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Endothelium-derived Relaxing Factor and Cyclic GMP-dependent Vasorelaxation in Human Chorionic Plate Arteries

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SUMMARY

Endothelium derived relaxing factor (EDRF), now widely believed to be nitric oxide (NO), may play an important part in the control of fetoplacental vascular tone. To further explore this role we have determined the relaxation responses to exogenous NO and examined the temporal relationship between intracellular concentrations of cyclic GMP and vascular tone in isolated ring segments of human chorionic plate arteries. We have also determined the dose relations for the contractile agonists serotonin and the thromboxane analog U46619. Lastly, we have explored the relaxation responses to a wide range of agents known to elicit EDRF release in other vascular beds. Chorionic plate arteries relaxed significantly to exogenous NO with concomitant increases in cyclic guanosine monophosphate over basal values. ED_{50}s for serotonin and U46619 were 1.48×10^{-6} M and 3.39×10^{-8} M respectively. The ED_{50} for NO derived from S-nitroso-N-acetyl-penicillamine was 1.28×10^{-6} M. Endothelium-intact segments of chorionic plate arteries pre-contracted with either serotonin or U46619 failed to relax significantly to acetylcholine, adenosine diphosphate, A23187, bradykinin, and histamine and only minimally to substance P. We suggest that EDRF is likely to be important in the control of placental vascular tone, but that it is not possible to demonstrate its action in an unperfused experimental system.

INTRODUCTION

Since the discovery of endothelium derived relaxing factor (EDRF) (Furchgott and Zawazki, 1980) and its likely identity as nitric oxide (NO) (Ignarro, 1989) there has been an explosion of interest in the actions of this substance in a wide range of vascular beds under physiological conditions and in a variety of disease states (Moncada, Palmer and Higgs,

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1991). The fetoplacental vasculature is not innervated (Fox and Khong, 1990) and thus relies entirely on autocrine and circulating vasoactive factors for its regulation. The importance of prostaglandins, in particular prostacyclin (PGI_2), and their interaction with thromboxane (TXA_2) in this vascular bed has been extensively reviewed recently (Boura and Walters, 1991). The role of EDRF, and indeed that of other endothelium-derived factors such as endothelin and endothelium-derived hyperpolarizing factor, is less certain.

The human placenta is a potentially rich source of information regarding regulation of the fetoplacental vasculature. However, early studies, prior to the realization of the importance of vascular enthothelium, produced some confusing results. The often conflicting results arising from the variety of techniques used by different investigators, including the use of isolated placental lobe perfusion, arterial rings and strips, and cell culture studies have further complicated an already complex area.

Several investigators have demonstrated that placental arteries are capable of releasing an EDRF-like substance in response to various stimuli (Van de Voorde, Vanderstichele and Leusen, 1987; Chaudhuri et al, 1991). Such studies have used superfusion cascades to detect the action of placentally-derived EDRF on an endothelium-denuded detector tissue. However, experiments intended to demonstrate the release by, and action of endogenous EDRF on placental arteries themselves have been less successful (Van de Voorde, Vanderstichele and Leusen, 1987; Griggs and McLaughlin, 1989; Monuszko et al, 1990). Placental arteries have been shown to relax in response to exogenous NO (Chaudhuri et al, 1991), thus the potential for a response to endogenous EDRF seems to be present in these vessels.

EDRF acts through the stimulation of vascular smooth muscle guanylate cyclase to produce increased intracellular levels of 3′-5′ cyclic guanosine monophosphate (cGMP). Increased levels of cGMP lead in turn to phosphorylation of G kinase and a subsequent series of, as yet undefined, steps, which result in a fall in intracellular calcium concentration and vasorelaxation (Lincoln, 1989).

