Physiol. Res. 53: 255-263, 2004

Endothelium-Dependent Responses in Small Human Mesenteric Arteries

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Received April 23, 2003
Accepted July 14, 2003

Summary

The aim of the present study was to investigate the endothelial function in human mesenteric arteries with specific reference to defining the role of endothelium-derived nitric oxide (EDNO) and the endothelium-derived hyperpolarizing factor (EDHF). Isolated segments of small human mesenteric arteries (225-450 μm inner diameter) were mounted in organ baths for recording isometric tension. In arteries precontracted with U46619 (thromboxane A_2 analogue, 10^{-7} M), endothelium-dependent relaxations were induced in a concentration-dependent manner by substance P and histamine. In normal Krebs solution the relaxations to substance P (10^{-9} M) and histamine (10^{-7} M) were not significantly affected by preincubation with N^{ω}-nitro-L-arginine (L-NNA, 10^{-4} M) or indomethacin (10^{-5} M). When the preparations were exposed to a solution containing 60 mM KCl, stable contractions were induced, but relaxations could still be induced by substance P and histamine. When the arteries were further preincubated with L-NNA, the relaxations were almost abolished. A combination of apamin (3 x 10^{-7} M) and charybdotoxin (10^{-9} M) almost abolished relaxations in normal Krebs solution. It is concluded that isolated human mesenteric arteries respond to substance P and histamine with relaxations that are endothelium-dependent. Synthesis of both EDNO and EDHF seem important for these relaxations, whereas prostaglandins seem to be of minor importance.

Key words
Human artery • Nitric oxide • EDHF • Potassium channels • High potassium solution

Introduction

The endothelium plays an important role in the control of vascular tone and microcirculation. This influence is exerted through the release of different substances from the endothelial cells, and these substances diffuse to the underlying vascular smooth muscles to influence the tone and/or membrane potential. The fascinating mechanism behind the ability of acetylcholine to induce endothelium-dependent relaxations was first elegantly demonstrated by Furchgott and Zawadzki (1980), and the factor(s) being released was named endothelial-derived relaxing factor (EDRF). Prostanoids are important vasoactive substances generated in the endothelium (Moncada and Vane 1978), although their relative importance seems to vary considerably in different vascular beds (Garland and McPherson 1992, Zygmunt *et al.* 1994, Murphy and Brayden 1995, Corriu *et al.* 1996, Shimokawa *et al.* 1996, Urakami-Harasawa *et al.* 1997). Even before nitric oxide (NO) had been established as being one of the components of EDRF, different studies had indicated that

acetylcholine-induced endothelium-dependent effects could be classified as a relaxing effect independent of membrane potential changes, and a hyperpolarizing effect that was unaffected by substances with a profound inhibitory effect on relaxations (Bolton and Clapp 1986). In some studies, endothelium-derived nitric oxide (EDNO) has been shown to act independently of membrane potential changes (Chen *et al.* 1988, Huang *et al.* 1988), although some exceptions also exist (Tare *et al.* 1990). It is therefore tenable to classify endothelial-dependent relaxations as being due to prostanoid synthesis, formation of EDNO, or to release of endothelium-dependent hyperpolarizing factor (EDHF). Due to the fact that both prostacyclin (Murphy and Brayden 1995) and nitric oxide (Garland and McPherson 1992) are capable of hyperpolarizing cell membranes, responses to EDHF are those elicited in the presence of agents blocking the formation of prostanoids and nitric oxide (Feletou and Vanhoutte 1996).

Mesenteric blood flow is very important from a clinical point of view, and endogenous factors regulating vascular tone in the mesenteric bed have therefore been studied extensively. Briefly, inhibitory effects attributable to both EDNO and EDHF have been demonstrated in mesenteric arteries of various experimental animals. Interestingly, the relative importance of EDHF in the rat mesenteric artery seems much greater than in femoral and intrarenal arteries (Zygmunt *et al.* 1995) and in the aorta (Shimokawa *et al.* 1996). Human gastroepiploic arteries show responses attributable to a release of both EDNO and EDHF (Urakami-Harasawa *et al.* 1997), while prostanoids seem to play no role in these vessels in contrast to findings in rabbit mesenteric arteries (Murphy and Brayden 1995), but confirming findings in rats (Parsons *et al.* 1994, Garland and McPherson 1992, Shimokawa *et al.* 1996, Chen and Cheung 1997).

