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Heterogeneity of glyceryl trinitrate response in isolated bovine coronary arteries

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Abstract

Factors determining heterogeneity of response to glyceryl trinitrate in coronary microvessels have been extensively documented in recent years, but determinants of heterogeneity between conduit and large resistance vessels are poorly understood. The current study has characterised heterogeneity to glyceryl trinitrate and other vasodilators in bovine isolated proximal (4.5 mm i.d.) and distal (0.5 mm i.d.) segments of left anterior descending artery. Compared with proximal segments, distal segments were less responsive to glyceryl trinitrate and sodium nitroprusside, equi-responsive to *S*-nitroso-*N*-acetylpenicillamine, and more responsive to isoprenaline. Heterogeneity to glyceryl trinitrate was unaffected by the presence of the thiols (cysteine or *N*-acetylcysteine, 100 μM). The results are interpreted as evidence that heterogeneity of vascular responsiveness to glyceryl trinitrate reflects impairment in the small artery of the cellular events which precede activation of the cyclic GMP pathway. An implication is that the impairment is not a consequence of limited thiol availability, and in this respect the cellular mechanism of heterogeneity differs from those proposed for the coronary microvasculature.

Keywords: Vascular heterogeneity; Coronary artery, bovine; Glyceryl trinitrate; *S*-Nitroso-*N*-acetylpenicillamine; Sodium nitroprusside; Isoprenaline

1. Introduction

It has long been recognised that the coronary vasodilator action of glyceryl trinitrate is more prominent in large epicardial arteries than in resistance vessels (Fam and McGregor, 1968; Winbury et al., 1969; Cohen and Kirk, 1973; Macho and Vatner, 1981; Simonetti et al., 1989). Recent studies on isolated coronary arteries from dog and pig heart have shown that there is also substantial heterogeneity of the response to glyceryl trinitrate between large and small resistance vessels, i.e., between microvessels with diameters of the order of 0.2–0.3 mm and ≤ 0.1 mm respectively (Sellke et al., 1990, 1991; Harrison and Bates, 1993; Wheatley et al., 1994). Since *S*-nitrosothiols did not display heterogeneity and the presence of a thiol (cysteine or *N*-acetylcysteine) largely restored the response of the smaller microvessels to glyceryl trinitrate, it was proposed that the weak response of the small microvessels to glyceryl trinitrate reflected impaired biotransformation to the active metabolite (nitric oxide or *S*-nitrosothiol) and that this was due to limited availability of thiols.

Other studies on isolated coronary arteries of dog heart have demonstrated considerable heterogeneity between epicardial proximal segments (2–3 mm i.d.) and distal segments (0.4–0.6 mm i.d.) (Schnaar and Sparks, 1972; Tsukada et al., 1984). Whether the mechanism responsible for heterogeneity at this more macro level of vascular diameter is similar to that proposed for the microvessels is uncertain, since responses to glyceryl trinitrate of the large microvessels in the studies of Sellke et al. (1990) were insensitive to thiols.

In the present study, we have explored the occurrence and mechanism of heterogeneity in bovine coronary arteries. To this end we have compared the effects of glyceryl trinitrate in proximal (termed large) and distal (termed small) segments of the left anterior descending artery with those of other nitric oxide donors (*S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside) and with an agent (isoprenaline) which uses the adenylate cyclase-cyclic AMP pathway instead of the guanylate cyclase-cyclic GMP pathway to elicit vasodilation.

The choice of bovine vessels was influenced by previous studies in this laboratory which had provided extensive documentation of the effects of the above agents on the large epicardial left anterior descending artery (Henry et al., 1989a,b; Zhang et al., 1994). However, heterogeneity

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in the bovine coronary vasculature has not previously been analysed.

