Biochemical Pharmacology, Vol. 35, No. 16, pp. 2645–2649, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

COMMENTARY

DESENSITIZATION OF PROSTACYCLIN RESPONSIVENESS IN PLATELETS

APPARENT DIFFERENCES IN THE MECHANISM *IN VITRO* OR *IN VIVO*

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Prostacyclin (epoprostenol, PGI_2) is an unstable metabolite of arachidonic acid metabolism [1]. It is synthesised in numerous tissues in man, including arterial endothelium [2, 3]. Prostaclycin is a potent inhibitor of platelet aggregation [1] and mediates relaxation of vascular smooth muscle [4]. The physiological role of this compound remains uncertain, but it is believed widely to include the limitation of platelet deposition at the site of vascular injury [5]. The biological effects of PGI_2 on each of its target tissues are mediated by activation of adenylate cyclase, and in platelets the PGI_2-dependent increase in intracellular cyclic AMP may be shown to reduce aggregation of platelets *in vitro* [6, 7].

The therapeutic use of PGI_2 is most widely accepted in the field of extracorporeal haemoperfusion systems [5, 8]. The techniques involved in these methods require complex machinery, and the deposition of platelets on dialysis membranes, charcoal columns and numerous filtration systems presents serious clinical problems. Predictably, the loss of platelets from blood returning to the circulation may result in an increase in the bleeding time. PGI_2 has been used alone in these circumstances, or in combination with heparin, to minimise this problem. Furthermore, the chemical instability of the PGI_2 molecule may be seen as an added advantage. PGI_2 survives a single passage through most extracorporeal haemoperfusion systems, but its chemical instability leads to rapid hydrolysis of the molecule and little residual effect in the blood as it returns to the general circulation.

The role of platelet aggregates in the pathogenesis of atheroma remains controversial, but it has been proposed that their deposition on the endothelial wall of larger blood vessels may be an essential step in the development of atheroma (review: see Ref. 5). These considerations have lead to much research in this area, and there are numerous reports in the medical literature of a beneficial effect of prostacyclin, or one of its more stable analogues, in the treatment of peripheral vascular disease (reviewed in Refs. 5 and 8). Arteriosclerosis is a major health problem and is associated with progressive obliteration of the lumen of large blood vessels. The ensuing hypoxia of the distal tissues results in myocardial infarction, cerebral thrombosis, muscle pains, ulceration of the skin, and finally peripheral gangrene. Numerous pharmaceutical companies have therefore been prompted by the promising early reports to develop stable PGI_2 analogues (see Fig. 1) for use in vascular disease. These compounds are administered by slow intravenous infusion that is sustained frequently for periods of several days. Thorough clinical evaluation of any therapeutic benefit of PGI_2 in these diseases is still awaited and lies beyond the scope of this review, but there seems to be some reason for cautious optimism.

It became apparent in the early clinical trials of prostacyclin that desensitization of platelet responsiveness to PGI_2 might be a problem [9]. An infusion of PGI_2 at a rate of 5 ng/kg/min results in a circulating concentration of 150–200 pg/ml PGI_2, at which concentration (approximately 0.5 nM) the fractional occupancy of platelet PGI_2 receptors would be <3% of the total. This calculation is based on the finding that the K_d for PGI_2 binding to its receptor is about 20 nM [10–12]. Nevertheless, under these circumstances, desensitization of up to 80% may be observed in platelet PGI_2 responsiveness [9]. This anomaly has prompted a line of research which provides some interesting insights into the problems of desensitization *in vivo*.

Desensitization of PGI_2 responsiveness has been observed in numerous tissues including human fibroblasts [13] and platelets *in vivo* [9] and *in vitro* [14], and in the NCB-20 cell line [15, 16]. Cells of the NCB-20 somatic hybrid [17] were derived by fusion of the N18TG2 mouse neuroblastoma [18] and brain cells from hamster (18 days *in utero*). These cells express PGI_2 receptors [11, 19] that are indistinguishable pharmacologically from those of human platelets [6, 7] or blood vessels [20, 21], and the general availability of these cells and ease of culture make them a suitable system for detailed biochemical examination of the PGI_2 receptor. They have provided also some insights into the mechanisms of PGI_2 desensitization.