Identification of responses attributable to EDHF seems to require electrophysiological measurements. Numerous studies have shown that hyperpolarizations and relaxations induced by EDHF are due to opening of potassium channels, and some blockers of these channels are indeed efficient inhibitors of EDHF. Moreover, it has turned out to be experimentally possible to distinguish responses attributable to EDNO and EDHF by studying the inhibitory events in solutions containing elevated concentrations of potassium, since this disables the potassium channel-dependent action of EDHF (Adeagbo and Triggle 1993, Cai *et al.* 1994, Hecker *et al.* 1994, Zygmunt *et al.* 1995, Kamata *et al.* 1996, Kamata and Makino 1997, McCulloch *et al.* 1997, Randall *et al.* 1997, Zygmunt *et al.* 1997)

When studying these events in microvessels, it is important to realize that both vessel type and vessel size seem to have impact on the relative importance of the above mentioned factors. By comparing the responses in different segments of the rat mesenteric artery, an increasing importance of EDHF was found as vessel size decreased (Shimokawa *et al.* 1996). The aims of the present study were to study endothelial function in small human mesenteric resistance arteries with specific reference to defining relaxations attributable to the release of EDNO and EDHF.

Methods

Small arteries (inner diameter 225-450 μm) were obtained from segments of colon with attached mesentery removed from patients with bowel cancer. The specimens were taken from macroscopically normal tissue, and care was taken not to include vascular beds supplying the area of the tumor. After removal, the tissue was immediately placed in cold Krebs solution (for composition, see below) and transported to the laboratory. The vessels were located in the mesentery by the aid of a stereo microscope, and besides selecting arteries of a distinct size we always selected arteries with a definite course in the bowel wall. Ring preparations (length approx. 2 mm) of the vessels were suspended in small organ chambers (Hogestatt *et al.* 1983) containing calcium free solution (for composition, see below). Isometric tension was measured by Grass FT.03 transducers, and registered on a Grass Polygraph (model 7E). The arteries were stretched until a stable passive tension level of 3 mN was obtained. After an equilibration period of up to one hour, the calcium-free solution was replaced by a calcium containing Krebs solution (for composition, se below). In separate experiments, the endothelium was removed by rubbing the interior of the vessel gently by a horse hair. The functional stability of the vessels was tested prior to the experiments by repeated exposure to a solution containing 124 mM potassium. This resulted in monophasic contractions that were reproducible after several exposures.

Composition of solutions (mM)

Krebs solution: 119 NaCl, 4.6 KCl, 15 $NaHCO_3$, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, and 11 glucose. *Calcium-free solution:* 119 NaCl, 4.6 KCl, 15 $NaHCO_3$,

1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11 glucose, and 0.01 EGTA (ethylene glycol-bis (ß-aminoethyl ether)-N,N,N',N'-tetracetate). *124 mM potassium solution:* 124 KCl, 15 $NaHCO_3$, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, and 11 glucose. *60 mM potassium solution:* 64 NaCl, 60 KCl, 15 $NaHCO_3$, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, and 11 glucose.

Drugs

N^{ω}-nitro-L-arginine (L-NNA, Sigma, St. Louis, MO, USA), carbamoylcholine chloride (carbachol, Sigma), apamin (Sigma), substance P (Sigma), U46619 (9,11-dideoxy-11,9,epoxy-methano-prostglandin F_2, UpJohn), charybdotoxin (Sigma). All dilutions were made in 0.9 % NaCl containing 1.0 mM ascorbic acid, except apamin and charybdotoxin that were both dissolved in distilled water containing 0.05 % albumin. Ultrasonic disruption of L-NNA powder was necessary for dissolving this agent.