2. Materials and methods

2.1. Materials

Bradykinin acetate, L-cysteine, (−)-isoprenaline HCl, *N*-acetyl-L-cysteine, sodium nitroprusside and U46619 (9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$) were purchased from Sigma (St. Louis, MO, USA). Glyceryl trinitrate was purchased from Fisons (Australia) and *S*-nitroso-*N*-acetylpenicillamine from Colour your Enzyme (Bath, Ontario, Canada). Stock solutions were made up in either ethanol (glyceryl trinitrate, U46619, *S*-nitroso-*N*-acetylpenicillamine) or distilled water (bradykinin, cysteine, isoprenaline, *N*-acetylcysteine, sodium nitroprusside) and stored at −20°C. Dilutions of the stock solutions were made in distilled water and maintained on ice.

The Krebs solution was gassed with carbogen (95% O_2/5% CO_2) and was of the following composition (mM): NaCl (118), KCl (3.89), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01) and glucose (5.56), pH 7.4. High K^+ solution, referred to as potassium physiological salt solution (KPSS), was obtained by replacing the NaCl in Krebs solution with iso-osmolar KCl.

2.2. Artery preparation

Fresh bovine heart was transported from the abattoirs to the laboratory in ice-cold Krebs solution. Vessels were dissected immediately and stored in ice-cold Krebs solution. Large artery rings (approximately 3 mm long) were dissected from the left anterior descending artery. Two stainless steel wires were inserted through the lumen and the preparation was bathed in Krebs solution in a 15 ml glass organ bath. One of the wires was fixed and the other attached to a Grass FTO3 isometric transducer. Small artery rings (approximately 2 mm long) were dissected from intramural branches of the left anterior descending in the apical region of the heart. Two stainless steel wires (0.04 mm diameter) were inserted through the lumen and the segment bathed in Krebs solution in a Mulvany stainless steel microbath (12 ml volume). One of the wires was fixed and the other attached to the myograph force transducer (DSC-6/MMH).

2.3. Baseline tension

The large artery rings were maintained under a tension of 3 g in early experiments, but in latter experiments they were 'normalised' to a tension level (4.6 ± 0.2 g in 22 arteries) where the circumference was 90% (IC_{90}) of the value at 100 mmHg. The normalising procedure of Mulvany (1992) (see also Mulvany and Aalkjaer, 1990) was used, i.e., the artery was progressively stretched to obtain a length-tension curve which was then analysed by the Basic program 'Normalisation' to obtain values of the diameter at 100 mmHg and of the tension corresponding to the IC_{90}. The two conditions (3 g or normalised) are not distinguished in the results since paired comparisons ($n = 4–7$) failed to reveal significant differences between EC_{50} values or E_{max} values (glyceryl trinitrate, *S*-nitroso-*N*-acetylpenicillamine, sodium nitroprusside and isoprenaline tested).

Baseline tensions of all small arteries were normalised at their IC_{90} levels. The automated procedure of Mulvany (1992) (see also Mulvany and Aalkjaer, 1990) was used for this purpose.

2.4. Experimental procedure

After a period of 1–2 h when the preparations had equilibrated in Krebs at 37°C and baseline tensions were established, the small and large arteries were treated according to the following protocol.

(i) The contractile response to K^+ was determined by replacing the Krebs solution with KPSS. This was done to establish the viability of the preparation; preparations failing to respond were discarded.

(ii) The vasorelaxant effect of bradykinin was measured as an index of endothelial function. The artery was first contracted with U46619 (0.1 μM) and the bradykinin applied cumulatively. The results refer only to those vessels displaying relaxation to bradykinin of 45% or greater; in most experiments the maximum relaxation exceeded 80%.

(iii) U46619 was applied cumulatively, commencing with 0.003 μM, in order to establish a steady state level of contraction appropriate for measuring vasorelaxant activity. In most experiments the concentration of U46619 required was 0.03 or 0.1 μM. These concentrations were in the high but submaximal range (see Fig. 1 in Section 3). The relative levels of the responses to U46619 in the small and large arteries were compared by expressing the levels as a percentage of the second contractile response to K^+ elicited at the completion of each experiment.

