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Hemoglobin causes both endothelium-dependent and endothelium-independent contraction of the pig coronary arteries, independently of an inhibition of EDRF effects

J.-L. Bény, P. C. Brunet and V. Van der Bent

Département de Biologie Animale, Université de Genève, 3, place de l'Université, CH-1211 Genève 4 (Switzerland)
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Summary. Hemoglobin is widely used as an inhibitor of EDRF effects. Hemoglobin contracts pig coronary arteries in vitro. However, during this contraction, effects of substance P and bradykinin which act via the EDRF are not inhibited. This means that the hemoglobin contraction is not caused by inhibition of the EDRF. This contraction is caused by a substance released from the endothelium, and by eicosanoïds released from the smooth muscles.
Key words. Coronary artery; oxyhemoglobin; endothelium; EDRF; electrophysiology; eicosanoïds.

Oxyhemoglobin has been shown to be a vasoconstrictor for cerebral arteries [1, 2]. Consequently, oxyhemoglobin could be the cause of cerebral vasospasms which follow subarachnoid hemorrhage. The contraction caused by oxyhemoglobin is more pronounced for cerebral arteries, yet it also contracts other arteries, among them the coronary arteries [1]. These observations were made before the discovery of the role played by the endothelium in vasodilation [3]. This implies that the studies undertaken previously were done without considering the possible role of a functional endothelium. Since the discovery of the endothelial-derived relaxing factor (EDRF), oxyhemoglobin has been extensively used as an inhibitor of the vasodilation caused by EDRF [4]. In this context, vasoconstriction caused by oxyhemoglobin on many arteries has been interpreted as a result of suppression of the relaxation caused by the EDRF [5]. Yet destruction of the endothelium in cerebral arteries does not inhibit the vasoconstriction caused by oxyhemoglobin [6]. This demonstrates that oxyhemoglobin may induce arterial constriction by at least two distinct mechanisms: an inhibition of the EDRF and a direct action on smooth muscles.

In the present study we investigate whether oxyhemoglobin contracts pig coronary arteries by inhibiting EDRF or by a direct action on smooth muscle, or both together. The endothelium-dependent relaxation in pig coronary arteries is characterized by a concomitant hyperpolarization [7, 8]. We used this hyperpolarization as an indication of endothelium-dependent relaxation. Thus measurement of smooth muscle membrane potential together with isometric tension were performed in this study.

We report here that oxyhemoglobin contracts pig coronary arterial strips in vitro in two ways: by an action on smooth muscles via eicosanoïds and by the release from the endothelium of a contracting substance, and not by inhibiting the EDRF. The existence of both an endothelium-dependent and an endothelium-independent vasoconstriction of coronary arteries caused by oxyhemoglobin could be important in cardiac physiopathology.

Materials and methods. Preparation of tissues. The anterior descending branches of coronary arteries were obtained from freshly killed pigs. The coronary lumen was rinsed by injection of cold oxygenated Krebs solution (mM: NaCl 118.7, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 24.8, $MgSO_4$ 1.2, glucose 10.1; 4 °C; pH 7.3). Segments of the coronary artery were cleaned of all adherent fat and connective tissue. Then they were cut into 2-mm-wide rings which were cut to give strips of about 5 mm in length. For some experiments, the endothelium was removed by gently rubbing the internal face of the strip with a cotton-tip. To check that the endothelium was removed by this procedure, a 0.5% w/v solution of Evans blue in Krebs solution was applied for 10 s to the strip, which was then washed with Krebs solution [9]. The luminal face of a strip with intact endothelium remained white whereas a de-endothelialized strip was colored blue. The response of the strip to substance P produces a marked endothelial-dependent relaxation of precontracted smooth muscle [10]. The absence of such a response, plus the positive Evans blue staining, was taken as evidence for the complete removal of the endothelium. When intact and de-endothelialized strips were compared as in figure 1, they were from adjacent coronary rings.

Two types of in vitro experiment were performed. In each type of experiment, changes in tension were measured isometrically (Grass force displacement transducer FT03C) and amplified (Lectromed 3559). Contractile responses were recorded on chart paper with polygraphs (W + W Electronics).

In each experiment, Krebs solution was pumped to the preparation from a siliconized glass or plastic beaker with a peristaltic pump. Oxyhemoglobin and peptides were applied to the preparations by diluting them directly in the beaker containing the superfusion solution. To avoid the production of mechanical artifacts during the experiment all changes in perfusate were achieved without the introduction of bubbles into the tissue chamber [7].