Statistical considerations

All data are given as means ± S.E.M. A paired or unpaired Student´s t test was used where appropriate, and ANOVA test was applied for a comparison of more than two groups. In case of significant difference detected with the ANOVA test ($p<0.05$), Student-Newman-Keuls test was applied for multiple comparisons. N denotes the number of patients studied in each experimental series. One vessel from each patient was used in each series.

Results

The inner diameter of these vessels varied between 225 and 450 µm (unstretched). After the preparations had been suspended under a passive tension of 3 mN and allowed to equilibrate in normal Krebs solution, the exposure to 124 mM potassium solution resulted in a tonic contraction, occasionally with superimposed phasic contractions of relatively low amplitude. In paired studies, the amplitude of the contractions amounted to 9.3±0.5 mN in arteries with intact epithelium, and to 6.2±0.4 mN in endothelial-denuded preparations ($p<0.05$, N=5). The appearance of the contractile response of the vessels when exposed to the solution containing 60 mM of potassium was indistinguishable from the response in 124 mM potassium, but the amplitude was significantly lower (8.3±0.7 mN vs. 7.3±0.7 mN in 124 mM and 60 mM potassium solution, respectively. $p<0.05$, N=9)

The thromboxane A_2 analogue, U46619, induced concentration-dependent contractions of the arteries. Maximal contractions amounted to 9.4 ± 2.3 mN (N=13) corresponding to 152±12 % of the response to 124 mM potassium solution. EC_{50} was 1.3 x 10^{-8} M (pD_2 = 7.88±0.15). For experiments where a submaximal stimulation was intended, a concentration of 10^{-7} M was chosen (yielding approximately 90 % of maximal contraction).

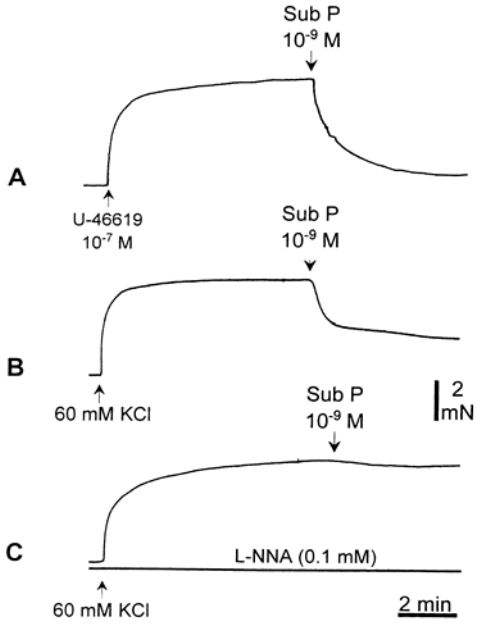

Fig. 1. Tracings illustrating the effect of substance P on mesenteric arteries contracted with U46619. In **A**, the artery was contracted with U46629 in normal Krebs solution. In **B** and **C**, the arteries were contracted in a solution containing 60 mM of potassium. Furthermore, the baths were preincubated with L-NNA (10^{-4} M) in C.

Endothelium-dependent relaxations

In arteries precontracted with U46619 (10^{-7} M), carbachol 10^{-6} M induced only very modest, and quite variable relaxations. Higher concentrations of carbachol (up to10^{-5} M) turned out to be as incapable of relaxing the arteries. Substance P induced long-lasting, concentration-dependent relaxations (Figs 1A and 2). The maximal response obtained in experiments, where a cumulative addition of substance P was used, amounted to 78.1±5.8 % of the contraction induced by U46619. With single exposures to 10^{-9} M of substance P the relaxation amounted to 74.8±7.7 % of U46619-induced tension (Fig. 3), and the magnitude of this relaxation was not changed with the second exposure to substance P (time control, Fig. 3). The relaxation was significantly attenuated in endothelium-denuded preparations (Fig. 3).