(iv) The vasorelaxants in the sequence, glyceryl trinitrate, *S*-nitroso-*N*-acetylpenicillamine or sodium nitroprusside and isoprenaline were applied cumulatively, commencing with a concentration of 0.001 or 0.0001 μM. The above order of sequence was based on earlier evidence that there is little if any cross-tolerance to *S*-nitroso-*N*-acetylpenicillamine or sodium nitroprusside following glyceryl trinitrate (Henry et al., 1989b; Zhang et al., 1994) and that neither of these agents produces cross-tolerance to an agent (theophylline), which like isoprenaline acts by mechanisms not involving nitric oxide or guanylate cyclase activation (Henry et al., 1989b).

In a number of experiments, the effect of thiols (cysteine and *N*-acetylcysteine; each 100 μM) on the vasore-

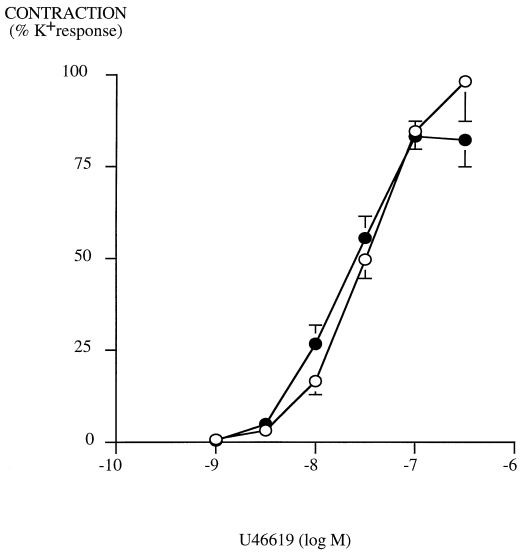

Fig. 1. Concentration-contractile response curves to U46619 in small (○) and large (●) arteries, $n = 25$ except at the highest concentration (0.3 μM) where $n = 7$. The contractile response is expressed as a percentage of the response to K^+ elicited after U46619 washout.

laxant response to glyceryl trinitrate was examined. Artery rings were exposed to the thiols 30 min prior to addition of glyceryl trinitrate.

2.5. Data analysis

The response to the relaxant agent was expressed as percent decrease in contractile tone. Sigmoid curves of best fit to the concentration-response curves for relaxants, from which EC_{50} and E_{max} values were calculated, were obtained using the non-linear regression programs Graphpad Prism 1.01 and Kaleidagraph 3.0.1. The concentration-response curves to glyceryl trinitrate in small arteries tended to be biphasic and to possess an intermediate plateau. Where the plateau was well defined, the curve up to this level (termed phase-1) was analysed as above to establish whether it conformed to a sigmoid relationship. If so an 'EC_{50}' and a phase-1 maximum were determined.

Significance of differences between EC_{50} values (in log units) or E_{max} values in small and large arteries were determined by Student's paired *t*-test. *n* refers to the number of animals unless otherwise stated.

3. Results

3.1. Artery diameters

Intraluminal diameter of artery rings estimated at 100 mmHg distending pressure were:- small 0.52 ± 0.02 mm, $n = 49$ arteries; large, 4.54 ± 0.11 mm, $n = 37$ arteries.

3.2. Contractile response to U46619

Small and large arteries displayed similar sensitivities to U46619 with thresholds and maxima in the vicinity of 0.003 and 0.3 μM respectively (Fig. 1). The mean levels of contraction at which relaxant responses were measured, when expressed as a percentage of the final response to K^+, were $71 \pm 7\%$ in the small arteries and $86 \pm 3\%$ in the large arteries ($n = 25$). The somewhat lower levels in the small arteries reflected a tendency for the response to U46619 to decline after reaching its peak level; usually the decline persisted for 10–20 min before the response became steady.

3.3. Relaxant response to bradykinin

Most arteries relaxed in response to bradykinin. Small arteries were less sensitive to bradykinin (Table 1) but their maximum responses ($83 \pm 2\%$, $n = 49$ arteries) were similar to those in large arteries ($86 \pm 1\%$, $n = 86$ arteries).