In the present study, we have examined the vasorelaxation responses of endothelium-intact isolated ring segments of chorionic plate arteries to exogenous NO derived from S-nitroso-N-acetyl-penicillamine (SNAP) (Ignarro et al, 1981) and have determined the temporal relationship between cGMP and those responses. Additionally we have determined the effects of both receptor-dependent and receptor-independent agents known to elicit EDRF release in other mammalian vascular tissues. Our intention was to assess the ability of these arteries to respond to exogenous NO/ERDF and to seek to demonstrate the magnitude of EDRF release in response to one or more of the agents tested.

MATERIALS AND METHODS

We obtained placentae from normal term vaginal deliveries. We specifically excluded patients with hypertension or diabetes, those known to smoke or use drugs and patients with growth retardation or other fetal abnormality. The placentae were obtained immediately following delivery and placed on ice. Following transport to the laboratory, we rapidly dissected individual chorionic plate arteries (average external diameter 1 mm) from the surrounding tissue and placed them in oxygenated Krebs solution. Up to 32 artery segments were obtained from each placenta, and a total of 36 placentae yielded 424 segments. When a single protocol was run on multiple segments from the same placenta, we averaged the results into a single value prior to analysis. Thus all reported values of n refer to the number of individual placentae, not the number of segments used.

Initial treatment of the arteries was similar regardless of the later experimental protocol, and has been described in detail elsewhere (Pearce et al, 1991). In brief, we cut the arteries into 3 mm-long ring segments and mounted them on paired wires between a force transducer (Kulite BG-10) and a post attached to a micrometer used to vary resting tension. During all experiments we continuously digitized, normalized, and recorded contractile tensions using an on-line computer.

We equilibrated the arteries at 37.5°C for at least 1 h in a bicarbonate Krebs solution, containing (in mM), 122 NaCl, 25.6 $NaHCO_3$, 5.56 dextrose, 5.17 KCl, 2.49 $MgSO_4$, 1.60 $CaCl_2$, 0.114 ascorbic acid, 0.027 disodium EDTA, 0.3 L-arginine and 0.001 indomethacin, continuously bubbled with 95% O_2, 5% CO_2. During this time we adjusted the resting tension to 1 g, previously determined to be optimum for these vessels. We then contracted the arteries by exposure to an isotonic potassium Krebs solution containing 122 mM K^+ and 31 mM Na^+. After peak tensions were reached, we washed the arteries with normal sodium Krebs solution and allowed them to return to baseline tension for 1 h. Treatment of the artery segments thereafter varied with each protocol.

We conducted five experimental protocols. First, we determined the dose response relation for the contractile agonists serotonin (5HT) and the thromboxane mimetic U46619 (9,11-dideoxy-11 α, 9 α-epoxymethano-prostaglandin F2α, Upjohn, Kalamazoo, Michigan). Second, we determined the dose response relation for the vasorelaxant SNAP. Third, we measured the relaxation time-course for SNAP at a dose of 10 μM. Fourth, we determined the relations between the levels of the intracellular second messengers cGMP and adenosine cyclic monophosphate (cAMP), and the duration of exposure to SNAP. Finally, we determined the dose–response relation for a battery of endothelium-dependent vasorelaxants, comprising the receptor-dependent agents: acetylcholine; adenosine diphosphate (ADP); bradykinin; histamine and substance P and the receptor-independent agent calcium ionophore (A23187).

Following the initial regimen described above we added cumulative doses of either serotonin or U46619 to the tissue baths. Both agents were given in ½ log increments to yield bath concentrations between 10^{-10} and 10^{-3} M for serotonin and 10^{-10} and 10^{-5} M for U46619. We calculated and expressed contractile responses as percentages of the maximum response achieved for each agent. The pD_2 value for each dose–response relation was determined by fitting the normalized dose–response relation with the logistic equation using computerized non-linear regression.

For the SNAP dose–response experiments we again initially treated these arteries as above. We then induced a second contraction using 10^{-7} M U46619. Once stable tone was established we added cumulative doses of SNAP to the tissue baths, to yield bath concentrations between 10^{-10} and $10^{-3.5}$ M. Since SNAP is inherently unstable in solution, it was prepared immediately before use and held on ice. We calculated and expressed relaxation responses as percentages of the maximum response achieved. We determined the pD_2 value for SNAP by fitting the normalized dose–response relation with the logistic equation using computerized non-linear regression.