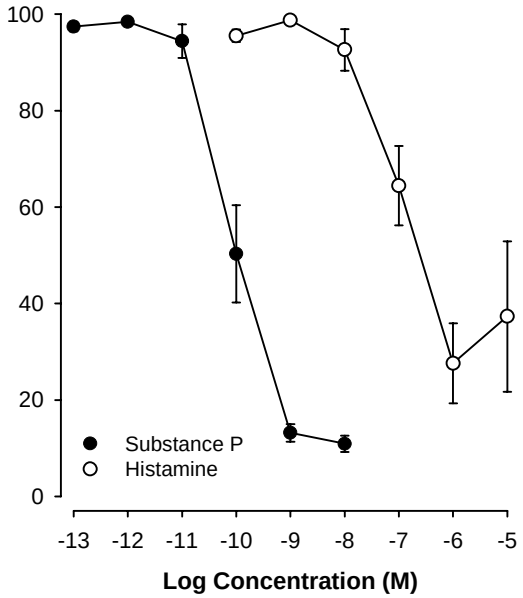

Fig. 2. Inhibitory effects of substance P (N=6) and histamine (N=6) in isolated mesenteric arteries. The arteries were precontracted with U46619 (10^{-7} M), and substance P or histamine were added in cumulatively increasing concentrations. Values are mean ± S.E.M.

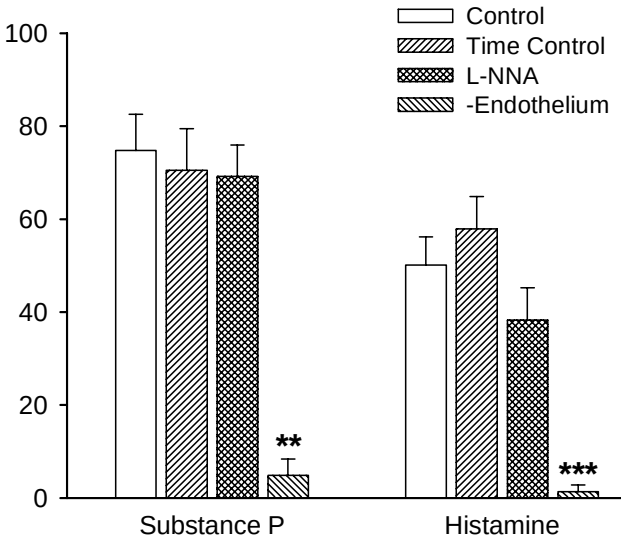

Fig. 3. Effects of substance P (10^{-9} M, N=7)) and of histamine (10^{-7} M, N=7)) in vessels precontracted with U46619 (10^{-7} M). Control responses denote responses to first exposure to substance P or histamine, while responses grouped as time control are responses to second exposure to the these inhibitory substances. L-NNA (10^{-4} M, N=7)) was added to the baths at least 10 min before a second exposure to substance P or histamine. ** p<0.01, *** p<0.001. Values are mean ± S.E.M.

U46619-precontracted arteries were also relaxed by histamine in a concentration-dependent manner (Fig. 2). Relaxation amplitude in response to 10^{-6} M histamine amounted to 50.1±6.1 % of U46619-induced tension (Fig. 3). This response was unchanged at the second exposure (time control), and it was almost abolished in endothelium-denuded preparations (Fig. 3). Preincubation with indomethacin (cyclooxygenase inhibitor, 10^{-5} M) had no influence on relaxations induced by substance P and histamine (data not shown).

Effect of L-NNA

Pretreatment with L-NNA (inhibitor of nitric oxide formation, 10^{-4} M) did not change the contractile response to U46619. L-NNA did not significantly change relaxations induced by substance P or histamine, although the response to histamine seemed attenuated (Fig. 3, time control vs. L-NNA: 58.8±8.2 % vs. 36.8±8.0 %, p=0.06). Thus a substantial relaxation was evident in the presence of L-NNA (10^{-4} M).

Relaxations in 60 mM potassium solution

In this solution, where actions mediated through potassium channels are inactivated, histamine (10^{-7} M) induced relaxations amounting to 20.9±4.9 % (N=5), which is significantly lower than in U46619-precontracted vessels. When the vessels were also preincubated with L-NNA (10^{-4} M), the exposure to histamine (10^{-7} M) resulted in a very poor relaxation amounting to 5.2±2.0 % (Fig. 4).

