725 (0.25 + 1.0; 0.1 + 0.5, and 0.05 + $0.5\,\mu g\,kg^{-1}$ min^{-1}), respectively. The numbers inside the columns represent the number of experiments. $*P < 0.05$ and $**P < 0.01$, *versus* vehicle.

Infusion of vehicle did not change MABP, whereas infusion of HL 725 or PGI_2 alone produced a dose-dependent decrease in MABP. The maximum infusion rates of PGI_2 and HL 725 were chosen to decrease MABP by less than 20%. The three different combinations of HL 725 and PGI_2 tested led to more pronounced MABP decreases (Table 1). In this regard, single infusions of HL 725 ($0.5\,\mu g\,kg^{-1}\,min^{-1}$) and PGI_2 ($0.1\,\mu g\,kg^{-1}\,min^{-1}$) decreased MABP by 4 ± 2 and 7 ± 2 mm Hg, respectively, whereas a combined infusion of the two compounds was followed by a fall of 22 ± 7 mm Hg ($P < 0.05$). Thus, infusion of a combination of PGI_2 and HL 725 resulted in a marked depressor response (Table 1).

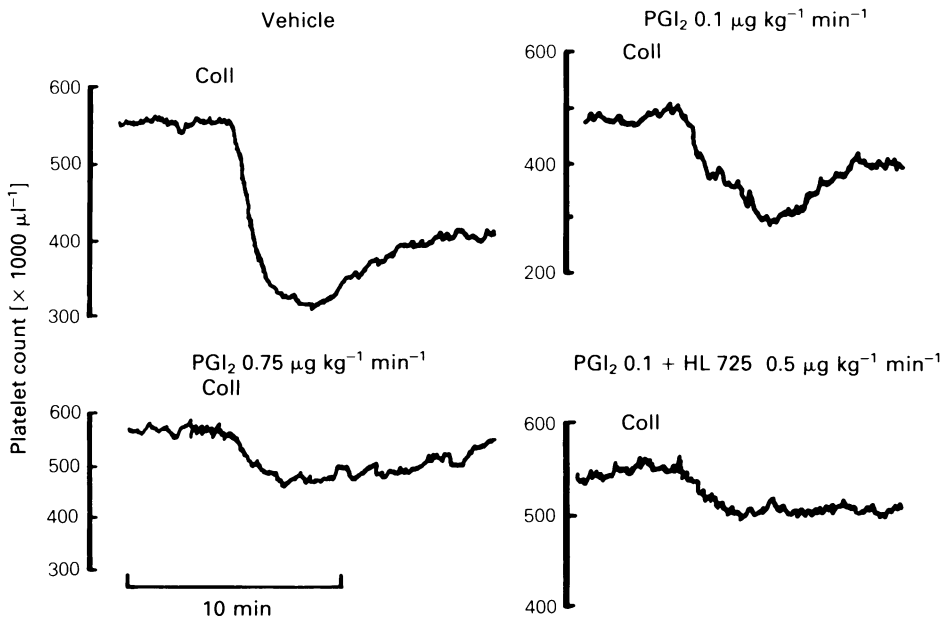

Figure 4 Original recordings of collagen (Coll; $100\,\mu g\,kg^{-1}$) induced decrease in peripheral platelet count. Infusion of prostacyclin (PGI_2) $0.1\,\mu g\,kg^{-1}\,min^{-1}$ slightly diminished the decrease. Combined infusion of PGI_2 0.1 and HL 725 $0.5\,\mu g\,kg^{-1}\,min^{-1}$ showed comparable inhibitory effects on platelet count decrease to PGI_2 $0.75\,\mu g\,kg^{-1}\,min^{-1}$.