For the SNAP relaxation experiments we followed the initital regimen and then contracted these arteries with 10^{-7} M U46619 and allowed stable tone to develop. We then added 10 μM SNAP to the baths and recorded the resultant changes in tension. We defined the magnitude of relaxation as the maximum per cent relaxation observed after the addition of SNAP. All relaxation responses were normalized relative to maximum initial contractile tension.

We performed the next experiments to determine the relationships between cGMP,

cAMP and the duration of exposure to 10 μM SNAP. Eight artery segments from each placenta were studied simultaneously. We contracted the arteries with high potassium Krebs, washed them and contracted them again with 10^{-7} M U46619. Following establishment of stable tone, we froze one segment by rapid immersion in liquid nitrogen. This segment was used to determine the basal levels of cGMP and cAMP. We then added 10 μM SNAP to the remaining segments and flash froze in sequence up to a maximum of $t = 720$ s. Thus each series yielded a set of artery segments whose treatment differed only by the duration of exposure to SNAP.

We stored arteries designated for cyclic nucleotide analysis at −80°C until assay, at which time they were individually homogenized in 1 ml ice-cold 6% trichloroacetic acid. After centrifuging the homogenates for 60 min at 3000 *g*, we reserved the resultant pellet for protein determination and decanted the supernates for subsequent cyclic nucleotide assay. We determined protein contents using an extraction designed to exclude connective tissue and structural proteins (Pearce et al, 1991). Proteins were quantified using the Bradford Coomassie Brilliant Blue assay.

We extracted the reserved supernates with water-saturated diethyl ether, lyophilized aliquots of the aqueous phase, reconstituted them in 50 mM acetate buffer, and assayed for cyclic nucleotides using standard radioimmunoassay techniques. We determined both cGMP and cAMP content for each sample using commercially available kits (RPA 525 and RPA 509, Amersham Corp.). Both cGMP and cAMP values were normalized relative to vessel protein content and were expressed as pmol/mg vessel protein. Changes in cyclic nucleotide content were expressed as fold increases over basal values.

In a further set of arteries we determined the relaxation responses to a battery of agents known to produce entothelium-dependent relaxation in other vascular beds. Following the usual equilibration, potassium contraction, wash and re-equilibration, we contracted these arteries with either 3×10^{-7} M serotonin or 3×10^{-8} M U46619. These agonist concentrations were chosen because they produced sub-maximal, yet stable, responses. Following the establishment of stable tone we added cumulative doses of each vasorelaxant to the tissue baths, to yield bath concentrations as follows: acetylcholine, 10^{-10}–10^{-4} M, A23187, ADP; bradykinin and histamine, all 10^{-10}–10^{-5} M and substance P as a single dose of 10^{-7} M. Each agent was added in ½ log increments, and only a single agent was used for each artery segment. Following the last dose of each agent we added 10 μM SNAP to each bath. We calculated and expressed relaxant responses for each agent as a percentage of the agonist-induced tension remaining following each addition of the vasorelaxant. Only segments showing 30% or more relaxation to 10 μM SNAP were used in subsequent analysis.

Following completion of this protocol every segment was assessed for anatomical endothelial integrity using en-face silver staining (Poole, Sanders and Florey, 1958). We carefully removed each segment from its mounting wires, and opened the lumen with fine scissors. We then treated each segment as follows: 5% dextrose wash, 0.25% $AgNO_3$ (3 min), 5% dextrose wash, 3% $CoBr_2$ in 1% NH_4Br (10 min), 5% dextrose wash, 4% formaldehyde. We then mounted each segment in glycerin and examined them using light microscopy under medium power. We discarded all data from artery segments showing damaged or absent endothelium.