Prolonged culture of NCB-20 cells in the presence of saturating concentrations of carbacyclin or iloprost (both stable analogues of PGI_2) results in substantial loss of subsequent sensitivity to PGI_2, iloprost, carbacyclin or PGE_1 [15, 16]. Desensitization is effected by a reduction in the maximum PGI_2-dependent

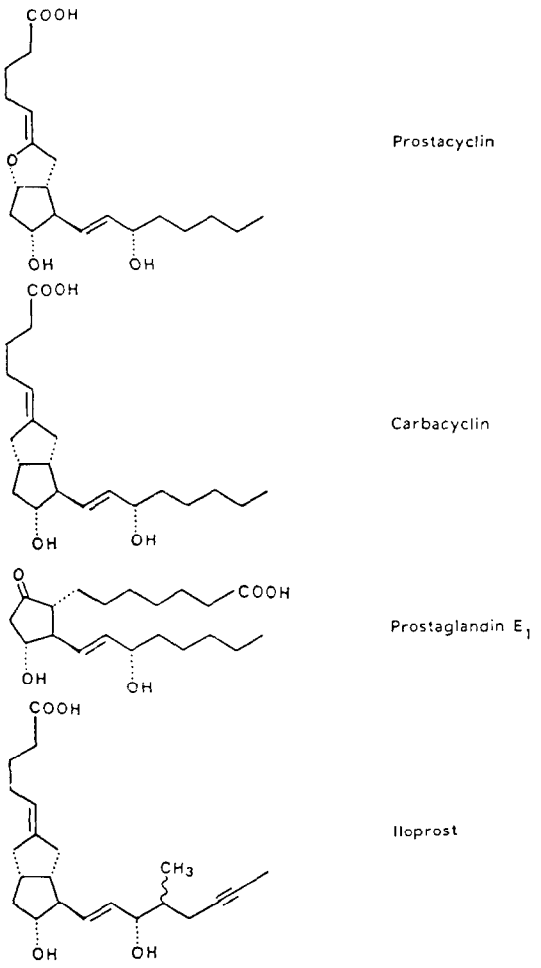

Fig. 1. Structures of prostaglandin E_1, prostacyclin and two stable prostacyclin analogues.

increase in adenylate cyclase activity [41.2 ± 1.0 reduced to 15.1 ± 2.6 pmoles cyclic AMP · min^{-1} · (mg protein)$^{-1}$]. There is simultaneously an increase in the K_{act} value for PGI_2 (K_{act} = concentration required for half-maximum enzyme activation) from 64.1 ± 1.6 to 174 ± 9.2 nM. Desensitization of PGI_2 responsiveness in NCB-20 cells was not accompanied by alteration in their sensitivity to other hormones or neurotransmitters that regulate adenylate cyclase in these cells (e.g. opiates, adenosine, serotonin or noradrenaline). Further experiments have revealed that the degree of desensitization is closely linked to the fractional occupancy by agonist. The close association between these two events is seen in most other examples of homologous desensitization.