Table 1 Maximum decrease in mean arterial blood pressure (mmHg) during infusion of vehicle, prostacyclin (PGI_2) and HL 725 alone or in combination

		PGI_2 ($\mu g\,kg^{-1}\,min^{-1}$)			
	Vehicle	*0.05*	*0.1*	*0.25*	*0.75*
Vehicle	2 ± 2	5 ± 0	7 ± 2	—	15 ± 3
HL 725 0.2 ($\mu g\,kg^{-1}\,min^{-1}$)	2 ± 2	—	—	—	—
" 0.5 "	4 ± 2	15 ± 2	22 ± 7	—	—
" 1.0 "	10 ± 0	—	—	17 ± 4	—
" 3.0 "	20 ± 3	—	—	—	—

All values are means ± s.e.means for 3–9 rabbits.

Injection of collagen resulted in an abrupt marked decrease in MABP in all animals studied. The effect occurred immediately after the addition of collagen, peaked after 1 min and recovered to the initial value within 10 min after injection. In vehicle treated animals, the MABP decreased by 50 ± 2, 52 ± 5 and 50 ± 5 mmHg during the three consecutive injections of collagen. Infusion of PGI_2 or HL 725 alone, and of the two agents combined, significantly inhibited the MABP decrease induced by collagen. The maximum effect of PGI_2 at 0.75 $\mu g\,kg^{-1}\,min^{-1}$ was comparable to the effect of a combination of HL 725, 1.0 $\mu g\,kg^{-1}\,min^{-1}$, and PGI_2, 0.25 $\mu g\,kg^{-1}\,min^{-1}$. In these groups, the depressor response was diminished by approximately 50%. The inhibitory effects of the combinations were greater than when either of the compounds was infused on its own (Figure 5).

Discussion

Moncada & Korbut (1978) concluded from their experiments that phosphodiesterase inhibitors potentiate the effects of endogenous, circulating PGI_2 on platelet cyclic AMP concentrations and may thereby act as potent anti-aggregatory agents *in vivo*. In contrast to this are the findings of other groups. For example, Smith *et al.* (1978) demonstrated that basal blood pressure in cats is unchanged by infusion of antibodies which antagonize the effect of prostacyclin. They concluded that intravascular PGI_2 does not contribute to persistence of vascular tone. Christ-Hazelhof & Nugteren (1981) were unable to detect 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) levels in plasma or whole blood of volunteers, although the detection limit of the method used was 20 pg ml^{-1}. More

