Editorial

EDHF and residual NO: different factors

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See article by Ge et al. [61] (pages 547–556) in this issue.

1. Introduction

The endothelium plays a pivotal role in the control of vascular tone and blood pressure. In response to a variety of physiological stimuli, such as bradykinin, acetylcholine, histamine, substance P, shear stress and pulsatile stretch, endothelial cells release vasodilator substances. Those include prostacyclin [1] and endothelium-derived relaxing factor (EDRF) [2]. The latter has been identified as nitric oxide (NO) [3]. This labile compound is continuously synthesized from L-arginine by the nitric oxide synthase (NOS) enzyme constitutively expressed in the endothelial cell. It acts by direct stimulation of the soluble guanylyl cyclase in vascular smooth muscle cells. Since the elucidation of the biosynthesis of NO, L-arginine analogues such as \( \text{N}^2 \)-monomethyl-L-arginine (\( \text{L}-\text{NMMA} \)) and \( \text{N}^3 \)-nitro-L-arginine (\( \text{L}-\text{NA} \)) and its methyl ester (\( \text{L}-\text{NAME} \)) have been used as inhibitors of NOS to assess the contribution of the NO/cGMP pathway in endothelium-dependent responses.

In the presence of inhibitors of both cyclo-oxygenase and NOS, residual endothelium-dependent relaxations were observed in a variety of preparations. Moreover, direct measurements of the smooth muscle membrane potential showed agonist-induced hyperpolarizations which disappeared after removal of the endothelium. Hyperpolarization of the vascular smooth muscle cells is known to cause relaxation by decreasing the open-probability of voltage-dependent calcium channels and by interfering with intracellular calcium release mechanisms. The presence of haemoglobin, which binds and inactivates NO, or of methylene blue, which inhibits guanylyl cyclase and destroys NO, did not inhibit the endothelium-dependent hyperpolarization in the rat aorta and pulmonary artery [4], the rabbit femoral artery [5] or the dog mesenteric artery [6]. Therefore, the existence of another relaxing factor, unrelated to NO and prostanoids, was proposed to contribute to endothelium-mediated relaxations [4,7,8]. This factor has been called endothelium derived hyperpolarizing factor (EDHF). Later reports showed that inhibition of NOS had no effect on endothelium-dependent hyperpolarization in the coronary artery [9,10], the rat mesenteric artery [11] and femoral vein [12]. The importance of EDHF in vasorelaxation was described to depend on the stimulus, the kind of vascular bed and the species under study. In several vascular beds, it has been shown that endothelium-dependent EDHF-mediated relaxations increase with decreasing size of the vessel [13]. Up to now, however, the chemical nature of this factor remains elusive. Among possible candidates are epoxyeicosatrienonic acids (EETs) [14], which are cytochrome P450-derived metabolites of arachidonic acid, arachidonoyl-ethanolamide or a related cannabinoid [15], or potassium ions [16]. Recent evidence suggests that an EDHF diffuses to the vascular smooth muscle cells via heterocellular gap junctions [17].

2. Nitric oxide causes hyperpolarization

During the past decade, the existence of a separate EDHF has been repeatedly challenged. Thus, while earlier studies had shown that NO itself did not influence the resting membrane potential of vascular smooth muscle cells [6,18], a key observation questioning the existence of EDHF was the fact that, at least in some vessels such as the guinea-pig uterine artery, exogenous NO caused hyperpolarization [19]. Also in rat small mesenteric arteries, NO applied either as NO gas or acidified \( \text{NaNO}_2 \) was shown to cause hyperpolarization [20]. While these studies seemed at first to be contrasting, it was later found that the membrane electrical response to NO in guinea-pig cor-
onary arteries was dependent on the degree of passive membrane stretch [22], becoming progressively larger at increasing muscle tension. Moreover, differences were described between EDHF- and NO-induced hyperpolarizations in that the former occurs, although with varying magnitude, independently of passive or active tone [19]. In addition, EDHF- and NO-induced hyperpolarizations were shown to be differently affected by K⁺ channel inhibitors. In rat small mesenteric arteries [20] and the guinea-pig coronary artery [22], the NO-induced but not the acetylcholine-evoked change in membrane potential was completely blocked by glibenclamide, an inhibitor of ATP-sensitive K⁺ (KATP) channels.

