EDRF Increases Cyclic GMP in Platelets During Passage Through the Coronary Vascular Bed

U. Pohl and R. Busse

It was investigated whether endothelium-derived relaxing factor (EDRF) increases cyclic GMP (cGMP) content in platelets passing through the coronary bed. Boluses of washed platelets from healthy human donors were injected into the aortic perfusion line of isolated, saline-perfused rabbit hearts under constant flow conditions (28±2 ml/min). The coronary effluent was collected over 5 seconds, and the cGMP content of platelets was determined by radioimmunoassay. Platelet cGMP amounted to 0.34±0.11 pmol/mg protein after passage through the unstimulated coronary bed. During stimulation with acetylcholine (1 μM), it increased to 1.6±0.5 pmol/mg (p<0.01; n=14). Simultaneously, the platelet recovery (measured over 20 seconds after injection) was enhanced (by 45±11%; p<0.01) during endothelial stimulation with acetylcholine. Treatment with the EDRF inhibitor hemoglobin (6 μM) completely abolished the increase in platelet cGMP (p<0.01; n=11) as well as the enhanced platelet recovery (n=8). Inhibition of EDRF by hemoglobin reduced also the basal platelet cGMP content to 0.17±0.11 pmol/mg (p<0.01). The data indicate that basally released EDRF is able to increase cGMP in platelets during a single passage through the coronary bed. The enhanced recovery of platelets after EDRF stimulation, which coincides with an increase of platelet cGMP, suggests that EDRF plays an important role as inhibitor of platelet activation in the coronary circulation. (Circulation Research 1989;65:1798-1803)

Endothelium-derived relaxing factor (EDRF), which is likely to be identical with nitric oxide,1 exerts its vasodilator effects by a direct stimulation of soluble guanylate cyclase in smooth muscle cells.2 The fact that EDRF is also released toward the vascular lumen and that there is a high activity of soluble guanylate cyclase in platelets3 has led to the concept that EDRF may be involved in the control of platelet activity.4 Several in vitro studies have now established that EDRF is able to inhibit platelet aggregation and adhesion.5-8 An increase of platelet cyclic GMP (cGMP) has also been observed in the intact animal after prolonged (5 minutes) systemic exposure to the EDRF-stimulator carbachol.9 However, it is still unclear whether the basal release of EDRF in an intact vascular bed is sufficiently high to affect platelet cGMP levels. Moreover, it is unknown whether under conditions of high flow, EDRF can affect platelet cGMP level during a single passage through an organ. This would be a prerequisite for a physiological role of EDRF as antiplatelet agent in the intact circulation. We therefore studied whether EDRF-induced changes of cGMP levels can be observed in platelets after a single passage through the coronary beds of isolated perfused rabbit hearts. Furthermore, the effect of stimulation of EDRF release on platelet recovery in the coronary effluent was examined, which could be taken as an indicator of adhesion and aggregation in the coronary bed.

Materials and Methods

Langendorff Preparation

Mongrel rabbits (1.0–1.5 kg) of either sex were anesthetized with sodium pentobarbital (30–50 mg/kg). After anticoagulation with heparin (5,000 units/kg), one carotid artery was transsected. The heart was excised immediately after administration of a second (lethal) dose of sodium pentobarbital (50 mg/kg). Constant flow perfusion was performed by means of a roller pump (Heidolph RGL 85, Kelheim, FRG) through a cannula inserted into the aortic stump. The perfusion solution gassed with 95% O₂-5% CO₂ to maintain a pH of 7.4, consisted of a modified Krebs-Henseleit buffer as described

From the Institute of Applied Physiology, University of Freiburg, Freiburg, FRG.
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Address for correspondence: Dr. U. Pohl, Institut f. Angewandte Physiologie, Hermann-Herder-Str. 7, D-7800 Freiburg, FRG.
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earlier. Test solutions and platelet boluses were administered through a Y-shaped connector in the aortic perfusion line. Coronary perfusion pressure was measured with a pressure transducer (Gould-Statham P 2310, Oxnard, California) through a side-branch of the aortic perfusion cannula. A fluid-filled latex balloon was inserted into the left ventricle through a pulmonary vein and connected to a second pressure transducer (Statham CP-01) to measure isovolumetric left ventricular pressure and heart rate, derived from the pressure signals by means of a cardiotachometer. The coronary effluent was collected from the coronary sinus or the right atrium respectively. Passage through the right ventricle was prevented by distraction of the atrioventricular valve and ligation of the pulmonary artery.

**Preparation of Platelets**

Blood from healthy human donors who had not received any medication was collected into 3.8% sodium citrate (20% of final volume). Platelet-rich plasma (PRP) was obtained by centrifugation at 200g for 20 minutes. After administration of acid citrate/dextrose (20 vol%) and prostacyclin (300 ng/ml), the PRP was centrifuged again and the pellet finally suspended in a modified Tyrode’s solution containing indomethacin (1 μM) to obtain a final platelet count of 2–3 × 10⁶ platelets/μl. Experiments were finished within 180 minutes after the end of platelet preparation.

