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Evidence that mechanisms dependent and independent of nitric oxide mediate endothelium-dependent relaxation to bradykinin in human small resistance-like coronary arteries

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1 The effects of the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine (L-NOARG), the NO scavenger, oxyhaemoglobin (HbO) and high extracellular K^+ upon endothelium-dependent relaxation to bradykinin were investigated in human isolated small coronary arteries.

2 Endothelium-dependent relaxations to bradykinin were compared in vessels contracted to ~50% of their maximum contraction to 124 mM KCl Krebs solution, regardless of treatments, with the thromboxane A_2 mimetic, U46619 and acetylcholine. All relaxations were expressed as percentage reversal of the initial level of active force.

3 L-NOARG (100 μM) caused a small but significant, 12% ($P<0.01$), decrease in the maximum relaxation (R_{max}: 91.5±5.4%) to bradykinin but did not significantly affect the sensitivity (pEC_{50}: 8.08±0.17). Increasing the concentration of L-NOARG to 300 μM had no further effect on the pEC_{50} or R_{max} to bradykinin. HbO (20 μM) and a combination of HbO (20 μM) and L-NOARG (100 μM) reduced R_{max} to bradykinin by 58% ($P<0.05$) and 54% ($P<0.05$), respectively. HbO (20 μM) and L-NOARG (100 μM, combined but not HbO (20 μM) alone, caused a significant 11 fold ($P<0.05$) decrease in sensitivitiy to bradykinin. HbO (20 μM) decreased the sensitivity to the endothelium-independent NO donor, S-nitroso-N-acetylpenicillamine (SNAP), approximately 17 fold ($P<0.05$).

4 Raising the extracellular concentration of K^+ isotonically to 30 mM, reduced the R_{max} to bradykinin from 96.6±3.1% to 43.9±10.1% ($P<0.01$) with no significant change in sensitivity. A combination of HbO, L-NOARG and high K^+ (30 mM) abolished the response to bradykinin. High K^+ did not change either the sensitivity or maximum relaxation to SNAP.

5 In conclusion, L-NOARG does not completely inhibit endothelial cell NO synthesis in human isolated small coronary arteries. By comparison, HbO appeared to block all the effects of NO in this tissue and revealed that most of the relaxation to bradykinin was due to NO. The non-NO -dependent relaxation to bradykinin in the human isolated small coronary arteries appeared to be mediated by a K^+-sensitive vasodilator mechanism, possibly endothelium-derived hyperpolarizing factor (EDHF).

Keywords: Endothelium; nitric oxide; hyperpolarization; human coronary artery

Introduction

The endothelium plays an important role in the control of vascular tone via the release of a number of vasodilator substances including endothelium-derived relaxing factor (EDRF, Furchgott & Zawadzki, 1980), identified as nitric oxide (NO, Palmer *et al*., 1987), prostacyclin (PGI_2; Moncada *et al*., 1976) and endothelium-derived hyperpolarizing factor (EDHF, for review see Taylor & Weston, 1988; Komori & Vanhoutte, 1990; Garland *et al*., 1995). In the coronary vasculature, there is evidence to suggest that endothelium-dependent relaxations of resistance-like arteries are mediated predominantly by non-NO endothelium-derived relaxing factors. Specifically, endothelium-dependent relaxations in resistance-like coronary arteries from the pig (Tschudi *et al*., 1991), rat perfused heart (Baydoun & Woodward, 1991; Fulton *et al*., 1994) and intact dog heart (Sudhir *et al*., 1994) are relatively resistant to L-arginine analogue inhibitors of nitric oxide synthase (NOS) such as N^G-nitro-L-arginine (L-NOARG), its methyl ester, L-NAME, and N^G-monomethyl-L-arginine (L-NMMA). The lack of effect of these NOS inhibitors together with the ability of K^+ channel inhibitors to block endothelium-dependent responses in coronary resistance-like arteries, at least in the rat (Fulton *et al*., 1994), has been attributed to the release from the endothelium of EDHF.