It should be remarked, however, that NO has been reported to activate hyperpolarizing mechanisms independent of KATP channels. In smooth muscle cells of the rabbit cerebral artery, NO and a membrane permeable form of cGMP activate Ca²⁺-dependent K⁺ (KCa) channels [23]. Also in the rat pulmonary artery, NO and cGMP have been shown to enhance KCa channel activity and to induce a charybdotoxin-sensitive relaxation [24]. In this respect, it can be remarked that stretching the membrane patch of voltage-clamped rabbit mesenteric artery cells increased the activity of the large conductance KCa (BKCa) channels [25]. Moreover, a direct enhancing influence of NO on the activity of KCa channels, not requiring the cGMP pathway, has been demonstrated [26]. In addition, it was documented in rabbit thoracic aorta that NO and the NO-donor sodium nitroprusside (SNP) caused a time- and concentration-dependent stimulation of ouabain-sensitive ⁸⁶Rb uptake [27]. The mechanism for this NO-induced stimulation of Na⁺-K⁺-ATPase was independent of the ability of NO to increase intracellular cGMP levels but might involve activation of the Na+-H exchanger [27].

Also prostacyclin, which induces an increase in muscular cAMP levels, has been described to cause hyperpolarization in some arteries [21]. It was stressed, therefore, that the EDHF-mediated relaxations and hyperpolarizations should always be measured in the continuous and combined presence of high concentrations of inhibitors of cyclo-oxygenase and NOS.

### 3. Residual NO

A second challenge for the existence of a separate EDHF resulted from the application of NO-microsensors to isolated blood vessels. With this method it became possible to directly measure the endogenous release of NO during physiological stimulation of the endothelium. In the rabbit carotid artery stimulated with acetylcholine, it was shown with this technique that the application of l-NAME, in a concentration generally considered to be sufficient to inhibit NOS (30 μM), diminished but did not completely inhibit NO release [28]. In these experiments, the additional application of l-NA (300 μM) further reduced, but could not completely abolish, the release of NO. Moreover, the hyperpolarization and relaxation of these vessels to acetylcholine decreased after additional application of l-NA, and both phenomena remained closely correlated quantitatively. It was suggested, therefore, that NO was the only mediator of both the relaxation and the hyperpolarization in this artery [28]. It should be remarked that in the combined presence of these inhibitors, relaxations and hyperpolarizations, although drastically diminished, were not completely inhibited. Moreover, the membrane potential of this preparation seemed to be very sensitive to NO (48 mV hyperpolarization to exogenous NO applied as 10 μM of the NO-donor SIN-1).

At about the same time, Kemp and Cocks [29] reported that treatment with 100 μM of l-NA did not completely inhibit endothelial cell NO synthesis of human isolated small coronary arteries, since the additional application of the NO scavenger oxyhaemoglobin (HbO) further reduced l-NA resistant vasodilator responses to bradykinin. An additional observation in this study [29] was that increasing the concentration of l-NA from 100 to 300 μM had no larger effect than observed with the lower concentration. Since half maximal inhibition of purified constitutive NOS was reported to occur at 25 nM of l-NA [30], and maximal effects of l-NA have been obtained at concentrations of about 10 μM in porcine large coronary arteries [31], the possibility that the applied 100 μM of the inhibitor was not sufficient to inhibit NOS was considered as unlikely. Alternative options, such as release of NO from a source other than l-arginine, an excess of l-arginine present in the endothelial cells, or impaired uptake or increased metabolism of l-NA, were suggested in this study. It should be pointed out, however, that in the combined presence of HbO and NOS inhibitor some endothelium-dependent relaxation to bradykinin persisted. The remaining HbO-insensitive relaxation was considered to be caused by the EDHF, since it was abolished by high K⁺ solution [29]. Membrane potential was not measured in this study.