**Platelet Cyclic GMP Content**

The coronary effluent was collected over a period of 5 seconds after the injection of platelets into a tube containing trichloroacetic acid (TCA; final concentration 7%). After centrifugation, TCA was four times extracted from the supernatant with water-saturated ether, and the samples were kept frozen (−20° C) until analysis. Cyclic GMP was determined in the acetylated samples using a commercially available radioimmunoassay (New England Nuclear). The release was expressed in picograms 6-keto prostaglandin F₃α per milliliter effluent and was normalized to a perfusion rate of 30 ml/min.

**Experimental Protocol**

After a 30-minute equilibration period, platelet boluses (300 μl) were injected in 10-minute intervals. Injections were performed under control (unstimulated) conditions and two minutes after the beginning of continuous administration of acetylcholine or adenosine (in random order) to the perfusate (final concentration 1 μM each). The same protocol was repeated in the presence of hemoglobin. The coronary effluent was collected over 5 seconds after platelet injection and was assayed for cGMP content. In an additional series, the platelet passage through the coronary bed was studied under control conditions and during endothelial stimulation with acetylcholine. To this end, the protein content of the coronary effluent, collected over a period of 20 seconds after platelet injection, was assayed. The recovery of platelet protein in the effluent was expressed in percent of the amount of platelet protein administered during each bolus injection. The platelets were activated by 40 mU thrombin 20 seconds before bolus injection. Thrombin in this concentration did not induce any detectable effect on the coronary perfusion pressure. The experiments were performed in control hearts as well as in hearts exposed to indomethacin (30 μM for 30 minutes) or hemoglobin (6 μM).

**Statistics**

Data are presented as means±SEM. Comparisons were performed by means of paired or unpaired t test. Differences were considered significant at a value of p<0.05.

**Drugs**

Adenosine and acetylcholine-hydrochloride were obtained from Sigma, Deisenhofen, FRG, and freshly dissolved in Krebs-Henseleit buffer. Prostacyclin was a generous gift from Dr. Gertrud Schröder (Schering AG, Berlin). Indomethacin was dissolved in ethanol/0.1 NaHCO₃, 1:3 vol/vol, and freshly diluted by Krebs-Henseleit buffer. Bovine hemoglobin (purchased from Sigma) was dissolved in 0.1 M phosphate buffer under nitrogen. After addition of sodium dithionite (10 mM), the stock solution was passed through a column packed with sepharose (CL-6B, Pharmacia, Freiburg, FRG). The hemoglobin fraction was collected in liquid nitrogen and kept frozen (−70° C) until the experiment was started. All drugs were infused through a Y-shaped connector in the aortic perfusion line at dilation induced by acetylcholine (1 μM). The samples were kept frozen until assayed for the content of 6-keto prostaglandin F₃α, the stable metabolite of prostacyclin, by means of a commercially available radioimmunoassay (New England Nuclear). The release was expressed in picograms 6-keto prostaglandin F₃α per milliliter effluent and was normalized to a perfusion rate of 30 ml/min.
In contrast, acetylcholine did not induce any change of platelet cGMP as observed during passage through the heart. Likewise, neither adenosine nor hemoglobin affected the platelet cGMP content during in vitro incubation (data not shown). By contrast, sodium nitroprusside induced a similar increase of platelet cGMP as observed during passage through the coronary bed (1.72±0.4 pmol/mg; n=3).

In a separate series of experiments the recovery of platelets in the coronary effluent (determined as protein) was investigated. Under control conditions, 35±4% of the injected platelets could be detected during the collection period. In the presence of acetylcholine, the recovered fraction increased significantly (Figure 2). This increase was independent of concomitant changes in perfusion pressure since it was also observed during continuous infusion of papaverine, preventing further reduction of the perfusion pressure by acetylcholine (Table 2). In contrast, in the presence of hemoglobin, no significant increase of the platelet recovery was observed (n=8) (Figure 2).

Acetylcholine induced a significant increase of the 6-keto prostaglandin F1α concentration in the coronary effluent, from 303±56 pg/ml to 966±225 pg/ml (p<0.01; n=5). After 30 minutes' infusion of indomethacin (30 μM) the release of prostacyclin was virtually completely suppressed (12±7 pg/ml) and no increase was observed during administration of acetylcholine (n=3). Nevertheless, there was still an increase of platelet recovery after administration of acetylcholine in hearts pretreated with indometh-
TABLE 2. Coronary Perfusion Pressure and Platelet Recovery in Control and Predilated Hearts

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<tr>
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<td>PRC (%)</td>
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ACh, acetylcholine; basal, before injection of platelets; peak, during injection of platelets; CTL, control hearts (n=4); CPP, coronary perfusion pressure; PRC, platelet recovery; PAV, hearts predilated with papaverine (3 μM; n=4).

*P<0.01, significantly different from the value in unstimulated hearts.