RESULTS

The dose responses for 5HT and U46619 are shown in Figure 1. The pD_2 for U46619 was 7.47 ± 0.07 and that for 5HT, 5.83 ± 0.13, corresponding to ED_{50}s of 3.39×10^{-8} M

and 1.48×10^{-6} M respectively. Thus the 3×10^{-7} M concentration of U46619 used in subsequent experiments was about ED_{75}. The concentrations of agonists used for the endothelium-dependent relaxation experiments, 3×10^{-8} M for U46619 and 3×10^{-7} M for 5HT were clearly sub-maximal, and equivalent to the ED_{45} and ED_{20} for each drug respectively.

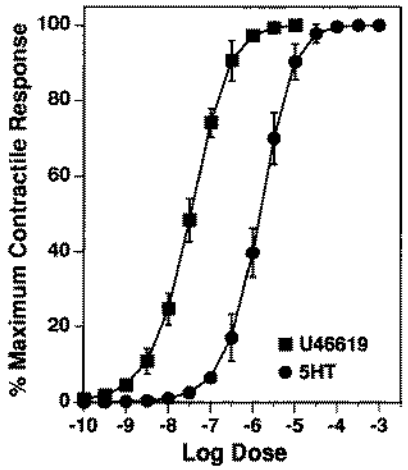

Figure 1. Cumulative dose responses to U46619 and serotonin (5HT) are shown. Average contractile responses are given together with standard errors where these exceed the size of the symbols used in the figure. Arteries from eight placentae were used for U46619 and from seven placentae for 5HT.

The dose response relation for SNAP is shown in Figure 2. The pD_2 value for SNAP was 5.90 ± 0.15, corresponding to an ED_{50} of 1.28×10^{-6} M, thus the 10 μM concentration used in later experiments was equal to the ED_{85} or greater.

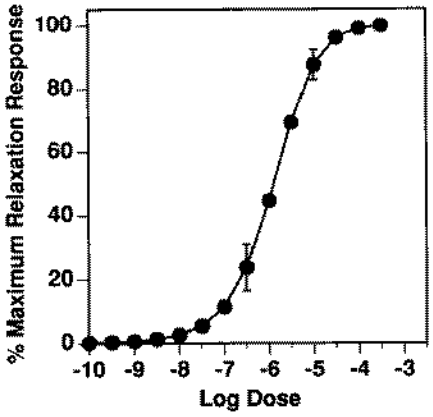

Figure 2. The cumulative dose response to SNAP was determined in arteries pre-contracted with 0.1 μM U46619. Average relaxation responses are shown together with standard errors where these exceed the size of the symbols used in the figure. Arteries from a total of six placentae were used.

Relaxation to SNAP began within 20 s of exposure (Figure 3), and required up to 600 s to reach a maximum. Such maximal relaxation averaged 54.2 ± 7.0% in arteries pre-contracted with 10^{-7} M U46619.

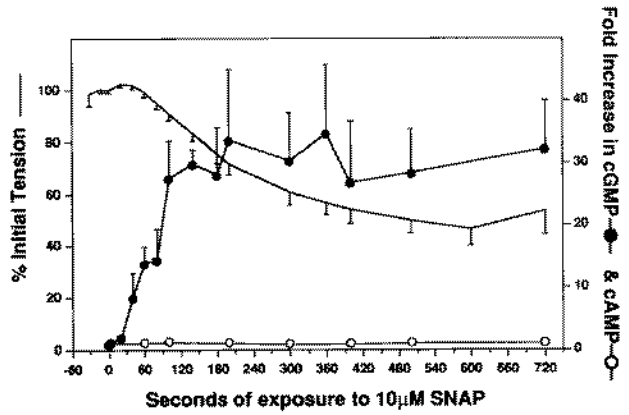

Figure 3. Relaxation response to 10 μM SNAP was determined in arteries pre-contracted with 0.1 μM U46619. Levels of cGMP and cAMP are shown as fold increases over basal values in pre-contracted artery segments, flash frozen 20–720 s after exposure to 10 μM SNAP. Mean values are given together with standard errors where these exceed the size of the symbols. Twelve placentae were used for the relaxation responses and 10 for the cyclic nucleotide measurements.