Recently, simultaneous measurements of NO concentration and relaxation were performed in rat superior mesenteric arteries [32]. In the presence of 100 μM of l-NA, acetylcholine evoked small increases in NO concentration, accompanied with relaxations. At the highest concentrations of acetylcholine, transient relaxations of about 40% of the pre-existing tone were noted, and were temporally related with the small and transient increases in measured NO. Since HbO reversed these l-NA resistant relaxations, it was suggested that the l-NA resistant relaxations observed in this preparation [32,33] were due to residual NO. Moreover, it was suggested [32] that the transient hyperpolarizations measured in the rat main mesenteric artery [34], the magnitude of which was completely unaffected by NOS inhibitors and indomethacin [34], were due to residual NO. However, in this study [32], direct measurements of the membrane potential, confirming this suggestion, were lacking.
In the rat aorta and pulmonary artery, haemoglobin inhibited the acetylcholine-induced relaxation, but did not change the transient hyperpolarization [4], directly showing the existence of a NO-independent hyperpolarizing mechanism. Moreover, if residual NO would be responsible for the endothelium-dependent hyperpolarization in the presence of NOS inhibitors, as suggested by Simonsen et al. [32], one should be able to induce comparable hyperpolarizations with exogenous NO applied in physiological (nanomolar) concentrations. In the rat aorta, it was shown that nitroglycerin (NTG), applied at 10 μM, caused only a hyperpolarization of about 2 mV [35]. Another NO-donor, SIN-1 (3 μM) hyperpolarized the membrane by 1 mV, and direct application of NO by infusion of acidified NaNO₂ (3.3 mM HNO₂ in the experimental chamber) changed the membrane potential by –4 mV. This is only a fraction of the transient membrane potential change induced by acetylcholine applied to the same vessel in the same conditions [35]. Comparable observations were made in the main mesenteric artery [34–36]. Moreover, the acetylcholine-induced hyperpolarizations in the rat aorta [35] and in the main mesenteric artery [37] were unaffected by the continuous presence of NO donors, or of 8-Br-cGMP, which by itself caused a barely detectable change of the membrane potential [35]. Using the NOS inhibitors l-NAME (100 μM), l-NMMA (100 μM), and l-NA (200 μM) we were able to dissect out the component of acetylcholine-induced hyperpolarization due to endogenous NO as a small steady state membrane potential change being largely obscured by the much larger and transient EDHF-mediated hyperpolarization. The NO-induced component became only apparent during the later phase of the response to acetylcholine [35,38]. It should be remarked that, in the rat aorta, the endothelium-dependent relaxation induced by acetylcholine is totally abolished by 200 μM l-NA, functionally showing the absence of residual NO in this condition. Moreover, while the membrane potential of the smooth muscle cells of the rat aorta and the main mesenteric artery apparently share the same (low) sensitivity to exogenous NO, the EDHF-mediated hyperpolarization elicited by acetylcholine is much more expressed in the mesenteric artery than in aorta [34–36]. In addition, it was shown that the EDHF-mediated hyperpolarization was inhibited by increasing the extracellular K⁺ concentration [34,37] while it is unlikely that this experimental manoeuvre inhibits NO release. A further argument in favour of the existence of a separate EDHF is the fact that in small mesenteric arteries in certain conditions (after repeated application of NTG), a small depolarization is sometimes induced by this NO donor, whereas acetylcholine consistently produces a transient peak hyperpolarization before the membrane potential shows a steady state depolarization in the prolonged presence of the vasodilator [36]. Thus, in these conditions, NO and EDHF produce even opposite changes in membrane potential [36].

### 4. Porcine coronary arteries

The pig coronary artery does not relax in response to prostacyclin [31]. Moreover, it has been observed that exogenous NO does not influence the smooth muscle cell membrane potential of this vessel [39]. Several studies showed that the endothelium-dependent relaxation elicited by bradykinin in this artery was only slightly affected by NOS inhibitors as well as by methylene blue and by the NO scavenging haemoglobin [10,40–42]. Also the bradykinin-induced hyperpolarization was little affected by indomethacin and l-NA [43]. The main part of the relaxation induced by the kinin in these conditions, therefore, was considered to be due to a factor different from NO [10,40–43]. In coronary resistance arteries of the pig heart, bradykinin-mediated relaxations were similarly resistant to l-NMMA (10⁻⁵ M) as well as to methylene blue treatment [44]. In addition, the NOS inhibitor was shown to completely prevent the bradykinin-induced increase in muscular cGMP levels [44]. Since the apparent K_m of NO for guanylyl cyclase is in the low nanomolar range [45], this latter finding is indicative for the absence of residual NO after bradykinin-induced stimulation in the presence of l-NMMA.

It should be pointed out that the relative importance of EDHF-mediated relaxations, expressed after inhibition of the NO/cGMP pathway, is not necessarily the same as in normal conditions. This has been clearly demonstrated in porcine coronary arteries [31]. Indeed, the EDHF system might become upregulated under conditions of impairment of the NO/cGMP pathway and serve as a backup mechanism to maintain endothelium-dependent vasodilator function under conditions of impaired NO release [31]. In normal physiological conditions, NO could account for the largest part of the agonist-induced dilatations regardless of whether hyperpolarization is present or not [31]. In the pig coronary artery endothelium, indeed, the production of EDHF is damped by NO [46].