Similarly, *in vivo* studies in man have suggested that non-NO factors may also contribute to endothelium-dependent relaxation in coronary resistance-like arteries. Thus, Lefroy *et al*. (1993) found that L-NMMA did not prevent the ACh-induced increase in coronary blood flow and thus vasodilatation of coronary resistance vessels in man. However, the nature of this non-NO response in human coronary resistance arteries remains unknown.

Therefore, the aim of the present study was to assess the relative contribution of NO and non-NO, non-prostanoid mechanisms to endothelium-dependent relaxation in human isolated small resistance-like coronary arteries. In order to achieve this, we evaluated the effect of two independent inhibitors of NO, the L-arginine analogue, L-NOARG, and the NO scavenger, oxyhaemoglobin (Martin *et al*., 1986), both alone and in combination upon the response to the endothelium-dependent vasodilator, bradykinin. Furthermore, the contribution of EDHF to bradykinin-mediated relaxation was assessed by raising the extracellular concentration of K^+ in order to inhibit K^+ channel activity and thus hyperpolarization (Chen & Suzuki, 1989). Our results indicate that L-NOARG does not completely inhibit NO synthesis in human small coronary arteries. Furthermore both NO and a K^+ channel-dependent relaxation mechanism, possibly EDHF, mediate endothelium-dependent relaxation in human coronary resistance-like arteries.

Methods

Tissue source

Small coronary arteries were obtained from the discarded tip of the right atrial appendage from patients (63.0±1.7 years; 25

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male, 4 female) undergoing mitral valve ($n=2$), aortic valve ($n=4$) or coronary bypass graft surgery ($n=23$). Following surgical removal, the atrial appendage was placed in cold oxygenated Krebs solution and transported to the laboratory. The segment of atrial appendage was viewed under a dissecting-light microscope and small coronary arteries carefully freed of surrounding tissue.

Mounting of vessels in the myograph

Coronary arteries were cut into 2 mm lengths and mounted on 40 μm wires in a small vessel Mulvany-Halpern myograph as previously described (Angus *et al.*, 1988). Briefly, one wire was attached to a force transducer to measure changes in isometric tension which were recorded on dual channel flatbed recorders (W & W Scientific Instruments, Basel, Switzerland) and the other wire to a micrometer which was used to measure the distance between the two wires. Vessels were maintained in physiological Krebs-solution at 37°C and continuously oxygenated with carbogen (95% O_2, 5% CO_2). The Krebs solution was composed of (in mM): Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11, pH 7.4. Some arteries were revolved around the support wires a number of times in order to remove the endothelium.

Normalization

After a 30 minute equilibration period, vessel rings were maximally relaxed with the β-adrenoceptor agonist, isoprenaline (1 μM), and then set to passive tensions equivalent to that required to produce 90% of their internal circumference when exposed to a transmural pressure of 100 mmHg (refer to Mulvany & Halpern, 1977; Angus *et al.*, 1986). In brief, a passive length-tension curve was constructed in each vessel. From this curve, the effective transmural pressure was calculated and the vessel set at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg (D_{100}).

Experimental protocol

Following normalization, vessel segments were washed three times and left to equilibrate for 30 min. Indomethacin (3 μM) was then added to inhibit the release of prostanoids (PGI_2) and 30 min later vessels were contracted with a depolarizing physiological salt solution (KPSS) containing isotonic 124 mM KCl. Once the KPSS-induced contraction had reached a plateau ($KPSS_{max}$), the tissues were washed, indomethacin (3 μM) reapplied and the force allowed to return to baseline. Isoprenaline (1 μM) was then added to reduce the high level of inherent tone which is characteristic of these arteries. This allowed the full range over which the arteries could contract to be determined. Subsequently, either sequential concentration-relaxation curves to bradykinin or single relaxation responses to S-nitroso-N-acetylpenicillamine (SNAP) were constructed in vessel segments. All concentration-response curves were obtained in the presence of isoprenaline (1 μM) in order to prevent the spontaneous contraction of these arteries.