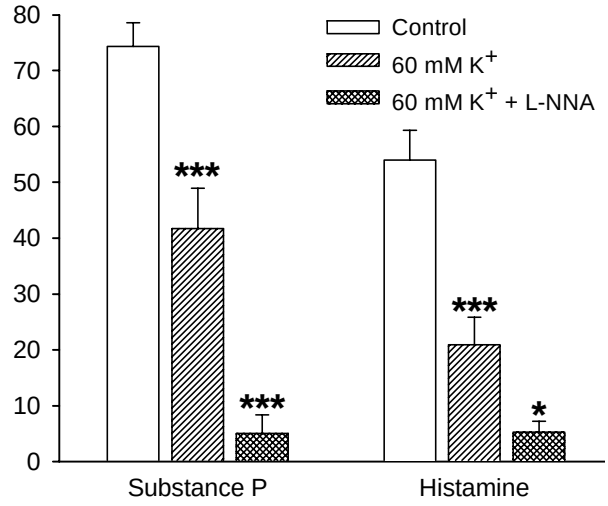

Fig. 4. Effects of substance P (10^{-9} M, N=5) and of histamine (10^{-7} M, N=5) in vessels precontracted with either U46619 (10^{-7} M, control) or 60 mM KCl. L-NNA (10^{-4} M) was added to the baths at least 10 min before the exposure to substance P or histamine. * p<0.05, *** p<0.001. Values are mean ± S.E.M.

Substance P was also capable of inducing considerable relaxations in 60 mM potassium solution (Figs 1B and 4), although the relaxations in paired experiments were of lower amplitude than in U46619-

precontracted vessels (74.4±4.2 % vs. 41.2±7.2 %, N=5). L-NNA preincubation (10^{-4} M) resulted in a pronounced inhibition of the relaxation induced by substance P in this solution with a residual relaxation amounting to 5.0±3.3 % (Figs 1C and 4).

Effects of potassium channel blockers

Addition of apamin alone to the baths did not change resting tension or the U46619-induced tension. Relaxations in response to substance P (10^{-9} M) and histamine (10^{-7} M) were unchanged in the presence of apamin 10^{-6} M. After adding both apamin ($3x10^{-7}$ M) and charybdotoxin (10^{-9} M), the tension level achieved after addition of U46619 was also unchanged. Relaxations induced by substance P (10^{-9} M) and histamine (10^{-7} M) in the presence of apamin and charybdotoxin were almost abolished (Fig. 5).

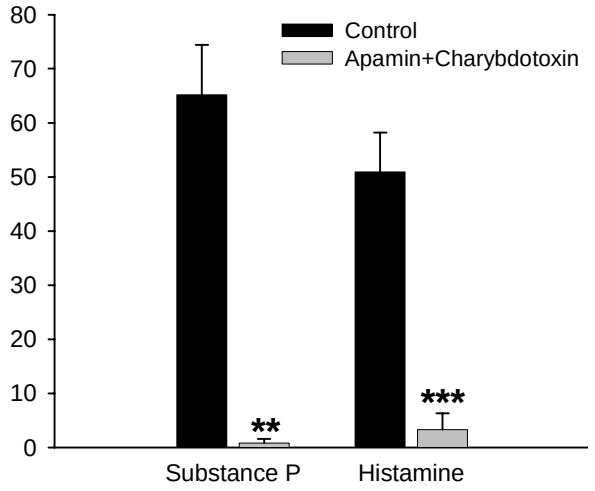

Fig. 5. Effects of apamin ($3x10^{-7}$ M) and charybdotoxin (10^{-9} M) on relaxations induced by substance P (10^{-9} M, N=5) and histamine (10^{-7} M, N=5) in isolated mesenteric arteries. The arteries were precontracted with U46619 (10^{-7} M). ** p<0.01, *** p<0.001. Values are mean ± S.E.M.