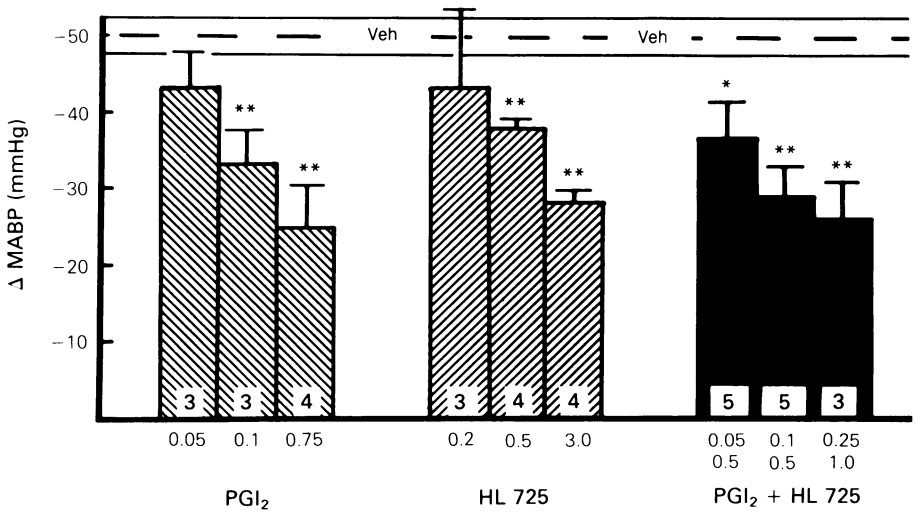

Figure 5 Collagen (100 $\mu g\,kg^{-1}$) induced abrupt decreases in mean arterial blood pressure (MABP) (50 ± 2 mmHg in vehicle treated animals, bar on top). Infusion of prostacyclin (PGI_2) 0.05, 0.1 and 0.75 $\mu g\,kg^{-1}\,min^{-1}$, HL 725 0.2, 0.5 and 3 $\mu g\,kg^{-1}\,min^{-1}$ or combinations of PGI_2 and HL 725 0.05 + 0.5, 0.1 + 0.5, and 0.25 + 1.0 $\mu g\,kg^{-1}\,min^{-1}$ dose-dependently reduced the mean arterial blood pressure. The numbers inside the columns represent the number of experiments. $*P < 0.05$ and $**P < 0.01$, *versus* vehicle.

recently, Blair *et al.* (1982) confirmed these findings and measured a 6-oxo-$PGF_{1\alpha}$ level of $3\,pg\,ml^{-1}$ in plasma of healthy volunteers. Thus, no definitive evidence exists for PGI_2 being a circulating hormone and for an *in vivo* potentiating effect of phosphodiesterase inhibitors and endogenous PGI_2.

Intense clinical investigation with synthetic PGI_2 and its chemically stable analogues has revealed that side-effects due to blood pressure lowering activities influence the subjective well-being of volunteers and patients. Thus, the idea of potentiating the *in vivo* platelet inhibitory effects of synthetic PGI_2 without increasing its cardiovascular effects by additional administration of a phosphodiesterase inhibitor is still attractive and a potentially important approach to the clinical use of prostacyclin as an effective anti-platelet therapeutic agent.

During systemic platelet activation, HL 725 and PGI_2, acted together to produce greater inhibition than when either compound was used on its own, as evidenced by inhibition of collagen induced platelet aggregate formation. This interaction was also observed with the inhibitory effects of PGI_2 and HL 725 on the depressor responses of a bolus injection of collagen. Vasodilator platelet release products such as ADP or ATP, may be involved in this circulatory reaction to collagen injection. Finally, both drugs also interacted *in vivo* to lower the blood pressure, although the absolute changes observed were in a narrow range. The experiments were designed to decrease MABP by less than 20% to avoid sympathetic counter-regulation and thereby catecholamine induced platelet activation. Thus, this approach to inhibit platelets *in vivo* via cyclic AMP elevation by simultaneous activation of adenylate cyclase and inhibition of phosphodiesterase leads to a higher efficacy of the drugs in inhibiting collagen induced platelet aggregation. Additionally, the compounds act synergistically with respect to their vasodilator activities, indicating that a specificity for platelet adenylate cyclase and phosphodiesterase does not exist in the intact rabbit.

Interesting results were recently obtained by Romson *et al.* (1983) in a model of local thrombosis induced by electrical irritation of a coronary vessel wall. Aminophylline, used as a phosphodiesterase inhibitor, at $20\,\mu g\,kg^{-1}\,min^{-1}$ infused together with PGI_2 at $25\,ng\,kg^{-1}\,min^{-1}$, resulted in a more than additive inhibition of thrombus formation. In contrast, the effects of a combination of aminophylline and PGI_2 ($50\,ng\,kg^{-1}\,min^{-1}$) were only additive when compared to the effects of the drugs infused alone. Although potentiating effects are difficult to evaluate in a model where the response is of the all-or-none type, these authors were able to demonstrate an antithrombotic efficiency of a phosphodiesterase inhibitor with low doses of PGI_2 *in vivo*. These combinations induced only moderate depressor effects compared to single PGI_2 infusions with identical protective effects studied in the same model (Romson *et al.*, 1981).

In conclusion, the combination of HL 725 and PGI_2 prevents platelet activation *in vivo* but this was associated with a significant increase in the cardiovascular effects (i.e., depressor effect). The results of Romson *et al.* (1983) suggest that the most effective combination of a phosphodiesterase inhibitor and PGI_2 or one of its analogues has not yet been found. Therefore, the combination of drugs like PGI_2 acting on platelet cyclic AMP metabolism by adenylate cyclase activation, and phosphodiesterase inhibitors may still offer an opportunity of effective antithrombotic and platelet protective therapy, while reducing the number and severity of drug-related side-effects.