Bradykinin relaxations

Responses to the endothelium-dependent vasodilator, bradykinin, were compared in vessels contracted to approximately 50% of their maximum contraction to KPSS with titrated concentrations of the thromboxane A_2 mimetic, U46619 (0.001–0.9 μM). If U46619 did not cause a sufficient contraction then acetylcholine (ACh) was additionally applied (0.0001–4.5 μM). ACh was used as it is a constrictor of human small coronary arteries and fails to cause endothelium-dependent relaxation in these vessels (Angus *et al.*, 1991). Once the U46619 and ACh contraction had reached a plateau, cumulative concentration-relaxation curves to bradykinin were constructed. Vessels were then washed, indomethacin (3 μM) reapplied and 30 min allowed to elapse before vessels were again maximally relaxed with isoprenaline (1 μM), precontracted with U46619 and ACh and a second concentration-dependent relaxation curve to bradykinin constructed.

Between the first and second concentration response curves to bradykinin, vesssels were either left untreated or treated with inhibitors of NO and non-NO mediated relaxation as outlined below. Thus, each vessel served as its own control.

Effect of L-NOARG and HbO

Vessels were treated with L-NOARG (100 or 300 μM), HbO (10 μM) or a combination of L-NOARG (100 μM) and HbO (10 μM) for 20 min before precontraction with U46619 and ACh. Once a stable level of active force has been reached, tissues which had been treated with HbO (10 μM; either alone or in combination with L-NOARG) were treated with a further concentration of HbO (10 μM) to compensate for any denaturing of the protein that may have occurred during precontraction.

Effect of high extracellular K^+

The contribution of K^+ channels to bradykinin-mediated relaxation was assessed by using a high K^+ (30 mM) isotonic Krebs solution either alone or in combination with L-NOARG (100 μM) and HbO (20 μM). In the high K^+ Krebs solution, 25 mM NaCl was substituted with 25 mM KCl to produce a final K^+ concentration of 30 mM.

SNAP relaxations

Following normalization and contraction with KPSS, isoprenaline (1 μM) was added and vessels were left untreated or treated with 30 mM KCl Krebs or HbO (10 μM). Vessels were then precontracted to approximately 50% of their maximum contraction to KPSS with titrated concentrations of U46619 (0.001–0.8 μM) and ACh (0.02-0.1 μM). Once the U46619 contraction had reached a plateau, extra HbO was added and cumulative concentration-dependent relaxation curves to SNAP were then constructed.

Drugs

Drugs used and their sources were: U46619 ([1,5,5-hydroxy-11α, 9α-(expoxymethano)prosta-5Z, 13E-dienoic acid], Upjohn, Kalamazoo, MI, U.S.A.); acetylcholine bromide, ionomycin, indomethacin, (−)-isoprenaline bitartrate salt, N^G-nitro-L-arginine (L-NOARG), bovine haemoglobin (Sigma, U.S.A.); S-nitroso-N-acetylpenicillamine (Sapphire Bioscience, NSW, Australia). Stock solutions of U44619 (1 mM) were made up in absolute ethanol, L-NOARG (100 mM) in 1 M $NaHCO_3$ and indomethacin (100 mM) in 1 M Na_2CO_3. Haemoglobin was dissolved in 0.9% NaCl to make up a 1 mM stock solution. The stock solution was subsequently reduced to HbO by the addition of a small amount (<0.1 g) of sodium dithionite. Excess sodium dithionite was extracted by running the solution through a sephadex (PD-10) column equilibrated with 0.9% NaCl. All subsequent dilutions of stock solutions were in distilled water and all other drugs were made up in distilled water.

