The action of diaspirin cross-linked haemoglobin blood substitute on human arterial bypass conduits


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Received 11 October 1999; received in revised form 1 March 2000; accepted 7 March 2000

Abstract

Background: Immediately available blood substitutes could transform medicine. In coronary artery surgery, vasoconstriction induced by some of these agents could have serious implications. We have examined some of the vasoactive effects of one of these blood substitute, diaspirin cross-linked haemoglobin (DCLHb), on isolated rings of human arterial conduits. Methods: Sections of human left internal mammary artery (LIMA) and radial artery (RA) were cut into 3-mm rings, mounted in individual organ baths containing aerated (95% O2/5% CO2) Krebs–Henseleit solution at 37°C and attached to isometric strain gauge for measurements of tension. All rings were tested for the presence of endothelium by addition of carbachol to rings pre-contracted with phenylephrine. The relative importance of nitric oxide (NO) in contraction mediated by the addition of DCLHb was studied. Results: Carbachol relaxed phenylephrine precontracted LIMA by 72.3 ± 1.7% and RA by 97 ± 0.7% confirming the presence of a functional endothelium. Sodium nitroprusside (SNP) caused complete relaxation of LIMA with an EC50 value of 2.0 ± 0.1 × 10−8 M and RA with an EC50 value of 1.9 ± 0.1 × 10−8 M. In the presence of DCLHb (10−7 M), carbachol-induced relaxation was significantly reduced to 46.3 ± 0.7% (P < 0.01) and the BC50 value for SNP relaxation increased to 1.2 ± 0.1 × 10−7 M (P < 0.01). DCLHb caused rings to contract in the absence of phenylephrine with EC50 values of 1.6 ± 0.1 × 10−7 M (LIMA) and 1.8 ± 0.1 × 10−7 M (RA). Presence of L-NAME (300 μM) caused no alteration in DCLHb-induced contraction. Conclusion: In this study of isolated rings of human vessels, DCLHb causes a significant reduction in relaxation mediated by carbachol and SNP, which is likely to be due to its ability to bind NO. However, it is possible that other mechanisms might contribute to the vasoconstrictor effects of DCLHb and these might be amenable to anti-vasospastic strategies. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Blood substitute; Coronary artery surgery; Internal mammary artery; Radial artery

1. Introduction

Immediately available blood substitutes could transform all branches of medicine. Difficulties with obtaining donated blood and its storage along with the risk of transmissible diseases such as hepatitis, HIV, new variant Creutzfeldt–Jacob disease and as yet unknown transmissible agents make the development and use of such products an important goal. The other major application of such oxygen carrying and releasing substitutes would be as immediately available O2 delivery vehicles in resuscitating patients with severe haemorrhage. Other potential applications exist in reperfusion of ischaemic tissue, perfusion of extra-corpooreal organs before transplantation, in sickle cell anaemia, tumour therapy and chronic anaemia [1].

The hypertensive effect of haemolyzed blood and Hb-based blood substitutes such as diaspirin cross-linked haemoglobin (DCLHb TM) are poorly understood. The use of such products in coronary surgery would be precluded if this effect caused spasm in the conduits utilized. Routine use of the left internal mammary artery (LIMA) and increased use of other arterial conduits with a known propensity for vasoconstriction could have deleterious consequences for such patients. Intravenous administration of DCLHb to rats produces a dose-dependent increase in mean arterial pressure (MAP) and reflex bradycardia that remains poorly understood [2]. A similar hypertensive effect has been observed in human subjects [3]. As yet, no study has been performed in isolated human bypass conduits to determine the effect or elucidate the reason of this vasoconstriction. This study provides an in vitro inves-
tigation of the effects of DCLHb on human arterial bypass conduits.

2. Subjects and methods

2.1. Patients

Patients (n = 32) referred for surgical coronary artery revascularization (CABO) to a single surgical group in an age range (48–69 years) gave informed consent for 3-mm sections of arterial bypass conduits to be harvested for surgery. All patients underwent a routine procedure utilizing one surgical technique. Arterial sections were harvested in a fully heparinized normothermic patient before the induction of cardiopulmonary bypass. The conduits, from which the rings for the study were harvested, were all utilized for the construction of bypass grafts. Informed consent was obtained from patients for the use of their tissue in research and the study was approved by our local Ethics Committee.