3.4. Relaxant responses to glyceryl trinitrate and effect of thiols

Small arteries were less responsive than large arteries to glyceryl trinitrate (Fig. 2A). The difference was characterised by a tendency for the small but not the large arteries to display a biphasic concentration-response curve, whose first phase (termed phase-1) plateaued at about 50% relaxation in the 0.3–3 μM range. At higher concentrations there was further relaxation (termed phase-2). In the 0.3–3 μM range, the large arteries were almost fully relaxed.

In contrast with the differences in magnitudes of relaxation in this concentration range, EC_{50} values based on the

Table 1
Vasodilator responses [a] of small and large arteries

Agent	*n*	EC_{50} [b] (nM)		Maximum [c] (%)	
		Small	Large	Small	Large
GTN	14	43 (29–63) [d]	30 (22–41)	52 ± 6 [d]	92 ± 2 [e]
SNAP	8	138 (55–348)	143 (58–351)	90 ± 2	92 ± 4
SNP	8	618 (164–2330)	112 (43–288) [e]	93 ± 2	93 ± 2
ISO	7	1.2 (0.64–2.1)	13 (6.5–28) [e]	96 ± 1	95 ± 1
BK	18	8.8 (4.9–16)	2.5 (1.6–3.8) [e]	88 ± 2	84 ± 3

[a] The data referred to arteries which displayed 45% or greater relaxation to bradykinin at the commencement of the experiment. [b] The EC_{50} results are expressed as mean (±95% confidence limits). [c] The percent maximum results are expressed as mean ± S.E.M. [d] The maximum and EC_{50} for glyceryl trinitrate in the small arteries refer only to the phase-1 response and the concentration required to achieve 50% of this response. All other data in the table refer to the maximum response and the concentration required to achieve 50% of the maximum response. [e] Difference between small and large vessels is significant ($P < 0.05$, paired *t*-test). Abbreviations: *n*, number of animals; GTN, glyceryl trinitrate; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; ISO, isoprenaline; BK, bradykinin.

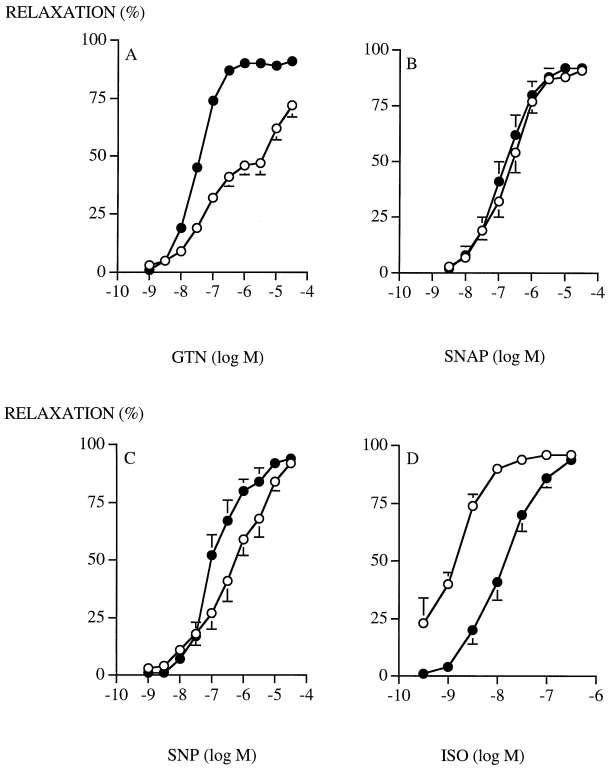

Fig. 2. Concentration-relaxant response curves to glyceryl trinitrate (GTN; A, $n = 25$), *S*-nitroso-*N*-acetylpenicillamine (SNAP; B, $n = 8$), sodium nitroprusside (SNP; C, $n = 8$) and isoprenaline (ISO; D, $n = 7$) in small (○) and large (●) arteries.

phase-1 maxima in the small arteries were little different from EC_{50} values based on maxima in large arteries (Table 1).