Pharmacological experiments. To measure the tension only, strips were suspended in a 85-µl bath [11] using two silk threads attached to the edges of the strips in parallel with the circular smooth muscles. Strips were continuously superfused with oxygenated Krebs solution (1.250 µl/min) main-

tained at 36 °C. For each strip, a tension of 10 mN was applied initially, and allowed to stabilize at about 5 mN before measurements. This usually required about 30–40 min. In order to establish the concentration-response curves, the agonists were superfused during 3 min with Krebs solution containing each concentration of the peptide in an ascending, non-cumulative manner with a 10-min wash-out between successive concentrations.

Electrophysiological experiments. Mechanical tension and transmembrane potential were measured simultaneously. The strip was incubated in a 100-µl perspex bath continuously perfused with oxygenated Krebs solution (1.250 µl/min) at 36 °C. One extremity of the strip was pinned to a silicon rubber surface with the intimal face facing up. The other extremity was fixed horizontally to the isometric force transducer. A force of 10 mN was applied to the strip by pulling the transducer with a micromanipulator. Experiments were begun after the tension stabilized at about 5 mN. The membrane potential was measured with a conventional glass microelectrode (80 MΩ) filled with 3 M-KCl. The cells were impaled near the fixed points of the tissue, i.e. near the pins, in order to reduce problems associated with muscle movement.

Preparation of peptides and chemicals. Substance P and bradykinin (Bachem Feinchemikalien, Switzerland) were prepared at a concentration of 1 mg/ml in 0.25% acetic acid. They were stored as aliquots or 50 µl and kept frozen at −20 °C until use. The peptides were diluted subsequently with Krebs solution to the desired concentrations.

Oxyhemoglobin was prepared by oxidizing bovine methemoglobin (Fluka) with sodium dithionite ($Na_2S_2O_4$)[4], followed by dialysis to remove sodium dithionite. The solution of oxyhemoglobin was frozen in aliquots and kept at −20 °C until used. To check that traces of sodium dithionite could not be responsible for the phenomena we studied, 0.1 µM sodium dithionite was applied to the coronary strip. No effects were observed in 4 independent trials.

Indomethacin (Sigma) was prepared at a concentration of 1 mg/ml in absolute ethanol. Nordihydroguaiaretic acid was from Fluka and acetylcholine (ACh) from Sigma.

Data analysis. Data were calculated as the mean ± standard error of the mean (SEM). Student's t-test was used to compare results; $p < 0.05$ was deemed to be significant.

Results. Oxyhemoglobin caused a long-lasting, concentration-dependent contraction of pig coronary artery strips with intact endothelium (fig. 1). The liminal concentration is 10 nM. A reliable ED_{50} cannot be calculated because the supramaximal concentration was not determined (more than 10 µM oxyhemoglobin foamed too much in the bubbled Krebs solution). In order to determine whether the endothelium could be implicated, the concentration-response curve was also determined using a de-endothelialized strip adjacent to the intact one. When the endothelium was removed, oxyhemoglobin still produced a contraction, but one which was more long-lasting (fig. 1). The amplitude of this endothelium-independent contraction was 45.4 ± 7.7% (n = 9) of the endothelium-dependent one (fig. 1). Since in pig coronary artery strips with intact endothelium, ACh causes an endothelium-independent contraction[7], we used this property to evaluate the effects of lesions caused by de-endothelialization. The latter reduced the ACh contraction by 13 ± 8% (n = 9). We concluded that the diminution of the oxyhemoglobin contraction caused by the de-endothelialization (45.4%) could not be entirely attributed to the lesions caused by the technique of de-endothelialization we used. Thus, at least one third (45% minus 13%) of the hemoglobin contraction could be attributed to the endothelium.