2.2. Preparation of human blood vessel rings

Sections of LIMA and radial artery (RA) were harvested at surgery. In the case of RA, sections were obtained from both proximal and distal ends of the vessel, after it had been removed for subsequent grafting. The vessels were immediately placed in glass bottles containing cold, aerated (with 95% O2/5% CO2) Krebs–Henseleit solution (composition in mM: NaCl 118, KCl 4.57, CaCl2 2.5, KH2PO4 1.10, MgSO4 1.19, NaHCO3 25 and glucose 5.55 at pH 7.4). The bottles were kept on ice during transportation to the laboratory. The total time taken for the vessels to arrive at the laboratory, after removal from the patient, was approximately 1 h.

On arrival at the laboratory, the vessels were placed in fresh, cold, aerated Krebs solution and were carefully dissected to remove all fat and connective tissue. Two to four rings of 3–4 mm in length were cut from the vessels and were each mounted between two L-shaped wire hooks in 10 ml Krebs-filled organ baths maintained at 37°C and continuously bubbled with 95% O2/5% CO2. One wire was in a fixed position within the organ bath and the other wire was attached to an isometric force transducer (Biegestab K30, Hugo Sachs Elektronik, Germany) linked via a Plugsys model 600 polygraph (Hugo Sachs Elektronik) to a Gould model TA4000 chart recorder (Gould Electronics Inc., Cleveland, OH). The distance between the wires was increased to provide passive tension on the vessels (3 g for arteries), which had been found in previous experiments to be the optimum tension to be applied to these vessels. Throughout all experiments, the cyclo-oxygenase inhibitor, indomethacin (1 × 10 μM), was present in the Krebs solution, to inhibit the production of prostanooids. Each ring was maintained at its appropriate passive tension and the Krebs solution was changed at 15-min intervals during an equilibration period of approximately 90 min for LIMA rings, and 120 min for RA rings.

2.3. Drugs

Drugs used and their sources were: diaspirein cross-linked haemoglobin (DCLHb TM) (a generous gift from Baxter Hemoglobin Therapeutics, USA), carbamyl choline chloride (carbachol) (BDH Ltd., Poole, Dorset), sodium dithio- nite, indomethacin, L-phenylephrine hydrochloride, sodium nitroprusside (sodium nitroferricyanide), 9,11-dideoxy-9α,11α-epoxymethano-prostaglandin F2α (U46619), Nω-nitro-L-arginine methyl ester (L-NAME) (all Sigma Chemical Co., Poole, Dorset). All other reagents were of analytical grade, and were obtained from Fisher Scientific UK Ltd. (Loughborough, Leicestershire).

DCLHb was diluted in distilled water to a known molar concentration, and aliquots (500 μl) were frozen −20°C until they were required.

Stock solutions of all other compounds were prepared each day. All were made up in distilled water, except for indomethacin (dissolved in 5% (w/v) sodium bicarbonate).

2.4. Experimental procedure

After the equilibration period, all blood vessel rings were contracted routinely with KCl, which was added in the form of a modified Krebs solution in which the K+ concentration had been increased to 50 mM by equimolar replacement of NaCl by KCl. This served to identify any potentially unresponsive vessels, and often helped to stabilize those rings which were showing spontaneous contraction.

The responses of each blood vessel type to the α1-adrenoceptor agonist, L-phenylephrine, were characterized by cumulative addition of the drug (concentration range 1×10−8–1×10−4 M). Contractile responses were observed and cumulative concentration–response data obtained. These data were used in subsequent experiments to select a suitable concentration of L-phenylephrine to be used to produce a submaximal contraction. On completion of the concentration–response curve, the bathing solution in the organ bath was changed three times, and once every 15 min thereafter, until the tension had returned to baseline. Typically, this took 30–45 min.

The ability of an endothelium-dependent vasodilator to cause a relaxation of the blood vessels was investigated. The rings were precontracted to a submaximal tension by the addition of L-phenylephrine (3×10−6 M). Carbachol was then added cumulatively (10−8–10−4 M), and the relaxations that occurred were plotted as concentration–relaxation curves for each blood vessel type. These data were used to select a suitable concentration of carbachol to be added in order to produce the maximum possible relaxation.

All subsequent blood vessel rings were routinely assessed for the amount of functional endothelium present. Following submaximal contraction with L-phenylephrine, a single concentration of carbachol (10−5 M) was added, and the degree of relaxation that occurred was noted.