Neither L-(−)-cysteine (100 μM, $n = 6$) nor *N*-acetylcysteine (100 μM, $n = 3$) augmented the responses to glyceryl trinitrate in small arteries (Fig. 3). In accord with earlier observations (Henry et al., 1989a), thiols were also without effect on glyceryl trinitrate responses in the large arteries.

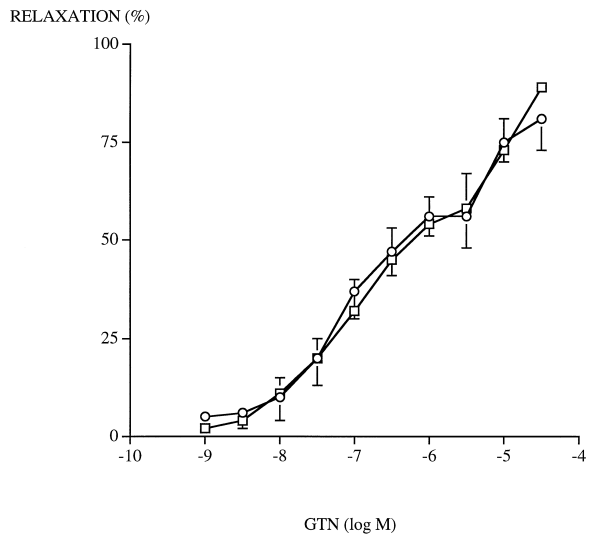

Fig. 3. Concentration-relaxant response curves to glyceryl trinitrate (GTN) in small arteries in the absence (○) and presence (□) of 100 μM cysteine ($n = 6$).

3.5. Relaxant response to S-nitroso-N-acetylpenicillamine

Responses to *S*-nitroso-*N*-acetylpenicillamine in small and large arteries were characterised by sigmoid concentration-response curves (Fig. 2B) with maxima in the 90–100% range; EC_{50} values did not differ significantly (Table 1).

3.6. Relaxant response to sodium nitroprusside

Small arteries were less responsive than large arteries to sodium nitroprusside. The difference was significant in the middle although not in near-threshold or maximum regions of the concentration-response curves (Fig. 2C). The net effect was that the EC_{50} was about 5-fold greater in the small arteries.

3.7. Relaxant response to isoprenaline

Responses to isoprenaline in small and large arteries displayed sigmoid concentration-response curves with maxima in the 90–100% range (Fig. 2D). The small arteries were more sensitive, the mean EC_{50} being about an order of magnitude less than in the large arteries (Table 1).

4. Discussion

Compared with the large arteries, the small arteries were less responsive to bradykinin, glyceryl trinitrate and sodium nitroprusside, equi-responsive to *S*-nitroso-*N*-acetylpenicillamine and more responsive to isoprenaline.

It is to be noted that bradykinin was used only to assess the functional state of the endothelium since it is known that the endothelium exerts an inhibitory effect on responses to nitric oxide donors (Shirasaki and Su, 1985; Pohl and Busse, 1987; Luscher et al., 1989; Busse et al., 1989; Tschudi et al., 1991). The decreased effect of bradykinin suggests that there may have been greater loss of endothelium function in the distal segment, but other explanations are possible, for example, a diminished effect of endothelium-derived relaxing factor on the smooth muscle. Our data do not permit these possibilities to be distinguished, but it is pointed out that loss of endothelium in the smaller segments would be expected to enhance rather than diminish the relaxant activity of the nitric oxide donor agents. Implications of a diminished effect of endothelium-derived relaxing factor, to heterogeneity to the exogenous nitric oxide donors, are obscured by evidence that a substantial part of the endothelium-derived relaxing factor released by bradykinin in the bovine coro-

nary artery consists of hyperpolarising factor rather than nitric oxide or congener (Holzmann et al., 1994).

