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In vivo EDRF activity influences platelet function

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The administration of carbachol to rabbits to stimulate the release of endothelium derived relaxing factor (EDRF) results in inhibition of platelet aggregation and elevation of platelet cyclic GMP content. These effects are reversed by simultaneous administration of the EDRF inhibitors methylene blue or haemoglobin. The data provide the first direct biochemical evidence of *in vivo* EDRF activity.

Introduction Endothelium derived relaxing factor (EDRF) and nitrovasodilators relax vascular smooth muscle (Furchgott & Zawadzki, 1980; Ignarro *et al.*, 1981; Griffith *et al.*, 1984) and inhibit platelet aggregation (Furlong *et al.*, 1987) *in vitro*. Both stimulate soluble guanylate cyclase and elevate cytosolic guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels (Ignarro *et al.*, 1981; Rapoport & Murad, 1983; Busse *et al.*, 1985). EDRF-mediated vasodilatation *in vivo* can be inferred from a number of observations, but biochemical confirmation of EDRF activity on vascular smooth muscle or platelet function *in vivo* is lacking.

In this study we have investigated the effects on platelet function of *in vivo* muscarinic stimulation in rabbits and, for comparison, of sodium nitroprusside.

Methods Cannulation of the ear artery for blood sampling from the whole animal and the major ear vein for drug administration was performed under 1% lignocaine local anaesthesia in conscious New Zealand White rabbits (2.5–3.5 kg).

Carbachol, to stimulate EDRF release (Bhardwaj & Moore, 1987), was given alone or with either methylene blue (infusion started 10 min before carbachol injection) or haemoglobin (infusion started 1 min before carbachol injection) to inhibit EDRF activity. Carbachol (50 μg in 0.2 ml) was given as a bolus. Methylene blue (2.5 ml of 1% solution) was infused over 10 min. Haemoglobin (0.3 ml of 10^{-3} M solution), prepared as previously described (Martin *et al.*, 1986), was infused over 30 s. Sodium nitroprusside (20 μg min^{-1}) was infused over 30 min.

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Arterial blood was drawn into a syringe containing anticoagulant (1:9, 3.2% trisodium citrate) and a cyclic GMP phosphodiesterase inhibitor (10^{-3} M M&B 22948 (2-O-propoxyphenyl-8-azapurin-6-one) dissolved in 1% triethanolamine). Samples were taken before and 5 min after administration of carbachol, shown from pilot studies to be the optimum time for sampling, with or without associated infusion of methylene blue or haemoglobin, and during the sodium nitroprusside infusion. On each occasion, 2 ml for aggregometry and 10 ml for measurement of platelet cyclic GMP levels were taken.

Aggregometry Arterial blood, 0.5 ml and 0.5 ml buffer (composition in mmol l^{-1}: NaCl 137, $MgCl_2$ 1.3, $CaCl_2$ 1.4, KCl 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.6, with heparin 2000 u l^{-1}) were placed in a siliconized cuvette. Platelet aggregation was measured by impedance aggregometry. The extent of platelet aggregation was measured as the increase in impedance 2 min after the addition of a threshold concentration of the aggregating agent, adenosine 5′-diphosphate (ADP).

Cyclic GMP content Platelet-rich plasma (PRP) was prepared from arterial blood by centrifugation at 560 *g* for 10 min at 40°C. One ml aliquots of PRP were further centrifuged at 2500 *g* at 4°C and the supernatant discarded. Then 1 ml 6% trichloroacetic acid was added to the pellet, vortexed for 2 min and the sample again centrifuged at 2500 *g* for 15 min at 4°C. The aqueous phase was assayed for cyclic GMP using a commercially available radioimmunoassay kit and expressed as pmol per 10^9 platelets.

Results are given as mean ± s.d., were compared by use of Student's *t* test for paired data and considered significantly different when $P < 0.05$.

Results *Aggregometry* (see Figure 1) Platelet aggregation in response to ADP was significantly less in blood samples from animals given carbachol than in blood from control animals. This difference was abolished by both methylene blue and haemoglobin. Carbachol (2.5 μg) added directly to arterial

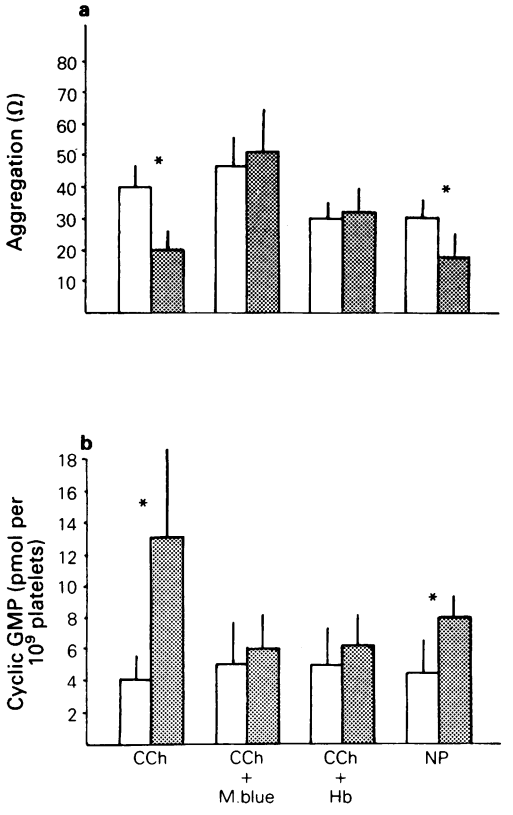

Figure 1 (a) Comparison of platelet aggregation (Ω) following treatment with carbachol (CCh), CCh and methylene blue (M. blue), CCh and haemoglobin (Hb) or sodium nitroprusside (NP). (b) Comparison of cyclic GMP levels following treatment with CCh, CCh and methylene blue, CCh and haemoglobin or nitroprusside. The open columns represent the pretreatment values and the stippled columns post-treatment values. Each column is the mean value ($n \geqslant 6$) and vertical lines indicate s.d. $^*P < 0.025$.

blood (0.5 ml) from control animals did not affect platelet aggregation.

Platelet aggregation to ADP was likewise significantly less in blood from animals given sodium nitroprusside than in blood from control animals.

Platelet cyclic GMP levels (see Figure 1) Platelet cyclic GMP content was significantly higher in blood from animals given carbachol than in blood from control animals. This difference was abolished by both methylene blue and haemoglobin.

Platelet cyclic GMP content was likewise significantly greater in blood from animals treated with sodium nitroprusside than in blood from control animals.

Discussion The data provide evidence for an *in vivo* action of EDRF and of sodium nitroprusside in platelets. They afford what is to our knowledge the first direct biochemical evidence of *in vivo* EDRF activity.

It is suggested that EDRF-mediated vasodilatation *in vivo* is localized to immediately subjacent vascular smooth muscle. This is in accord with the short half-life of EDRF as measured *in vivo* (Griffith *et al.*, 1984; Cocks *et al.*, 1985) and its deactivation by haemoglobin (Martin *et al.*, 1985), present as the haptoglobin-haemoglobin complex in plasma (Edwards *et al.*, 1986) and in red blood cells. The activity of EDRF within the vascular compartment is therefore likely to be very limited. The present study demonstrates an *in vivo* effect of intravascular EDRF on platelets which was inhibited by concurrent *in vivo* infusion of haemoglobin solution. This implies that there is some intravascular EDRF activity, though it may affect only those platelets passing in closed proximity to endothelium, and that intravascular inhibition of EDRF activity by endogenous haemoglobin is not maximal.

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