FIGURE 2. Increase in platelet recovery (percentage of injected platelet protein) in the coronary effluent after endothelium-derived relaxing factor stimulation with acetylcholine (ACh). After pretreatment with indomethacin (Indo; 30 μM), this increase is significantly reduced, whereas in the presence of hemoglobin (Hb; 6 μM) it is completely abolished. (n=8-11).

The EDRF inhibitor hemoglobin completely abolished this increase as it inhibited the acetylcholine-induced vasodilation. Earlier experiments have shown that the acetylcholine-induced dilation in the coronary microcirculation is mediated by EDRF. Moreover, in isolated hearts, an acetylcholine-induced release of EDRF (nitric oxide or its oxidation products) could be demonstrated directly.

It is important to note that the EDRF inhibitor hemoglobin not only blocked the cGMP increase during acetylcholine stimulation but also reduced the "unstimulated" cGMP level of platelets. This indicates that the cGMP level in platelets is already augmented when they pass the unstimulated heart and that the underlying mechanism can be inhibited by hemoglobin. Therefore, it can be concluded that the unstimulated coronary vasculature was able to release EDRF in considerable amounts. Our findings are consistent with observations in resistance vessels of the rabbit ear, in isolated guinea pig hearts, and isolated vessels, suggesting a basal release of EDRF. This continuous basal release might be sustained by physical stimuli-like flow, pulsatility, or low oxygen partial pressure and have considerable functional significance for the adjustment of blood flow to tissue metabolic demands.

The EDRF content of washed human platelets increased during a single passage through the coronary bed of isolated rabbit hearts. A roughly fivefold increase of cGMP was observed in the platelets when EDRF release from the coronary vasculature was stimulated by acetylcholine. Similar results were obtained with rabbit platelets (authors' unpublished data). The increase in platelet cGMP was most likely induced by EDRF, since treatment with the EDRF inhibitor hemoglobin completely abolished this increase as it inhibited the acetylcholine-induced vasodilation. Earlier experiments have shown that the acetylcholine-induced dilation in the coronary microcirculation is mediated by EDRF. Moreover, in isolated hearts, an acetylcholine-induced release of EDRF (nitric oxide or its oxidation products) could be demonstrated directly.

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Our experiments demonstrate for the first time that this release is sufficient to increase platelet cGMP and therefore add a further piece of evidence that the basal EDRF release is functionally important. The relative increase of platelet cGMP by EDRF in the resting heart far exceeds 25%, which was found to be a threshold increase for a detectable inhibition of platelet activation in vitro. Therefore, even the basal release of EDRF might exert a physiological function protecting against exaggerated platelet activity.

In contrast to experiments performed in test tubes, the EDRF effects on platelet cGMP were studied under conditions of high flow. The coronary flow (in milliliters per minute per 100 mg) was considerably higher than usually observed in animals in vivo and must have diluted the released EDRF substantially. Nevertheless, the increases in platelet cGMP were similar to those measured after exposure to 1 μM sodium nitroprusside, indicating that the amount of EDRF released was functionally equivalent to this high concentration of sodium nitroprusside.

One might infer that the inhibitory effect of oxygenated hemoglobin excludes such a role of EDRF under in vivo conditions since the concentrations of free hemoglobin in the plasma are in the range of 1 μM. However, our unpublished results as well as recent findings by Hogan et al also show an EDRF-mediated increase of platelet cGMP in vivo. The levels of free hemoglobin necessary to inhibit the EDRF-induced cGMP increase were much higher than physiological plasma concentrations. This is in accordance with observations in microvessels indicating that luminally released EDRF might contrib-
ute to downstream vasodilation at least over short distances. Moreover, EDRF effects on platelets in vivo may be facilitated by the presence of erythrocytes in blood that, compared with saline perfusates, enhance the margination, and hence, the endothelial contact, of platelets. The significant increase of platelet recovery during acetylcholine-induced EDRF stimulation is consistent with an increase of inhibition of aggregation and/or adhesion in the coronary bed. Oxyhemoglobin abolished this enhanced recovery just as it inhibited the increase in platelet cGMP. In contrast, the inhibition of prostaglandin I₂ formation by indomethacin did not abolish the enhanced platelet recovery in response to acetylcholine. This indicates that, in our preparation, prostaglandin I₂ was less important than EDRF as a single antiaggregating and antiadhesive factor. This is in accordance with recent in vitro findings.

Nevertheless, prostaglandin I₂ may have functional significance in potentiating the inhibitory effects of EDRF, since indomethacin reduced the increase of the acetylcholine-induced platelet recovery to some extent. This is consistent with in vitro observations showing a potentiation of the effects of EDRF and certain nitrovasodilators on platelets by prostaglandin I₂.

In summary, our study has shown that in the isolated rabbit heart there is a basal release of EDRF in amounts sufficient to increase platelet cGMP. The platelet cGMP can be further increased by pharmacological stimulation of the endothelium. The association of increased platelet cGMP levels with an augmented recovery of platelets in the coronary effluent suggests that EDRF plays an important role as inhibitor of platelet activation in the coronary circulation.

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