Discussion

The present study shows that the endothelium of small human mesenteric arteries supplying the colon can be stimulated *in vitro* to release relaxing factors. In contrast to findings in guinea pig (Bolton *et al.* 1984), rabbit (Murphy and Brayden 1995) and rat mesenteric arteries (Garland and McPherson 1992, Adeagbo and Triggle 1993, Zygmunt *et al.* 1995, Shimokawa *et al.* 1996, Chen and Cheung 1997, Hansen and Olesen 1997), human mesenteric arteries did not respond consistently to muscarinic receptor stimulation. A previous study on human gastroepiploic arteries has shown poor responses to acetylcholine (Urakami-Harasawa *et al.* 1997), indicating that this may be a common phenomenon in the human mesenteric bed. It was possible, however, to induce impressive relaxations by adding substance P or histamine, two other substances known for their ability to induce endothelium-dependent relaxations in vascular preparations. The disappearance of the relaxations in preparations, where the endothelium had deliberately been removed, clearly shows the endothelium-dependent nature of these responses.

Concerning the identification of possible factors involved in the endothelium-dependent relaxations in these arteries, prostanoids seemed of no importance, since indomethacin preincubation had no influence on the magnitude of relaxation. This is in agreement with the findings in rat mesenteric arteries (Garland and McPherson 1992, Parsons *et al.* 1994, Shimokawa *et al.* 1996, Chen and Cheung 1997, McCulloch *et al.* 1997), but in contrast to the findings in rabbit mesenteric artery (Murphy and Brayden 1995). Moreover, a previous study in human gastroepiploic arteries also failed to show any effect of cyclooxygenase inhibition (Urakami-Harasawa *et al.* 1997). Species and regional differences are well known in the control of mesenteric blood flow (Hansen *et al.* 1998).

In experiments where the synthesis of NO had been blocked by L-NNA, relaxations to substance P were unchanged, but those to histamine were slightly reduced. Under these experimental conditions, relaxations attributable to EDNO release seem to be of minor or no importance. A considerable part of the relaxations induced by substance P and histamine was accordingly not due to a release of a prostanoid or EDNO. This would definitely be in accordance with a large number of other studies in which EDHF were responsible for the major part of the endothelium-dependent relaxations. When interpreting the results, however, it should be kept in mind that the experimental conditions may affect results. Accordingly, impaired endothelial responses have been attributed in part to a cyclooxygenase constrictor substance in rats with experimental diabetes (Mayhan *et al.* 1991).

When trying to analyze these events further, the situation becomes more complex. Although a number of substances with inhibitory effects on different potassium channels is capable of inhibiting relaxations categorized as EDHF, it is difficult to be certain which is blocking the

formation of the substance or the effect. Thus, studies have shown that endothelial cells hyperpolarize in response to acetylcholine (Chen and Cheung 1992, Cheung and Chen 1992), and this hyperpolarization is indeed blocked by substances that are also blocking EDHF relaxations (Chen and Cheung 1992, Cheung and Chen 1992). Supposing that endothelial hyperpolarization is a part of the signal leading to EDHF formation/extrusion, it is very difficult to be sure where the effect is actually exerted as long as EDHF has not been identified and has become measurable.

The mechanism underlying the vascular hyperpolarization attributable to EDHF has been intensively studied. Initial experiments showed that membrane conductance increased during acetylcholine-induced hyperpolarization of different rat vessels. Moreover, the amplitude of the hyperpolarization was increased in low potassium solutions and decreased in high potassium solutions indicating a role of potassium channel opening in this response (Chen and Suzuki 1989). In the present study, the addition of two potassium channel blockers charybdotoxin and apamin, but not apamin alone, nearly abolished relaxations indicating that the response is caused by an agent acting on potassium channels. This factor or factors should consequently be classified as EDHF. The necessity of using the combination of two potassium channel blockers to completely inhibit EDHF is in full agreement with other studies (Corriu *et al.* 1996, Chen and Cheung 1997, Petersson *et al.* 1997, Plane *et al.* 1997, Chataigneau *et al.* 1998, Yamanaka *et al.* 1998), but in contrast to two studies in rabbit (Murphy and Brayden 1995) and rat (Adeagbo and Triggle 1993) mesenteric arteries, where apamin alone blocked EDHF (Murphy and Brayden 1995), and a study in rat mesenteric arteries, where iberiotoxin alone blocked the L-NNA-insensitive component of acetylcholine-induced relaxations (Hansen and Olesen 1997). In human mesenteric arteries, EDHF seems to act on a mixture of potassium channels, with small and large conductance Ca^{2+}-activated channels most likely being activated. Charybdotoxin has been shown to inhibit voltage-sensitive potassium channels (Grissmer *et al.* 1994) in addition to its effect on large conductance Ca^{2+}-activated channels (Strong 1990), and accordingly an effect of EDHF on this type of channel has to be considered (Petersson *et al.* 1997).