Endothelium-independent relaxation by an endogenous
nitric oxide donor was also studied. The rings were precontracted with L-phenylephrine, and sodium nitroprusside (SNP) was added cumulatively (10^{-9}–10^{-4} M).

To investigate the effect of DCLHb on the endothelium-dependent relaxation to carbachol, LIMA rings were first precontracted with L-phenylephrine. DCLHb was then added to two of the organ baths, at concentrations of 10^{-7} M. No additions were made to the other two baths. After 10 min, carbachol was added cumulatively to each bath until no further relaxation occurred in any ring. The same protocol was used, for each of the two blood vessel types, to study the effects of DCLHb on the endothelium-independent relaxations to both SNP.

The direct effects of DCLHb on blood vessel tone were then considered. Cumulative additions (10^{-9}–10^{-4} M) of the compound were made to rings at basal tension, and any contractions were recorded and plotted as concentration-response curves.

2.5. Data and statistical analysis

The data for responses to contractile agents were expressed as percentages of the maximum contraction of each blood vessel ring to the agent, since rings from adjacent parts of the same vessel could vary by up to 4-fold in the maximum tension generated. Relaxation responses to vasodilators were expressed as percentages of the initial level of precontraction, for each ring.

Log concentration–response data were plotted using KaleidaGraph software (Nbelbeck Software, version 3.05). Data were fitted to sigmoid curves by a non-linear, least squares regression method.

Data are presented as mean ± SD. Blood vessels from relatively few patients could be used for the various part of the study. For this reason, no error bars are shown on the figures, since the data cannot be assumed to reflect the results of a wider, more generalized population. It was possible, however, to calculate the statistical significance of any effect of DCLHb on vascular reactivity within each experiment without drawing assumptions about a wider population. As rings from different vessels and different patients had to be used for different part of the experiments, the unpaired Student’s t-test was applied to test the significance of any differences in EC_{50} values and maximum responses within a particular investigation, which was accepted at the P < 0.05 level.

3. Results

3.1. Vasoconstrictor responses to L-phenylephrine

Concentration-dependent increases in isometric tension were observed in the presence of L-phenylephrine in all blood vessel types. Calculated EC_{50} values were 3.7 ± 0.4 × 10^{-7} M for RA (n = 8) and 4.7 ± 0.3 × 10^{-7} M for LIMA (n = 8). From these curves, an appropriate concentration of L-phenylephrine was selected to produce submaximal (approximately 80% of maximum) contractions in subsequent experiments. The selected concentration was 3 × 10^{-6} M, for both RA and LIMA.

3.2. Vasorelaxant responses to carbachol, an endothelium-dependent vasodilator

Following preconstriction with a concentration of L-phenylephrine giving a submaximal response, concentration-dependent relaxation to carbachol was observed in LIMA and RA rings. Calculated EC_{50} values were 1.2 ± 0.2 × 10^{-7} M for LIMA (n = 2) and 2.6 ± 0.1 × 10^{-7} M for RA (n = 4). RA rings demonstrated a much greater maximum relaxation to carbachol (96.7 ± 0.7%) compared with the maximum response of LIMA rings to the same vasodilator (72.3 ± 1.7%), P < 0.0001. In contrast to RA rings, no LIMA ring demonstrated 100% relaxation of L-phenylephrine-induced tone in response to carbachol. A carbachol concentration of 10^{-5} M was identified as sufficient to induce near maximum relaxations and thus to serve as an indicator of the amount of functional endothelium present. Of RA rings, 73.7% had more than 60% of a functional endothelium, compared with only 39% of LIMA vessels.

3.3. Vasorelaxant responses to endothelium-independent vasodilators

SNP, a NO donor, induced full (100%), concentration-dependent relaxation of L-phenylephrine-induced tone, in all vessel types. The concentration-response curves obtained are shown for RA (n = 2) and LIMA (n = 7). Calculated EC_{50} values were 1.9 ± 0.1 × 10^{-8} M for RA and 2.0 ± 0.1 × 10^{-8} M for LIMA.

3.4. Effect of DCLHb on carbachol- and SNP-induced vasorelaxation

When DCLHb (10^{-6} M) was added to LIMA rings (n = 2) during carbachol-induced relaxation, a rapid reversal of the relaxation was observed and, typically, the tone of the ring was seen to rise above the initial level of L-phenylephrine-induced tension. Pretreatment of LIMA for 10 min with DCLHb (10^{-7} M) resulted in an inhibition of the relaxation induced by cumulative addition of carbachol (Fig. 1). In the presence of DCLHb (10^{-7} M), the maximum relaxation was decreased from 72.3 ± 1.7% to 46.3 ± 0.7%, which, for this particular investigation, was statistically significant (P < 0.01).