PGI_2 receptors have been identified by the binding of [^{3}H]PGI_2 [22] to several tissues including platelets [10, 23], vascular homogenates [21], and NCB-20 cells [11]. In most systems, the specific binding is complex with a single high-affinity receptor site and a second lower affinity site. More recently, [^{3}H]iloprost has become available commercially, and the binding of the [^{3}H]iloprost to membranes of control or desensitized NCB-20 cells revealed an interesting anomaly [16]. While the maximum binding capacity to the high-affinity site was reduced during desensitization from 431 to 200 fmoles/mg protein, the value of the equilibrium dissociation constant (K_d) remained essentially unchanged (9.55 nM in control cells and 14.8 nM in desensitized cells). Further experiments were performed using the technique of radiation inactivation. The application of "protein inactivation" by ionizing radiation in enzymology and cell biology has been reviewed recently [24]. Briefly, the method involves measurement of the loss of a protein function (e.g. catalytic activity of an enzyme) or identity (e.g. loss of antibody or ligand binding capacity) as a result of the total loss of structural integrity due to ionizing radiation in the beam of a linear accelerator. The measured loss of a protein, or complex of proteins, at known irradiation dosages allows an estimation of the "target size" and molecular weight. This technique has been used in an examination of the PGI_2 receptors on NCB-20 cells [25], with particular reference to its coupling to the N_s (GTPase) and C (catalytic subunit) of adenylate cyclase.

Application of this method has shown that the identity of the receptor remains unchanged during desensitization [16]. Specifically, the 82.8 K molecular weight polypeptide receptor revealed by radiation inactivation was unchanged during desensitization. In particular, there was no evidence for stable coupling of the receptor to other protein constituents of the membrane. In other experiments, the fluoride-dependent increase in adenylate cyclase activity was shown to be unchanged by desensitization, which suggested strongly that the coupling of the GTPase (N_s) to the catalytic subunit (C) of adenylate cyclase was also unaltered [16].

The anomalous increase in the K_{act} value for PGI_2-dependent activation of adenylate cyclase, without alteration of the K_d value for ligand binding, may be explained on the basis of spare receptors [26]. The concept is a simple one, although the literature appears a little confused in relation to what is meant by the term "spare receptors". In this context, it is proposed only that the receptor density [R] exceeds the number of adenylate cyclase complexes [N_sC] per unit area of membrane. A number of assumptions are implicit in the interpretation outlined below, and these include:

(i) The receptor [R] interacts with the complex [N_sC] with a 1:1 stoichiometry, and N_sC complexes are activated by an equal number of agonist-occupied receptors.

(ii) The probabilities of interaction with any particular N_sC complex and any receptor molecule are equal.

(iii) The enzyme exists in a "basal" or "activated" state, but in no intermediate states, and gradual changes in enzyme activity are mediated by sequential activation of N_sC complexes.

(iv) The level of enzyme activation is dependent on the number of occupied receptors per unit area of membrane, and not (necessarily) on the fractional occupancy of the receptors with agonist.

In the circumstances, if [R] exceeds [N_sC], it will be apparent that activation of all the [N_sC] complexes

will be effected before all of the receptors [R] are occupied by agonist. If during desensitization the value of [R] is reduced with no change in [N_sC], the value of K_{act} will increase until the concentration of [N_sC] is the same as [R]. It may be shown furthermore that $K_{act} = K_d/(2x - 1)$, where x is the factor by which [R] exceeds [N_sC]. This model for desensitization of PGI_2 responsiveness in NCB-20 cells has been validated experimentally by manipulating the relative concentrations of [R] and [N_sC] [16]. Culture of NCB-20 cells in the presence of cycloheximide, an inhibitor of ribosomal translation of mRNA, results in a relatively slow, but simultaneous loss of both PGI_2 receptors (identified by [^{3}H]iloprost binding) and adenylate cyclase complexes (identified as basal enzyme activity). As predicted, concentration curves of PGI_2-dependent activation of adenylate cyclase reveal a reduction in the maximum enzyme activity without an alteration in the K_{act} value. This result was anticipated by the model presented and is explained on the basis of a similar loss of both enzyme and receptors without an alteration in their relative concentrations. This result contrasts with the situation during desensitization, in which receptors are lost rapidly without an accompanying reduction in adenylate cyclase molecules. In experiments that measure the rate of desensitization in the absence or presence of inhibitors of protein synthesis, evidence was obtained that the loss of receptors from the cell surface of NCB-20 cells was accompanied by arrested transcription of the message coding the protein of the high-affinity receptor.