Statistical analysis

Responses to bradykinin and SNAP were expressed as a percentage reversal of the level of precontraction. Contractile responses were measured as a percentage of the maximum contraction to KPSS ($KPSS_{max}$). The individual relaxation curves were fitted (Graphpad Prism, version 1.00) to the sigmoidal logistic equation,

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}/1 + 10^{(pEC_{50}-X)\cdot n_H}$$

where X = the logarithm of the agonist concentration and Y = the response; Bottom = the lower response plateau, Top = the upper response plateau and pEC_{50} is the X value when the response is halfway between Bottom and Top. The variable Hillslope (n_H) controls the slope of the curve. From this relationship, computer estimates of pEC_{50} values were determined and expressed as $-\log$ M. pEC_{50} values could not be determined for bradykinin relaxation curves where the maximum relaxation was $<10\%$ reversal of the level of precontraction.

The significance of differences in mean pEC_{50} and maximum relaxation (R_{max}) values within tissues were tested by use of two-tailed Student's paired t test. Comparisons of pEC_{50} and R_{max} values between more than two experimental groups were performed by one way analysis of variance (ANOVA). If the F statistic exceeded the critical value, then Dunnett's modified t statistic was used to make comparisons between the control and treatment groups. Results are expressed as mean $\pm$ s.e. mean and statistical significance was accepted at the $P<0.05$ level.

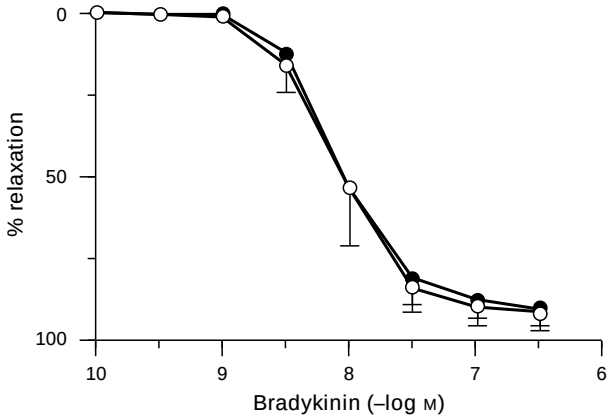

Figure 1 Consecutive relaxation curves to bradykinin in human isolated small coronary arteries ($n=4$). (○) and (●) represent the first and second concentration-response curves, respectively. The levels of precontraction with U46619 and ACh (expressed as $\%KPSS_{max}$) were (○) 57.2 ± 7.1 and (●) 63.4 ± 6.8. Responses are expressed as a percentage reversal of the initial level of precontraction to U46619 and ACh. Indomethacin (3 μM) was present throughout. Values are mean and vertical lines show s.e.mean; n = number of rings.

Results

Bradykinin relaxations

Bradykinin caused concentration-dependent relaxations ($R_{max}=91.5\pm5.4\%$, $pEC_{50}=8.08\pm0.17$) in small coronary arteries ($D_{100}=203.9\pm8.5$ μm) in which the endothelium was intact (Figure 1). No relaxation response was observed to either bradykinin or the calcium ionophore, ionomycin (0.3 μM), in vessels were the intima was damaged by mechanical abrasion (data not shown) and this was taken as evidence of endothelial cell removal. The maximum relaxation and sensitivity to bradykinin did not differ between arteries obtained from patients undergoing aortic or mitral valve replacement ($R_{max}=87.5\pm4.1\%$, $pEC_{50}=7.75\pm0.14$) and coronary bypass graft surgery ($R_{max}=92.8\pm2.2\%$, $pEC_{50}=7.98\pm0.09$). Concentration-relaxation response curves to bradykinin showed no time-dependent change in sensitivity or R_{max} between the first and second response curves (Figure 1).

The effect of L-NOARG and HbO on responses to bradykinin

L-NOARG (100 μM) caused a small but significant ($P<0.05$) reduction in the R_{max} to bradykinin from $90.6\pm4.6\%$ to $79.4\pm6.4\%$ (Figure 2a). L-NOARG also tended to reduce the sensitivity to bradykinin 8 fold but this change failed to reach statistical significance ($P=0.07$, Figure 2a). Increasing the concentration of L-NOARG to 300 μM had no greater inhibitory effect upon the response to bradykinin ($R_{max}=84.9\pm5.3\%$, $pEC_{50}=7.30\pm0.42$, $n=3$) than 100 μM L-NOARG. By contrast, both HbO (20 μM) and a combination of HbO (20 μM) and L-NOARG (100 μM) significantly decreased the R_{max} to bradykinin to $40.6\pm15.0\%$ and $39.3\pm15.1\%$, respectively (Figure 2b and c). The combination of HbO and L-NOARG but not HbO alone, reduced the sensitivity to bradykinin 11 fold ($P<0.05$) (Figure 2c).