Under the present experimental conditions, basal levels of cGMP and cAMP averaged 1.05 ± 0.19 pmol/mg protein and 2.73 ± 0.23 pmol/mg protein respectively. Following exposure to SNAP, cGMP concentrations rose rapidly, reaching peak values within 200 s (Figure 3). Maximal increases averaged 34.8 ± 11 times basal values, and were sustained over the 720 s sampling period. cAMP measured simultaneously in the same arteries did not vary significantly from basal levels at any time point.

The increase in cGMP with SNAP preceded the relaxation response. The arteries continued to relax after cGMP concentrations had reached a plateau. Indeed cGMP levels were near maximal at a time when the arteries had reached only around ~33% of their maximal relaxation response.

For the endothelium-dependent vasorelaxation experiments we used only artery segments relaxing >30% to 10 μM SNAP and showing intact endothelium by post-hoc en-face silver staining. Such segments relaxed an average of 44.6 ± 2.7% to SNAP when pre-contracted with 3×10^{-8} M U46619 and 35.1 ± 1.8% when pre-contracted with 3×20^{-7} M 5HT (Figure 4, panel G). A typical example of endothelial staining is shown in Figure 5.

Acetylcholine failed to produce a significant relaxant response over the dose range 10^{-10}–10^{-4} M in arteries contracted with either U46619 or 5HT (Figure 4, panel A). Similarly no significant response was seen to A23187 over the dose range 10^{-10}–10^{-5} M in either group of arteries (Figure 4, panel B). ADP also failed to elicit any significant responses (Figure 4, panel C).

Arteries pre-contracted with 5HT showed no response to bradykinin (Figure 4, panel D) whereas those pre-contracted with U46619 showed a small, dose-dependent increase in tension (to a maximum of 13.63 ± 5.4% above U46619-induced values). Histamine did not produce any significant alteration in tension in either group of arteries (Figure 4, panel E). A single dose of substance P produced a tiny relaxation response in both groups of

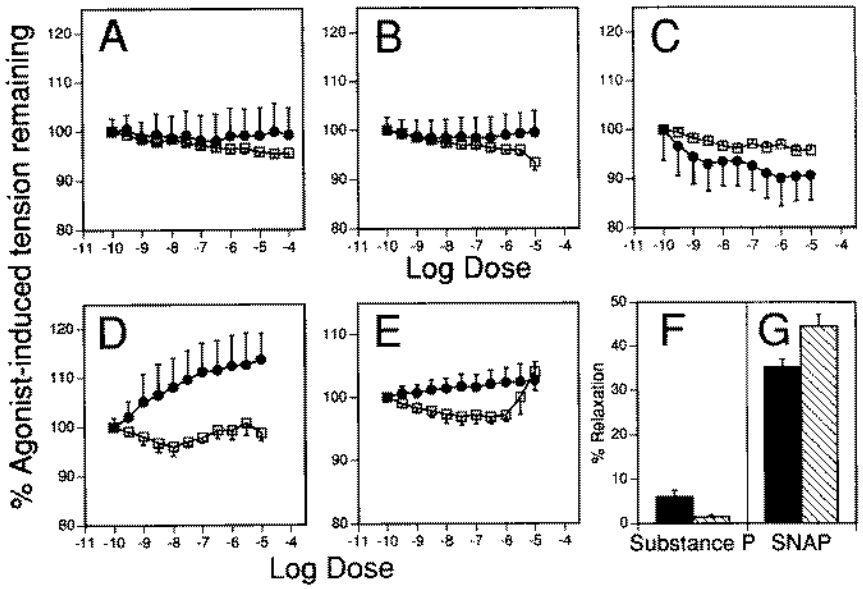

Figure 4. Cumulative dose responses to acetylcholine (panel A), A23187 (panel B), ADP (panel C), bradykinin (panel D) and histamine (panel E) are shown. Arteries were pre-contracted with either 0.3 μM 5HT, shown by open squares, or 30 nM U46619, shown by closed circles. Average responses are given together with standard errors where these exceed the size of the symbols. The responses to 0.3 μM substance P (panel F) and 10 μM SNAP (panel G) are shown (solid bars, arteries contracted with 0.3 μM 5HT; hatched bars, arteries contracted with 3 nM U46619). A total of 10 placentae were used for these responses.