Desensitization of PGI_2 responsiveness in human platelets is similar in many ways to that observed in NCB-20 cells. Incubation *in vitro* of platelet-rich plasma in the presence of selected concentrations of iloprost between 1.5 and 450 nM reveals once again that the degree of desensitization is linked closely to fractional occupancy of the receptor sites with agonist.* In our experiments, half-maximum desensitization at 30 hr was observed with 46% receptor occupancy. A comparison of iloprost-dependent activation of adenylate cyclase in membranes prepared from control or desensitized platelets revealed a loss of maximum iloprost-dependent activation of adenylate cyclase [83.3 to 33.3 pmoles cyclic AMP $\cdot \min^{-1} \cdot (\text{mg protein})^{-1}$]. Once again, there was an increase in the K_{act} value (7.2 to 33.9 nM), suggesting "spare" PGI_2 receptors on platelets. Platelets desensitized with iloprost showed decreased sensitivity to PGI_2, but no alteration in PGD_2-dependent activation of enzyme activity. This result confirms previous work [14].

The binding of [^{3}H]iloprost to membranes prepared from control or desensitized platelets revealed a reduction in the maximum binding capacity of the high affinity site from 439 fmoles/mg protein to 119 fmoles/mg protein. Once again there was no significant change in the K_d value (4.2 nM in control cells and 2.4 nM in desensitized cells).

An obvious different between control and desensitized PGI_2 responsiveness of platelets and NCB-20 cells relates to their capacity for recovery. The recovery in the NCB-20 cell line is dependent on *de novo* protein synthesis, and it is abolished totally when cells are cultured in the presence of cycloheximide or actinomycin D [16]. No evidence has been obtained for any recovery of PGI_2 responsiveness in mature human platelets, which have little or no capacity for *de novo* protein synthesis.

With the available data obtained from *in vitro* studies of desensitization in platelets or NCB-20 cells, there seemed no ready explanation for the 80% desensitization of platelet PGI_2 responsiveness observed following prolonged PGI_2 infusions, during which the plasma PGI_2 concentration would not be expected to exceed 150–200 pg/ml (0.5 nM). This paradox still remains to be resolved finally, but recent experiments† in this laboratory (performed in collaboration with scientists at Schering AG, Berlin) do provide a possible explanation.

Iloprost was infused into a peripheral forearm vein of the right arm of a healthy volunteer at a rate of 1.5 ng/kg/min. At this infusion rate, the subject became flushed secondary to peripheral vasodilatation, and he remarked on a migrainous headache. Blood samples were withdrawn from a remote vein (left arm) or 12 cm above the infusion site in the right arm (see Fig. 2). The iloprost concentration in the syringe was 76 μg in 50 ml, the subject weighed 83 kg, and the infusion rate was set at 5 ml/hr. Analysis of the blood samples obtained from the right arm revealed a rapid raise in iloprost concentration to 15 ng/ml (about 40 nM), while similar samples obtained from the remote vein revealed a plateau concentration of iloprost of 116 pg/ml. A comparison of the relative concentrations of iloprost in the syringe and in the vein draining the infusion site reveals a dilution factor of about 100. Since the infusion rate was at 5 ml/hr, it follows that the blood flow through the peripheral forearm vein *at rest* is very slow (about 500 ml/hr). This represents about 0.25% of cardiac output. It is proposed that, during the transit of platelets through the peripheral vein draining the infusion site in this experiment, the fractional occupancy of platelet PGI_2 receptors with iloprost was very high. This would be accompanied by substantial desensitization, with little or no capacity for recovery of that sensitivity. The sequential passage of blood cells through this small portion of the total vascular bed would result in an exponential decline in the number of cells that retain their original sensitivity to iloprost. Throughout the infusion, the iloprost concentration in blood samples obtained from the remote (left) forearm vein would not, of course, exceed the plateau level observed (about 2.5 nM), at which concentration the 80% desensitization of PGI_2 responsiveness that is seen following prolonged infusion appears inexplicable.