The effect of high K^+ on responses to bradykinin

Raising the extracellular concentration of K^+ to 30 mM significantly decreased the R_{max} to bradykinin from $96.6\pm3.1\%$ to $43.9\pm10.1\%$ ($P<0.01$) with no significant change in sensitivity (Figure 3a). In the presence of high K^+, the combination of L-NOARG and HbO abolished the response to bradykinin (Figure 3b).

The effect of high K^+ and HbO on responses to SNAP

The endothelium-independent NO-donor, SNAP, relaxed small coronary arteries $96.4\pm1.6\%$ ($pEC_{50}=6.55\pm0.25$)

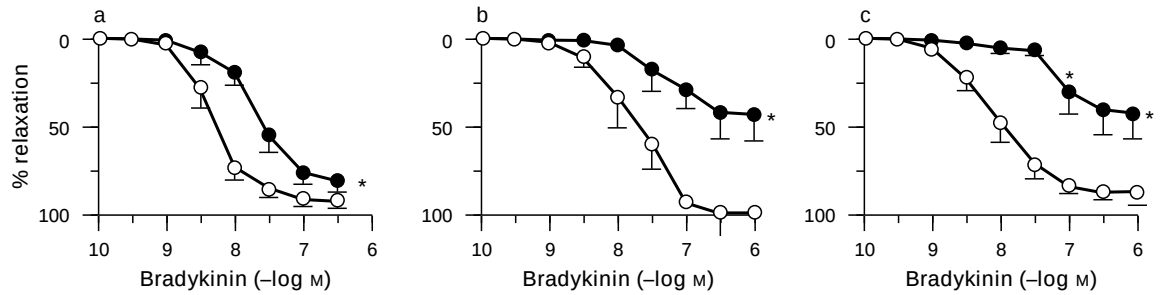

Figure 2 Relaxations to bradykinin in human isolated small coronary arteries in (a, $n=5$) the absence (○) and presence (●) of N^G-nitro-L-arginine (L-NOARG, 100 μM), (b, $n=6$) the absence (○) and presence (●) of oxyhaemoglobin (HbO, 20 μM) and (c, $n=6$) the absence (○) and presence (●) of a combination of HbO (20 μM) and L-NOARG (100 μM). The levels of precontraction with U46619 and ACh (expressed as % $KPSS_{max}$) were (O)47.7 ± 1.2 and (●) 66.4 ± 11.3 in (a), (○) 57.4 ± 5.8 and (●) 59.6 ± 9.1 in (b) and (○) 51.2 ± 4.7 and (●) 43.7 ± 3.5 in (c). Responses are expressed as a percentage reversal of the initial level of precontraction to U46619 and ACh. Indomethacin (3 μM) was present throughout. Values are mean and vertical lines show s.e.mean; n = number of rings. Asterisks indicate R_{max} or pEC_{50} values significantly different from control (*$P<0.05$ Student's paired t test).