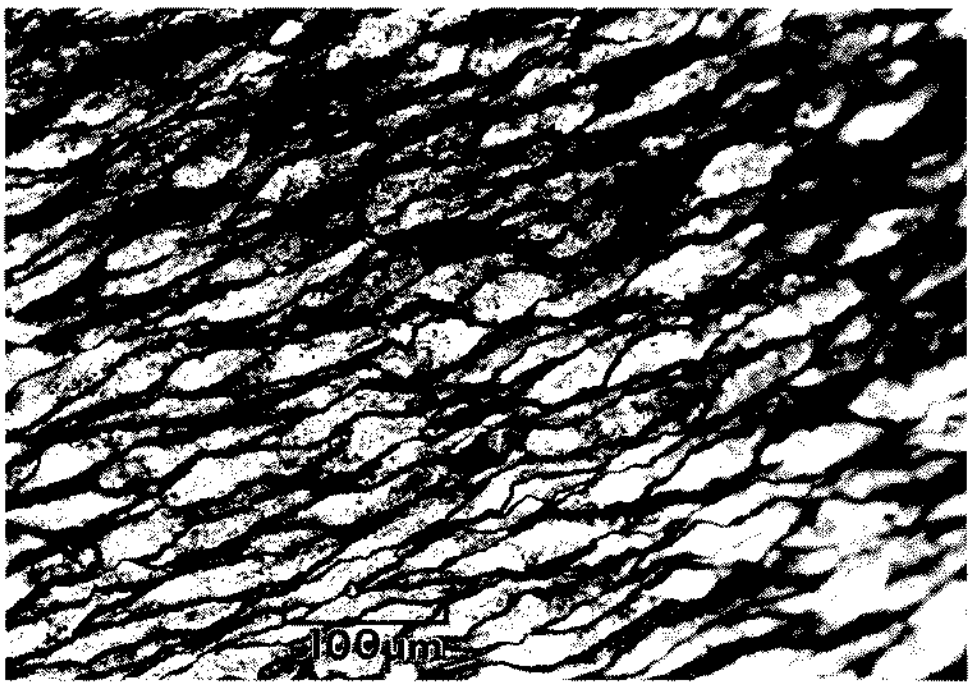

Figure 5. Typical appearance of chorionic plate artery endothelium after en-face silver staining.

arteries, averaging 1.4 ± 0.4% in those pre-contracted with U46619 and 6.1 ± 1.3% in those pre-contracted with 5HT.

DISCUSSION

Clearly, we have shown that human chorionic plate arteries have the capacity to relax to exogenous NO, as reported by others (Chaudhuri et al, 1991; Chaudhuri and Furuya, 1991). However, it appears that it is not possible to demonstrate the release of endogenous EDRF by these vessels by observing its action on their tone. Experiments using perfused placental preparations have shown that infusion of antagonists of EDRF synthesis, such as N-monomethyl-L-arginine and N-nitro-L-arginine, results in increased perfusion pressure (Gude, Boura and King, 1992; Myatt et al, 1992). Infusion of the guanylate cyclase inhibitor methylene blue produced similar results (Myatt, Brewer and Brockman, 1991; Gude, Boura and King, 1992). These findings, together with the superfusion studies referenced earlier (Van de Voorde, Vanderstichele and Leusen, 1987; Chaudhuri et al, 1991) provide good indirect evidence for the production and role of EDRF in the placental vasculature. Why then, is there this apparent discrepancy between these results and our findings?