These results raise an interesting general point about the suitability of intravenous infusion of receptor-activating drugs whose target tissues are any one of the cellular components of blood. In most circumstances, drugs infused into a peripheral forearm

* U. Alt, P. J. Leigh, A. J. Wilkins, P. K. Morris and J. MacDermot, *Br. J. clin. Pharmac.* in press.

† J. MacDermot, U. Alt, P. J. Leigh, P. K. Morris, A. J. Wilkins, M. J. Brown and I. A. Blair, manuscript in preparation.

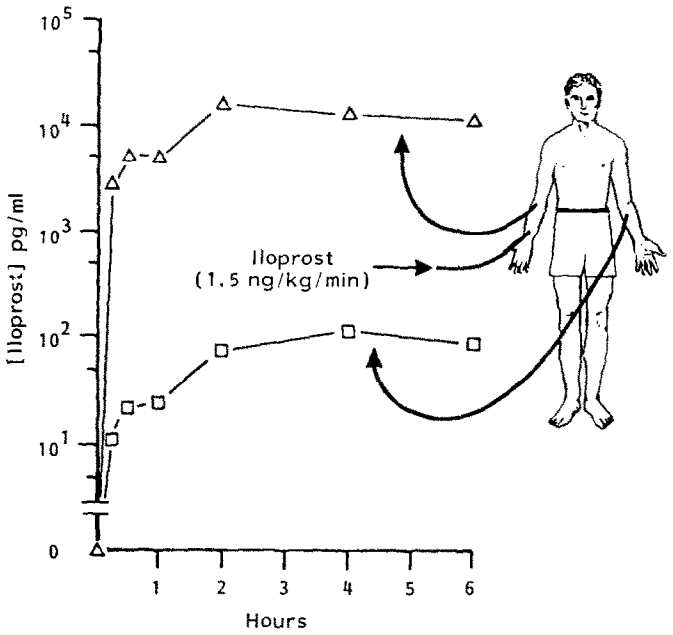

Fig. 2. Plasma concentrations of iloprost during intravenous infusion. Blood samples were taken 12 cm above the infusion site or from the opposite arm.

vein are administered because of a desired effect on a stationary (and remote) target tissue such as heart or lungs. If, however, the response of the drug is receptor-mediated, and the target tissue is (even temporarily) in close proximity to the infusion site, then prolonged infusion, even at relatively low rates, may be accompanied by very substantial receptor desensitization.

It should be possible to validate or refute the proposed explanation for *in vivo* desensitization of PGI_2 responsiveness following intravenous infusion. First, the hypothesis would predict that infusion of iloprost into a large central vein, in which more rapid dilution occurred, would result in little or no desensitization. It is important to note that the infusion referred to in Ref. 9 was intra-arterial, and the drug might, therefore, have been diluted rapidly. However, the infusion was given for peripheral vascular disease, in which arterial flow rates will certainly be reduced very substantially. Second, infusion at a much lower rate of a metabolically more stable PGI_2 analogue (with a plasma half-life that exceeded substantially that of iloprost) would result in a similar pharmacological response with correspondingly lower drug concentrations close to the infusion site. This would again be predicted to cause correspondingly less desensitization.

The case for prolonged infusions of PGI_2 (or one of its stable analogues) in peripheral vascular disease is not yet firmly established. However, numerous anti-platelet drugs, including those that activate PGI_2 receptors, are currently receiving much attention for a possible therapeutic role in peripheral vascular disease. The problem of *in vivo* desensitization of platelet responsiveness appears at first sight to limit substantially any therapeutic potential of these drugs, but an appreciation of the mechanism(s) involved may allow a more rational approach to therapy in these debilitating diseases.

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