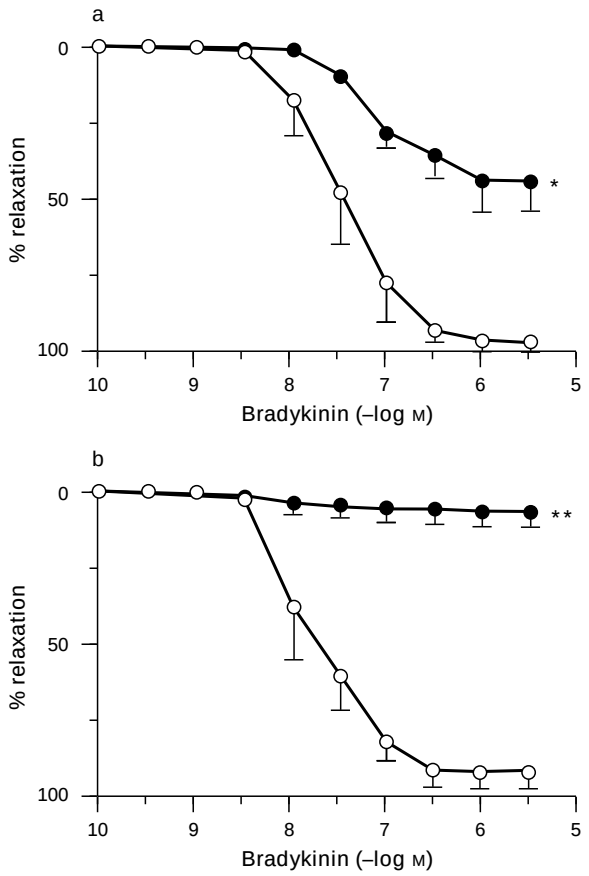

Figure 3 Relaxations to bradykinin in human isolated small coronary arteries in (a, $n=4$) the presence of normal Krebs (○) and 30 mM KCl Krebs (●) and (b, $n=5$) the presence of normal Krebs (○) and a combination of 30 mM KCl Krebs, N^G-nitro-L-arginine (L-NOARG, 100 μM) and oxyhaemoglobin (HbO, 20 μM; ●). The levels of precontraction with U46619 and ACh (expressed as % $KPSS_{max}$) were (○) 54.5 ± 7.0 and (●) 71.4 ± 5.4 in (a), and (○) 50.6 ± 6.1 and (●) 58.9 ± 4.6 in (b). Responses are expressed as a percentage reversal of the initial level of precontraction to U46619 and ACh. Indomethacin (3 μM) was present throughout. Values are mean and vertical lines show s.e.mean; n = number of rings. Asterisks indicate R_{max} values significantly different from control (*$P<0.01$; **$P<0.001$, Student's paired t test).

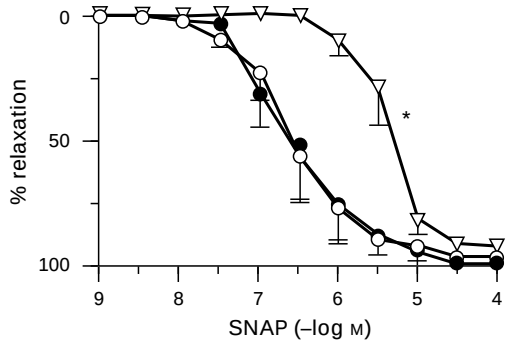

Figure 4 Relaxations to S-nitroso-N-acetylpenicillamine (SNAP) in human isolated small coronary arteries in the presence of normal Krebs (○, $n=4$), 30 mM KCl Krebs (●, $n=3$) and oxyhaemoglobin (HbO 20 μM; △, $n=3$). The levels of precontraction with U46619 and ACh (expressed as % $KPSS_{max}$) were (○) 47.7 ± 14.5, (●) 57.9 ± 18.3 and (V) 38.1 ± 8.2. Responses are expressed as a percentage reversal of the initial level of precontraction to U46619 and ACh. Indomethacin (3 μM) was present throughout. Values are mean and vertical lines show s.e.mean; n = number of rings. *Indicates pEC_{50} value significantly different from control ($P<0.05$, Dunnett's modified t-statistic).

(Figure 4). Raising the extracellular concentration of K^+ (30 mM) did not significantly change either the sensitivity ($pEC_{50}=6.56\pm0.30$) or R_{max} ($98.7\pm0.8\%$) to SNAP (Figure 4). By contrast, HbO (20 μM) caused a significant 17 fold decrease in sensitivity to SNAP ($pEC_{50}=5.33\pm0.15$, $P<0.05$) with no change in R_{max} ($92.3\pm1.7\%$) (Figure 4).