In our preparation of chorionic plate arteries the relaxant response to exogenous NO develops relatively slowly and is sustained for prolonged amounts of time (over 30 min—data not shown). Simultaneous measurements of cGMP show increases of over 30 times basal values, which are sustained for at least 12 min after exposure (Figure 3). Similar studies in other vascular beds do not show this pattern, usually a rapid rise in cGMP is followed by an equally rapid fall to baseline values. Since SNAP, the source of NO in these studies is very unstable in solution, it is unlikely to provide a continuing stimulus for cGMP synthesis. That levels remain elevated for so long suggests that cGMP degradation through cGMP phosphodiesterase takes place at a relatively low rate. If this is the case, then it would require a relatively low output of endogenous NO from the endothelium to produce relatively high levels of cGMP in the vascular smooth muscle, and thus low levels of vascular tone. The placenta certainly contains cGMP phosphodiesterases (Matsubara, Tamada and Saito, 1987) notable in the microvilli of the syncytriotrophoblast. However, their distribution and activity in placental vascular tissues is unknown. It is possible that the amount of NO used in these experiments is far in excess of that capable of being produced by the endothelium itself, thus 'overrunning' vascular smooth muscle cGMP synthesis and overwhelming its degradation capabilities. Nonetheless the target mechanism for EDRF action is intact and functional in chorionic plate arteries and it seems unlikely it should be physiologically redundant.

Our experiments to elicit endogenous EDRF-dependent relaxation in chorionic plate arteries were designed to optimize the conditions for such responses. Arteries were mounted under optimum tension and contracted with sub-maximal doses of agonist, both of which are necessary for optimum endothelium-dependent responses (Dainty et al, 1990). We used two different agonists, 5HT and U46619, which act on different receptor populations, in order to unmask any agonist-dependent effects on EDRF release. We supplemented our Krebs solution with 300 μM L-arginine to ensure an adequate supply of substrate for NO synthesis (Gold et al, 1990) and added indomethacin in adequate concentration to suppress endogenous cyclo-oxygenase activity and thus prostaglandin synthesis (MacLennan, McGrath and Whittle, 1988). Lastly, we included in our analysis only those

arteries which showed an adequate response to exogenous NO and a demonstrably intact endothelium. Despite all these efforts we were unable to demonstrate significant endothelium-dependent relaxation with any agent in any case.

Acetylcholine is the agent most commonly used to elicit EDRF release experimentally. It is present in enormous quantities in placental tissue, where its role is uncertain (King et al, 1991). Our findings confirm that it does not produce EDRF release in chorionic plate vessels (Van de Voorde, Vanderstichele and Leusen, 1987; Chaudhuri et al, 1991; King et al, 1991; Myatt et al, 1992).

A23187 has been shown to cause EDRF release in superfusion studies (Van de Voorde, Vanderstichele and Leusen, 1987; Chaudhuri et al, 1991), however it proved to be ineffective at causing vasorelaxation in both perfused placentae (Myatt et al, 1992) and our isolated vessel studies and those of others (Griggs and McLaughlin, 1989). A23187 acts by increasing calcium entry into the endothelial cells, thus driving Ca^{2+} dependent EDRF synthesis. It might be that the shear stress effect of flow present in the superfusion studies is required to promote the action of A23187; vascular strip preparations produce more reproducible results than vascular rings (Chaudhuri and Furuya, 1991), however this could not explain the lack of effect in perfused placental preparations. Although A23187 also causes PGI_2 synthesis all these studies were carried out in the presence of indomethacin, discounting this as an alternative explanation.

ADP produces EDRF release in several animal species, including the sheep and rabbit, but not in human chorionic plate arteries as shown here.