We also tested the non-oxidized form of hemoglobin, methemoglobin, which produced a smaller contraction than oxyhemoglobin: 61 ± 7% (n = 4) of the oxyhemoglobin

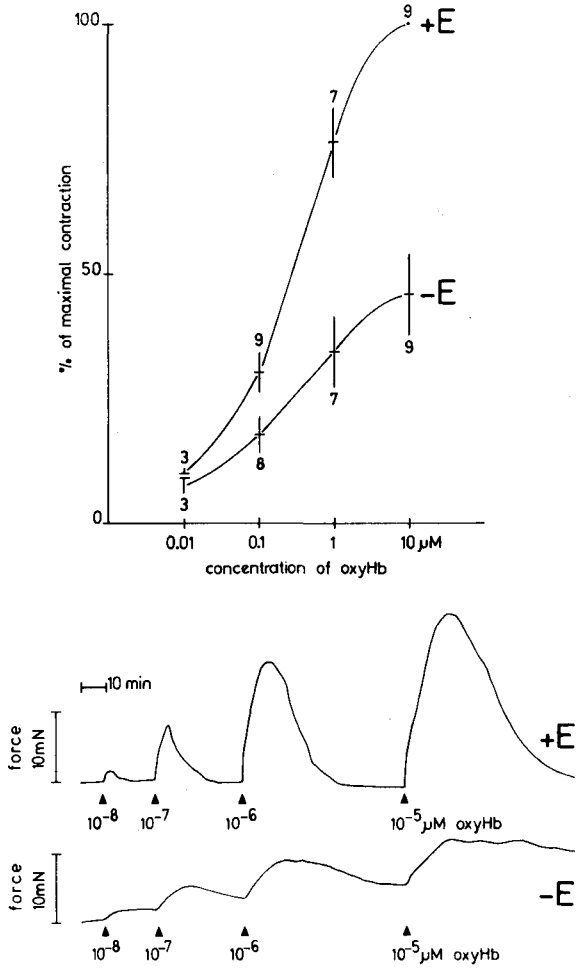

Figure 1. Effect of oxyhemoglobin (oxyHb) on the mechanical isometric tension of pig coronary artery transversal strip. Upper panel: concentration-response curve. The response of the strip is expressed in percent of the response for 10 µM oxyHb. Values are mean ± SEM (vertical bar). Number are numbers of experiments. +E: strip with intact endothelium; −E: strip without endothelium. Lower panel: original recording. The arrows indicate the time of oxyHb application. The isometric tension is expressed in millinewton (mN).

contraction for a strip with an intact endothelium and 68 ± 8% (n = 4) of the oxyhemoglobin contraction when the strip was de-endothelialized. The endothelium-dependent relaxation is characterized by a hyperpolarization of the smooth muscle[8, 12]. Therefore we checked whether oxyhemoglobin contraction was accompanied by an inhibition of the EDRF hyperpolarization. Oxyhemoglobin did not significantly ($p = 0.07$) change the smooth muscle membrane potential of a strip with intact endothelium: the membrane potential was − 44.6 ± 1.8 mV (n = 13) before application of oxyhemoglobin and − 43.5 ± 1.1 mV (n = 15) when oxyhemoglobin contracted the strip.

Substance P and bradykinin induced endothelium-dependent relaxations which were accompanied by hyperpolarizations of 10–17 mV (depending upon the state of contraction of the strip)[7, 8]. Thus, if the contraction caused by oxyhemoglobin treatment of the intact strip was produced by an inhibition of the EDRF, the effects of the two kinins would also be inhibited. Perfusion of oxyhemoglobin inhibited neither relaxations (fig. 2) nor hyperpolarizations caused by substance P and bradykinin. In the presence of oxyhemoglobin (10 µM) the hyperpolarization which accompa-

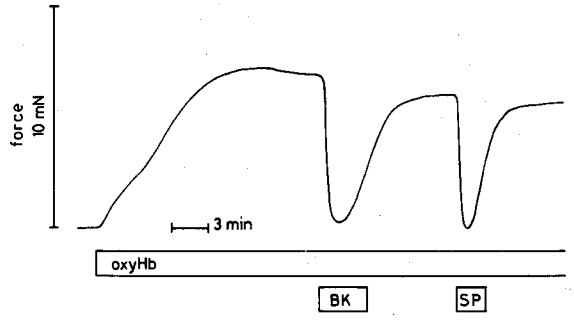

Figure 2. Original recording. When 10 μM oxyhemoglobin (oxyHb) had fully contracted the coronary artery transversal strip, substance P (SP) and bradykinin (BK) relaxed the strip via the endothelium. This demonstrates that the contraction caused by oxyhemoglobin is not due to an inhibition of the endothelial derived relaxing factor effect.