Interesting observations were made when studying relaxations in preparations exposed to 60 mM potassium solution. In this solution, cells are significantly depolarized and agents acting on potassium channels are no longer effective in relaxing/hyperpolarizing the muscle. Interestingly, pronounced relaxations were elicited by both substance P and histamine, indicating that these substances have a significant capability of releasing factors that may act by potential-independent mechanisms. Addition of L-NNA to the baths had a dramatic effect in 60 mM potassium solution, and was in contrast to the effect observed in normal Krebs as could be seen by virtual disappearance of relaxations. Thus, a component attributable to EDNO was much more apparent when studied under these circumstances. Similar findings have been made previously in a number of different preparations, and may reflect an interaction between EDNO and EDHF, as previously suggested (Hill *et al.* 1996, McCulloch *et al.* 1997). These authors used an analogue of guanosine 3',5'-cyclic monophosphate (cGMP) to demonstrate a possible modulatory effect on potassium channels expressed as an up-regulation of the EDHF/K^+ channel pathway on loss of NO, and this may functionally compensate the whole or a part of the loss of NO (McCulloch *et al.* 1997). It is interesting to note that in the internal anal sphincter (visceral smooth muscle), which is innervated by intrinsic nerves releasing both nitric oxide and a hyperpolarizing factor (possibly adenosine triphosphate, ATP), a similar interaction has been described (Knudsen *et al.* 1995). Thus the action of apamin, a potassium channel blocker, was significantly enhanced in the presence of L-NNA, indicating that potassium channel opening was facilitated as NO dropped out.

The finding that endothelium-dependent relaxations were still induced in 60 mM K^+ solution, indicate that this part of the response did not depend on membrane potential changes. L-NNA almost abolished this part of the response, strongly suggesting that it was due to release of EDNO. Consequently, NO must have actions independent of membrane potential changes as suggested in previous studies (Komori *et al.* 1988, Plane and Garland 1993). In conflict with this interpretation are the findings of the present study that apamin and charybdotoxin in combination almost abolished relaxations in response to substance P and histamine. The latter finding would be in agreement with an action of NO on charybdotoxin-dependent potassium channels as suggested in some studies (Plane *et al.* 1998, Bolotina *et al.* 1994). In our study, such an explanation is untenable since a prominent L-NNA sensitive component was evident in 60 mM K^+ solution.

The exact nature of EDHF remains debated. Edwards *et al.* (1998) were the first to suggest that EDHF is K^+ released into the myoendothelial space by the opening of endothelial potassium channels. More recently, it was shown that a high degree of coupling exists between the endothelial and the smooth muscle layers in some arterioles, and that endothelial hyperpolarization may spread electrotonically into the muscle from the endothelium (Coleman *et al.* 2001a,b).

To conclude, we have found evidence of EDNO and EDHF release in small human mesenteric arteries. The relative physiological importance of these factors cannot be assessed by experiments *in vitro*, because mesenteric blood flow is determined by a number of different influences such as shear stress, neuronal, hormonal, and paracrine influences etc., and these are not easily determined under *in vitro* conditions.

Abbreviations

EDHF: endothelium-derived hyperpolarizing factor,
EDNO: endothelium-derived nitric oxide,
EDRF: endothelium-derived relaxing factor,
L-NNA: N^{ω}-nitro-L-arginine.

Acknowledgements

This study was supported by the Novo Foundation and by Aarhus University Research Foundation.

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