Discussion

This study is the first to demonstrate that both NO and non-NO mechanisms mediate endothelium-dependent relaxation to bradykinin in human small isolated coronary arteries. The non-NO relaxations were mediated by a K^+-sensitive mechanism and thus may have been due to EDHF.

Relaxations to the endothelium-dependent vasodilator, bradykinin, were relatively resistant to the potent NOS inhibitor, L-NOARG (Mulsch & Busse, 1990), with 80% of the maximum response to bradykinin remaining in the presence of L-NOARG. Similarly, endothelium-dependent relaxations in pig isolated small coronary arteries (Tschudi *et al*., 1991), rat isolated perfused heart (Baydoun & Woodward, 1991) and in the intact human (Lefroy *et al*., 1993) and dog (Sudhir *et al*., 1994) coronary circulations have been shown to be largely unaffected by L-arginine analogue inhibitors of NOS. Taken together these findings suggest that non-NO factors mediate endothelium-dependent relaxations in resistance-like coronary arteries. However, we wished to establish that the lack of effect of L-NOARG upon bradykinin responses in human small isolated coronary arteries did not simply reflect incomplete inhibition of NOS. Thus, we examined the effect of the NO scavenger, HbO (Martin *et al*., 1986), upon the relaxation to bradykinin in the absence and presence of L-NOARG.

Unlike L-NOARG, HbO alone significantly decreased the maximum relaxation to bradykinin by 58%. Furthermore, HbO appeared to abolish the NO-mediated responses to bradykinin in human isolated small coronary arteries, as the combination of HbO and L-NOARG did not have a greater inhibitory effect than HbO alone and the same concentration of HbO caused an approximate 17 fold decrease in sensitivity to the NO-donor SNAP. Nevertheless, the ability of high concentrations of SNAP to overcome the block by HbO may indicate that high concentrations of NO are released by bradykinin and that the remaining response in the presence of HbO could still be due to NO. However, this is unlikely, as combined treatment with L-NOARG, which alone caused a significant inhibition of the response to bradykinin, gave no further block of the maximum relaxation than for HbO alone. Therefore, we suggest that under our bioassay conditions, 20 μM HbO appeared to block all the effects of any NO released by bradykinin. These findings contrast with those from a previous study in the rat isolated perfused heart in which HbO, at a concentration of 10 μM, failed to affect bradykinin-mediated vasodilatation, whereas L-NOARG reduced the duration but not the magnitude of the bradykinin response (Baydoun & Woodward, 1991). Such a discrepancy may reflect differences between species, experimental preparations, the higher concentration of HbO (20 μM) used in the present study or the attempt to limit protein denaturation in the organ bath by adding fresh HbO just before the construction of the bradykinin relaxation curve. The greater inhibitory effect of HbO compared with L-NOARG observed here did not reflect an impaired ability of the smooth muscle to relax, as an additional application of the β-adrenoceptor agonist, isoprenaline, or SNAP caused maximal relaxation in the presence of HbO. Thus, in the human small isolated coronary artery, L-NOARG does not completely inhibit NO synthesis and HbO is a more effective inhibitor of NO-mediated relaxation.

The reason for the apparent inability of L-NOARG to block completely relaxations to NO in the present study is unclear. It is unlikely that the concentration of L-NOARG (100 μM) employed was insufficient to inhibit NOS completely given that a concentration of 15 nM is required to achieve half-maximal

inhibition of purified constitutive bovine brain NOS (Furfine *et al*., 1993) and maximal effects of L-NOARG have been obtained at concentrations less than 10 μM in bovine cultured aortic endothelial cells (Stork & Cocks, unpublished observations) and pig isolated large coronary arteries (Kilpatrick & Cocks, 1994). Furthermore, in the present study, increasing the concentration of L-NOARG to 300 μM had no further inhibitory effect on bradykinin relaxations. Alternatively, the lack of effect of L-NOARG on endothelium-dependent relaxations may have occurred due to the release of NO from a source other than L-arginine, an excess of L-arginine in the endothelial cell, impaired uptake or increased metabolism of L-NOARG. Regardless of the underlying cause of the poor inhibitory ability of L-NOARG, these present findings highlight the potential for error if it is assumed these L-arginine analogues completely inhibit NOS.