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References

BLAIR, I.A., BARROW, S.E., WADDELL, K.A., LEWIS, P.J. & DOLLERY, C.T. (1982). Prostacyclin is not a circulating hormone in man. *Postaglandins*, **23**, 579–589.

CHRIST-HAZELHOF, E. & NUGTEREN, D.H. (1981). Prostacyclin is not a circulating hormone. *Prostaglandins*, **22**, 739–746.

DATA, J.L., MOLONY, B.A., MEINZINGER, M.M. & GORMAN, R.R. (1981). Intravenous infusion of prostacyclin sodium in man: clinical effects and influence on platelet adenosine diphosphate sensitivity and adenosine 3′:5′-cyclic monophosphate levels. *Circulation*, **64**, 4–12.

GORMAN, R.R., BUNTING, S. & MILLER, O.V. (1977). Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins*. **13**, 377–388.

JORGENSEN, K.A., DYERBERG, J. & STOFFERSON, E. (1979). Prostacyclin (PGI_2) and the effect of phosphodiesterase inhibitors on platelet aggregation. *Pharmac. Res. Commun.*, **11**, 605–615.

LEWIS,, P.J. & O'GRADY, J. (1981). *Clinical pharmacology of prostacyclin*. New York: Raven Press.

LONGMORE, D.B., GRAEME-BENNETT, J., HOYLE, P.M., SMITH, M.A., GREGORY, A., OSIVAND, T. & JONES, W.A. (1981). Prostacyclin administration during cardiopulmonary bypass in man. *The Lancet*, **I**, 800–804.

MILLS, D.C.B. & SMITH, J.B. (1971). The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3′,5′-cyclic monophosphate in platelets. *Biochem. J.*, **121**, 185–196.

MONCADA, S. & KORBUT, R. (1978). Dipyridamole and other phosphodiesterase inhibitors act as antithrombotic agents by potentiating endogenous prostacyclin. *The Lancet*, **I**, 1286–1289.

MONCADA, S., KORBUT, R., BUNTING, S. & VANE, J.R. (1978). Prostacyclin is a circulating hormone. *Nature*, **273**, 767–768.

ROMSON, J.L., HAACK, D.W., ABRAMS, G.D. & LUCCHESI, B.R. (1981). Prevention of occlusive coronary artery thrombosis by prostacyclin infusion in the dog. *Circulation*, **64**, 906–914.

ROMSON, J.L., HOOK, B.G. & LUCCHESI, B.R. (1983). Potentiation of the antithrombotic effect of prostacyclin by simultaneous administration of aminophylline in a canine model of coronary artery thrombosis. *J. Pharmac. exp. Ther.*, **277**, 288–294.

RUPPERT, D. & WEITHMANN, K.U. (1982). HL 725, an extremely potent inhibitor of platelet phosphodiesterase and induced platelet aggregation *in vitro*. *Life Sci.*, **31**, 2037–2043.

SMITH, G.M. & FREULER, F. (1973). The measurement of intravascular aggregation by continuous platelet counting. *Biol. Anat.*, **12**, 229–234.

SMITH, J.B., OGLETREE, M.L., LEFER, A.M. & NICOLAOU, K.C. (1978). Antibodies which antagonize the effects of prostacyclin. *Nature*, **274**, 64–65.

UBATUBA, F.B., MONCADA, S. & VANE, J.R. (1979). The effect of prostacyclin (PGI_2) on platelet behaviour. Thrombus formation *in vivo* and bleeding time. *Thromb. Haemost.*, **41**, 425–435.

VIGDAHL, R.L., MONGIN, J. JR. & MARQUIS, N.R. (1971). Platelet aggregation IV. Platelet phosphodiesterase and its inhibition by vasodilators. *Biochem. biophys. Res. Comm.*, **42**, 1088–1094.

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