Since the original proposal that, in bovine and in porcine coronary arteries, EDHF is a cytochrome P450-derived metabolite of arachidonic acid [14], substantial evidence has been accumulated that the EDHF liberated by bradykinin in the coronary vasculature is an EET or a mixture of such compounds [47–51]. EETs are produced in endothelial cells and have been shown to increase the activity of K_Ca channels [52,53]. Bioassay experiments from different laboratories clearly showed the release of a humoral hyperpolarizing substance liberated from donor pig coronary arteries [14,46,48,54], hyperpolarizing the membrane potential or increasing the open probability of K_Ca channels of detector vascular smooth muscle cells, as do EETs. Inhibitors of the liberation of arachidonic acid or of the cytochrome P450 pathway abolished detector responses [48,54]. Although in principle it can not be excluded that in these bioassay experiments residual (30 or 100 μM
1-NA-resistant) NO might account for the detector responses (especially since cytochrome P450 can generate NO in the presence of NOS inhibitors [55]), it was shown that the continuous presence of an NO-donor did not affect the bradykinin-induced hyperpolarization of the detector cells [46]. Moreover, induction of the cytochrome P450 in pig coronary artery endothelial cells by treatment with β-naphthoflavone increased the EDHF-mediated hyperpolarization in response to bradykinin [48,51]. In some studies, however, several cytochrome P450 inhibitors have been shown to be without specific effect on the bradykinin-induced EDHF-mediated relaxations in the pig coronary artery [56–58]. Moreover, some of these cytochrome P450 inhibitors directly affect K+ channel activation [59,60], thereby preventing the action of EDHF. In a recent non-pharmacological approach, however, antisense oligonucleotides derived from the cDNA of human cytochrome P450 2C, shown to downregulate the expression of the 2C isoform in pig coronary artery endothelial cells, blocked the bradykinin-induced hyperpolarization in segments of porcine coronary arteries [51].

5. The study of Ge, Zhang, Fung and He [61]

In the current issue of Cardiovascular Research, Ge et al. [61] further explore the 1-NA and indomethacin resistant responses to bradykinin in the pig coronary microcirculation. It is shown that the bradykinin-induced relaxation and hyperpolarization in the presence of indomethacin and 1-NA is sensitive to TEA, charybdotoxin and iberiotoxin, all inhibitors of BKCa channels although with varying selectivity. The blocker of small conductance KCa (SKCa) channels, apamin, has no influence when applied alone, but significantly enhances inhibition in the presence of charybdotoxin. The authors suggest, therefore, that both BKCa and SKCa channels mediate bradykinin-induced responses resistant to indomethacin and 1-NA, the former playing a relatively important role and the latter a rather compensatory role, as a backup mechanism under conditions of impaired BKCa channel activation [61].

In their investigations, the authors include measurements with NO-selective electrodes. The strength of the present study, therefore, is that it combines relaxation experiments with recordings of the membrane potential of the smooth muscle cells and of the NO concentration in vascular strips. The NO measurements clearly document that 300 μM 1-NA is not sufficient to completely prevent the liberation of NO from the pig coronary artery, as has been shown in the rabbit carotid [28] and the rat main mesenteric artery [32]. The additional inclusion of an NO scavenger seems necessary to completely block NO release. Moreover, even 1 mM of 1-NA cannot prevent some relaxation which is sensitive to additional application of HbO, and residual NO seems to account for a significant fraction of the indomethacin/1-NA-resistant relaxation in coronary microvessels [61]. Most importantly, however, in the additional presence of HbO, shown to completely eliminate the rise in NO concentration upon stimulation with bradykinin [61], the hyperpolarization of the membrane potential induced by the kinin is barely affected [61]. In conduit coronary arteries, the decrease in endothelium-dependent hyperpolarization just marginally reaches statistical significance, while in coronary microvessels the largest part of the hyperpolarization persists in the combined presence of indomethacin, 1-NA and HbO. This directly indicates that, although residual NO might be present in some conditions in which only NOS inhibitors and no additional NO scavengers are used, a separate NO-unrelated hyperpolarizing factor does indeed exist. Thus, the results of the study of Ge et al. [61] are straightforward in that they emphasize the importance of the use of NO-scavengers when measuring the 1-NA/indomethacin resistant relaxations to assess the importance of non-NO mediated mechanisms. On the other hand they demonstrate the existence of a separate, NO-unrelated, EDHF, which apparently makes an important contribution to the endothelium-dependent relaxation in porcine coronary microvessels at least after inhibition of the NO/cGMP pathway.

References

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