The weaker activity of glyceryl trinitrate in the small arteries was associated in most instances with a biphasic concentration-response curve, characterised by an intermediate plateau in the 0.3–3.0 μM range. There was little indication of a biphasic concentration-response curve in the large arteries where, in the same concentration range, relaxation usually exceeded 90% and was maximal. However, the results of earlier studies (Henry et al., 1989a,b) in which the effects of glyceryl trinitrate in bovine left anterior descending proximal segments were examined, provide clear evidence for a biphasic concentration-response curve under conditions (induction of tolerance) where the response to glyceryl trinitrate is depressed. Hence it is reasonable to regard the maximum response of the large artery as a phase-1 maximum whose magnitude made it difficult to detect any further relaxation. Despite the large difference between the phase-1 maxima, EC_{50} values derived from these maxima did not differ significantly between large and small arteries. An implication is that the heterogeneous action of glyceryl trinitrate in the bovine coronary artery reflects differences in efficacy rather than potency. In this respect the bovine artery appears to differ from the dog coronary artery where the results of Tsukada et al. (1984) indicate that a rightward shift of the concentration-response curve is the most prominent feature of the diminished response to glyceryl trinitrate in the distal segment.

The comparative effects of the nitrovasodilators, *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside, were examined to shed light on possible mechanisms of heterogeneity. Their selection was based on evidence that (a) they elevate cyclic GMP (Ignarro et al., 1981) and hence are likely to share with glyceryl trinitrate the nitric oxide activated guanylate cyclase-cyclic GMP pathway leading to relaxation and (b) they differ from glyceryl trinitrate with respect to the mechanism of nitric oxide generation. The generation of nitric oxide from the nitrovasodilators occurs spontaneously during decomposition as well as by membrane catalysed or enzymic processes (Kowaluk and Fung, 1990; Kowaluk et al., 1992). However, there is substantial evidence from studies on tolerance and cross-tolerance (Henry et al., 1989b) as well as recent direct evidence (McGuire et al., 1994) indicating that nitrovasodilators do not utilise the enzyme systems responsible for nitric oxide generation from glyceryl trinitrate. It follows from these considerations that the absence of heterogeneity of responsiveness to *S*-nitroso-*N*-acetylpenicillamine can be interpreted to mean that the activities of the cellular processes following guanylate cyclase activation are unimpaired in the small artery. Accordingly the effects of *S*-nitroso-*N*-acetylpenicillamine would appear to link heterogeneity with events preceding rather than following guanylate cyclase activation, hence favouring impaired biotransformation as the factor responsible for the weaker activity of glyceryl trinitrate in the small artery. However, this interpretation is complicated by the finding that sodium nitroprusside did exhibit significant heterogeneity. Bioconversion of sodium nitroprusside to release nitric oxide is also partially enzymatic (Kowaluk et al., 1992). Hence it is possible that there may also be relative impairment of this process in small arteries. Similar heterogeneity to the nitric oxide donors, sodium nitrite (Schnaar and Sparks, 1972) and SIN-1 (Coughlan et al., 1993) have been documented in dog coronary arteries (*S*-nitrosothiols not examined in those studies). Together these results suggest that there is impairment at the level of nitric oxide activation of guanylate cyclase, in the small artery. Further experiments using nitric oxide as the vasodilator may indicate whether this suggestion is correct, but if so, it is difficult to explain why this impairment is without effect on the responses to *S*-nitroso-*N*-acetylpenicillamine. It is conceivable that the difficulty reflects the simplistic nature of the assumption that the cyclic GMP pathway is common to the nitrovasodilators and to glyceryl trinitrate so that the difference between the vascular actions reflects differences in the cellular mechanisms which precede activation of the cyclic GMP–protein kinase G pathway. The assumption is now open to doubt in view of recent evidence that in rat aorta, a cyclic GMP analogue which inhibits protein kinase G abolishes the response to glyceryl trinitrate but has less effect on the response to sodium nitroprusside and is without effect on the response to *S*-nitroso-*N*-acetylpenicillamine (Brooks and Majewski, 1995). The result draws attention to the possibility, yet to be tested, that heterogeneity may reflect differing activities within the sequence of processes which follow guanylate cyclase activation. Also we cannot exclude the possibility that some of the relaxant effects of the nitric oxide donors used in this study are not mediated by cyclic GMP, in view of evidence of cyclic GMP-independent relaxation by nitric oxide in rabbit aorta (Bolotina et al., 1994) and in rat colon (Takeuchi et al., 1996).