Interestingly, in pig small isolated coronary arteries the guanylate cyclase inhibitor, methylene blue, has been found to impair bradykinin relaxations to a slightly greater degree than the L-arginine analogue, L-NMMA (Tschudi *et al*., 1991). Furthermore, in the human intact coronary circulation, ACh induced vasodilatation of coronary resistance vessels has been shown to be unaffected by L-NMMA (Lefroy *et al*., 1993) yet abolished by methylene blue (Hodgson & Marshall, 1989). Our present findings may offer an explanation for such a discrepancy. Specifically, complete block of NO-mediated vasodilatation in the coronary resistance arteries is achieved with inhibitors such as HbO and methylene blue but not with L-arginine analogues such as L-NOARG and L-NMMA.

Given the assumption that all NO is removed by co-treatment with HbO and L-NOARG, NO appeared to mediate most of the response to bradykinin over the concentration range which gave approximately 70% of the maximum response. At higher concentrations of bradykinin other non-NO mechanisms appeared to be activated and were able to mediate 42% of the maximum relaxation. The non-NO response to bradykinin was not due to prostacyclin since all vessels were pretreated with the cyclo-oxygenase inhibitor, indomethacin. However, the non-NO relaxation was abolished upon raising the extracellular concentration of K^+, which suggests it involved the opening of K^+ channels and subsequent smooth muscle hyperpolarization. In support of this hypothesis, bradykinin has been shown to cause endothelium-dependent hyperpolarization of vascular smooth muscle cells in human large coronary arteries (Nakashima *et al*., 1993). Although NO itself has been shown to cause hyperpolarization of vascular smooth muscle (Tare *et al*., 1990; Garland & McPherson, 1992; Plane *et al*., 1995) it is unlikely that NO-mediated hyperpolarization contributed to the relaxation in human small coronary arteries as raising the extracellular concentration of K^+ did not affect the response to the NO donor, SNAP.

A further consideration is that raising the extracellular concentration of K^+ may have decreased the release and synthesis of NO due to a decrease in the driving force for calcium entry into the endothelial cell (Luckhoff & Busse, 1990). However, this is unlikely, since in preparations such as the rabbit basilar artery (Plane & Garland, 1993) and thoracic aorta (Cowan *et al*., 1993), in which endothelium-dependent relaxations are mediated predominantly by NO, raising the extracellular concentration of K^+ to 65 mM and 25 mM, respectively, did not attenuate the response to ACh. Furthermore, cultured endothelial cells only release NO in response to bradykinin and this release is unaffected by high extracellular K^+ (67 mM) (Drummond & Cocks, unpublished data). Also, it is unlikely that high extracellular K^+ antagonized NO-mediated responses in human small coronary arteries since the response to the NO donor, SNAP, was unchanged in the presence of high K^+ Krebs.

Unlike previous findings in the pig (Kilpatrick & Cocks, 1994) and bovine large isolated coronary arteries (Drummond & Cocks, 1996), the K^+-dependent mechanism, 'EDHF', did not appear to function as a 'backup' mechanism for NO in the human small coronary arteries. Rather the observation that high K^+ alone attenuated the maximum response to bradykinin suggests that EDHF can mediate endothelium-dependent relaxation in these vessels in the presence of NO. The types of K^+ channels activated by EDHF and the mechanism via which the subsequent hyperpolarization mediates smooth muscle relaxation in human small coronary arteries remains to be elucidated.

In conclusion, this study highlights the importance of using different inhibitors of NO in order to assess the contribution of NO to endothelium-dependent relaxation. Furthermore, if the present findings can be extrapolated to the *in vivo* situation, then in man endogenously released bradykinin regulates coronary blood flow (Groves *et al*., 1995) via NO and a K^+-sensitive mechanism, possibly EDHF.

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