While bradykinin had no apparent effect on arteries pre-contracted with 5HT, there was a small increase in tension in arteries pre-contracted with U46619. The reason for this effect is unclear. Bradykinin is usually thought to be a vasodilator substance. Its role in placental vascular tone is uncertain, although it is known to be rapidly degraded, 98% being inactivated during a single passage through the placenta (Boura and Walters, 1991). It has been variously reported to have both a vasoconstrictor (Boura and Walters, 1991; Myatt et al, 1992) and a vasodilator action (Chaudhuri et al, 1991; Pinto et al, 1991). The actions of bradykinin are complex and include the release of EDRF, PGI_2 and superoxide anion (O_2^-) (Holland et al, 1990). Since O_2^- has been shown to degrade EDRF and inhibit PGI_2 formation, it may be that bradykinin stimulates the release of factors, which under some circumstances, cancel each other out. It is not, therefore, a 'clean' EDRF-producing agent.

Histamine has been shown to elicit EDRF release from umbilical artery endothelium (Van de Voorde, Vanderstichele and Leusen, 1987; Chaudhuri et al, 1991) but in perfused placental preparations causes vasorelaxation only in high concentration (10^{-6}–10^{-4} M) (Myatt et al, 1992). In our preparation no response was detected whereas others have shown a contractile response (Griggs and McLaughlin, 1989).

We were able to demonstrate a small relaxation response to substance P. We used a single dose to avoid the problems of tachyphylaxis associated with cumulative doses. Others (Hansen et al, 1988) have shown around 20% relaxation response in stem villous arteries with the same 0.1 μM dose, whereas our maximal relaxation was only around 6%. This difference in response may be due to differences in vessel size as is well known elsewhere (Pearce et al, 1991). Stem villous arteries are the resistance vessels of the feto-placental circulation and as such their responses may well be different to those of the larger chorionic plate and umbilical vessels.

In an isolated ring segment preparation there is little or no flow over the endothelium, thus it is subjected to little or no shear stress. It has been shown that strip preparations and ring preparations of umbilical arteries have different responses to agents which release

EDRF (Chaudhuri and Furuya, 1991). A strip preparation would certainly be subject to greater shear stresses from bubbling in the tissue bath than a ring preparation. Also, in ring preparations, agents are applied abluminally rather than intraluminally. This and other diffusional factors may affect responses to agents used to provoke EDRF release.

One possibility for the failure of external stimuli to produce EDRF in chorionic plate arteries might be that basal release is already near maximal, albeit low, leaving no margin for increase in response to agonists. Alternatively the total capacity for EDRF release may be limited to a level below which the underlying smooth muscle is unresponsive, although this seems unlikely.

It is important to recognize that the present experiments were designed to examine the release of EDRF from the endothelium in response to the agonists tested. Other vasoactive substances may be released in response to many of these agonists and their interactions are potentially complex. We used indomethacin to inhibit prostaglandin synthesis. Vasorelaxant responses obtained without this or some other inhibitor of cyclo-oxygenase in adequate dosage, may not be due to EDRF at all.

Almost all studies of placental vascular endothelial function, including the present one are performed at unphysiologically high pO_2 levels. Differences in cyclo-oxygenase activity and agonist responsiveness have been demonstrated at pO_2s closer to in vivo levels in umbilical vessels (MacLennan, Whittle and McGrath), it may be that EDRF synthesis and action is similarly altered. This is an as yet unexplored area.

The physiological significance of the release of EDRF from endothelium in response to various substances is unknown. EDRF is probably released continuously in most vascular beds, and is probably modulated via shear stress secondary to alterations in perfusion pressure (Moncada, Palmer and Higgs, 1991). This is likely to be the case in the placental vasculature. High basal release of EDRF, coupled with a high PGI_2/TXA_2 ratio would ensure the low vascular resistance which pertains in normal circumstances.

In summary, chorionic plate arteries have the capacity to relax to EDRF/NO, but it is not possible to demonstrate endogenous release of EDRF in a static unperfused system. Since functional assessment of vascular endothelium in such a system is not possible, investigators will have to rely on anatomical demonstrations of endothelial integrity.

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