nied the relaxation caused by bradykinin was 11.7 ± 1.3 mV (n = 4) and that which accompanied substance P-induced relaxation was 18.5 ± 4 mV (n = 4). These results suggest that oxyhemoglobin does not contract the intact strip by inhibiting the EDRF. An eicosanoïd could be the contracting factor released by the endothelium, because prostaglandins E_2 and $F_{2\alpha}$ both contract pig coronary arteries. To test this hypothesis, indomethacin (10 μM), an inhibitor of cyclooxygenase, and nordihydroguaiaretic acid (10 μM), an inhibitor of the 5-lipoxygenase, were used. The results are shown in figure 3. Inhibition of cyclooxygenase or of 5-lipoxygenase diminished the oxyhemoglobin contraction of a strip with and without endothelium to some extent. On the other hand, though the inhibition of cyclooxygenase plus 5-lipoxygenase did not totally inhibit the oxyhemoglobin contraction of strips with intact endothelium (black column, fig. 3), in this situation, removal of the endothelium abolished the contraction.

Discussion. Removal of the endothelium significantly depolarizes smooth muscles, by 3 mV in pig coronary arteries[8] and by 9 mV in rabbit aorta[12]. The lack of change of the membrane potential of the pig coronary artery smooth muscles when contracted by oxyhemoglobin is an indication that oxyhemoglobin does not contract coronary strip by inhibiting the effect of a continuous release of EDRF. Another evidence is that both the relaxations and the hyperpolarizations caused by substance P and bradykinin were not inhibited when oxyhemoglobin had maximally contracted the strip, even though substance P and bradykinin relax and hyperpolarize pig coronary arteries by releasing an EDRF[7,8]. These two arguments show that oxyhemoglobin contracts smooth muscles of pig coronary arteries by other mechanisms than an inhibition of the EDRF effect.

Oxyhemoglobin produced greater endothelium-dependent and -independent contractions than did methemoglobin. A possible explanation could be a configurational change that exists between these two forms, one oxidized and the other non-oxidized. Removal of the endothelium diminished the amplitude of the response by 32%. This shows that a component of the contraction caused by oxyhemoglobin is furnished by the endothelium. The existence of a diffusible vasoconstrictor substance has been proved in canine arteries, in particular in the coronary artery[13]. The release of this substance is stimulated by hypoxia[13]. Endothelial cells in culture also release into the culture medium a vasoconstrictor substance that contracts rabbit[14] and bovine coronary arteries[15]. This last substance is a peptide[15] which has been recently characterized[16]. The existence of an endothelium-derived constricting factor is thus well established. In the present study, at least a part of the endothelium-mediated contraction was not inhibited by inhibitors of prostanoid and leukotrien synthesis (black column, fig. 3). This suggests that a contracting substance released from the endothelium stimulated by oxyhemoglobin could be a substance other than leukotrienes and prostanoids, such as endothelin[16]. Another part of the contraction induced by hemoglobin is produced by direct action on smooth muscle, probably via eicosanoïds. A physiological role for such contracting substances released by oxyhemoglobin from the endothelium and the smooth muscle remains to be established and could be important in the context of cardiac pathology. In particular the effect of cyclooxygenase inhibitors could partially explain the protective effect of salicylate following infarction.

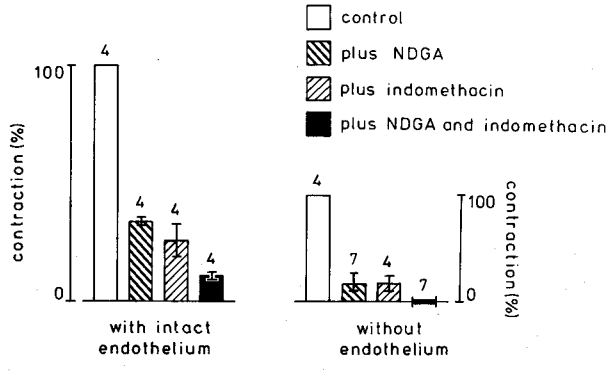

Figure 3. Effect of indomethacin and nordihydroguaiaretic acid (NDGA) on the contraction of isolated pig coronary arteries strip caused by 10 μM oxyhemoglobin. The contraction is expressed in percent of control. Number of experiments is indicated at the bottom of each column. The scale in the right panel is 45% smaller than that of left panel in order to respect the reduction of oxyhemoglobin contraction caused by the de-endothelialization (see fig. 1).

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