The role of thiols was explored in view of evidence that biotransformation of glyceryl trinitrate leading to guanylate cyclase activation in artery homogenates is thiol dependent, whereas activation by *S*-nitrosothiols is thiol independent (Ignarro et al., 1981). Hence it seemed possible that differing requirements for thiols contributed to the differing effects of glyceryl trinitrate in large and small arteries. However, the failure of both cysteine and *N*-acetylcysteine to affect the response to glyceryl trinitrate in the small arteries gives little credence to this possibility. These thiols in the same concentrations were shown by Sellke et al. (1990) and Wheatley et al. (1994) to restore the sensitivity of perfused small microvessels (approximately 0.1 mm i.d.) to glyceryl trinitrate although they were without effect on larger microvessels whose dimensions (approximately 0.3 mm i.d.) approached those of the small arteries used here. It would appear from their findings and ours that thiol availability is a factor which

distinguishes the mechanisms of heterogeneity prevailing at the microvascular level, from the mechanisms prevailing at the macrolevel epitomised by the proximal epicardial vessels and their distal branches used in the present study. Sellke et al. (1990) pointed out that their results were consistent with a role for *S*-nitrosothiols as an active intermediate in the vasorelaxation of glyceryl trinitrate, as proposed originally by Ignarro et al. (1981). Although this mechanism may apply in the microvessels, it is evident that our data offer no support for this possibility in the larger vessels. In this respect our results are consistent with other evidence against *S*-nitrosothiol formation as an obligatory step mediating glyceryl trinitrate-induced effects (Fung et al., 1992; Chirkov et al., 1993).

Isoprenaline was used as an example of an agent which does not utilise the cyclic GMP pathway and instead exerts its vasodilator action via cyclic AMP. The results are included in this report only because they provide some evidence, additional to that provided by the effects of *S*-nitroso-*N*-acetylpenicillamine, to show that terminal events in relaxation are probably intact in the small artery and hence unlikely to contribute to heterogeneity of response to glyceryl trinitrate. Although the greater potency of isoprenaline in the small than in large artery suggests that the cyclic AMP pathway is more active in the former, other factors may contribute. One is the distribution of β-adrenoceptors, since in dog heart there is histochemical evidence of an inverse relationship between β-adrenoceptor distribution and coronary vascular diameter (Muntz et al., 1984). Another is artery wall thickness, since there is evidence that loss of isoprenaline by uptake while diffusing to receptors, increases with vessel thickness (Guimaraes et al., 1974).

The results of these investigations indicate that the basis of heterogeneous vascular responsiveness varies between different blood vessels. The extent to which variation may be species related remains to be determined, since our study did not include resistance vessels of the arteriolar dimensions used by Sellke et al. (1990) and Wheatley et al. (1994) in their studies on pig and dog heart. The potential for species variability in heterogeneity is highlighted by evidence that there are multiple enzyme systems for converting glyceryl trinitrate to nitric oxide in vascular smooth muscle (reviewed by Bennett et al., 1994). Further studies using bovine microvasculature may shed light on the possibility raised by Sellke et al. (1990) that in perfused microvessels, vasodilator responses to glyceryl trinitrate may be achieved by formation of *S*-nitrosothiols, a process which appears to occur relatively slowly in human plasma and platelets (Chirkov et al., 1993). Interestingly the recent results of Wang et al. (1996) indicate that in dog coronary microvessels there is also heterogeneity of responsiveness to an organic mononitrate which is unaffected by the presence of a thiol ester in the molecule and which is not exhibited by sodium nitroprusside. In summary, our results demonstrate impairment of responsiveness both to glyceryl trinitrate and sodium nitroprusside, but not to *S*-nitrosothiols in bovine small coronary arteries. As the therapeutic utility of organic nitrates within the human coronary circulation depends largely on selectivity for large vessel dilatation and consequent avoidance of coronary 'steal', the present findings provide further impetus to carry out analogous investigations in human isolated coronary arteries.

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