In the RA (n = 2), pretreatment with DCLHb (10^{-7} M) for 10 min produced an inhibition of the endothelium-independent relaxation to SNP, with a parallel shift of the concentration–relaxation curve to the right (Fig. 2). In LIMA, pretreatment with DCLHb (10^{-5} M) caused a concentration-dependent inhibition of the relaxation induced by SNP, with parallel shifts of the concentration–relaxation curves to the right (Fig. 2). In LIMA (n = 3), the
3.5. Effect of DCLHb on basal tone.

Addition of DCLHb to RA and LIMA rings at basal tension resulted in a concentration-dependent contraction of the rings, which increased by a maximum of 3.54 ± 1.22 g in RA and 3.42 ± 0.49 g in LIMA. The concentration–response curve obtained for LIMA is shown (n = 6) in Fig. 3. Calculated EC50 values to DCLHb were 1.8 ± 0.1 × 10⁻⁷ M for RA and 1.6 ± 0.1 × 10⁻⁷ M for LIMA. Following addition of DCLHb, the tension was not easily brought back to baseline, despite repeated washouts, and it was often necessary to add SNP to cause a relaxation of the vessels.

3.6. Effect of l-NAME on DCLHb-induced vasoconstriction

Pretreatment with l-NAME for 30 min failed to abolish the vasoconstrictor effects of DCLHb (10⁻⁶ M) on RA and LIMA rings. In fact, the presence of l-NAME tended to potentiate the contractile response, although such an effect failed to reach statistical significance (n = 6).

4. Discussion

In this study, we have demonstrated an inhibition of carbachol-induced relaxation of phenylephrine pre-constricted arterial conduits, which supports the concept that DCLHb binds endothelium-derived NO to cause vasoconstriction. In addition, a small but significant degree of relaxation remained, in both LIMA and RA rings, when the production of NO had been blocked by the inhibitor, l-NAME. This provides evidence to suggest the involvement of a separate endothelium-derived factor, distinct from NO. We have demonstrated that DCLHb causes a concentration-dependent increase in basal tension of isolated LIMA and RA rings, which could contribute to a hypertensive effect if used in vivo.

This study utilized an in vitro model system to determine the vasoconstrictor responses of isolated rings of human arterial bypass conduits. The vasopressor response mechanism responsible for increases in mean arterial pressure when DCLHb is infused into humans has been proposed to be due to the involvement of NO [4,5], adrenoreceptors [6] and endothelin [4]. The ability of DCLHb to bind or inactivate NO has been demonstrated in porcine pulmonary artery and vein [7]. Carbachol induces relaxation by binding to G-protein-linked muscarinic receptors on the surface of the endothelial cell, thus increasing the synthesis of NO [8]. Inhibition of carbachol-induced relaxation supports the concept of DCLHb binding of endothelium-derived NO to induce vasoconstriction. We noted also that the isometric tone of each ring overshoot the level present before the addition of carbachol, which suggests the possibility of an additional NO-independent action. Not all of the relaxation induced by carbachol is mediated through NO because when production of NO is blocked by the presence of the inhibitor, l-NAME, a small but significant degree of relaxation remains in LIMA and RA rings. This supports further the concept of the release of an additional factor from the endothelium [9].

The vasoconstrictor effects of DCLHb have been shown...
induced contraction in dog basilar artery [11]. The elucidation of a mechanism for such observations could have important clinical implications in the development of anti-vasopressor strategies for all arterial conduits during CABG.

In conclusion, the shifts in concentration response relations to carbachol and SNP in this study suggest that DCLHb binds to NO, but NO-independent mechanisms cannot be ruled out. We have confirmed that DCLHb causes a concentration-dependent increase in basal tension for human arterial bypass conduits, which could contribute to the known hypertensive effects of DCLHb infusion in humans. The goal of clinically available blood substitutes remains a reality with immense potential for all aspects of medicine. Elucidation of the mechanism and effects of DCLHb in relation to arterial bypass conduits for use in coronary artery surgery provides insight into mechanisms and future clinical applications.

Acknowledgements

This work was supported in part by Baxter Hemoglobin Therapeutics, 2545 Central Ave., Suite FD1, Boulder, CO 80301, USA.

References