# The endothelium-derived hyperpolarizing factor: does it play a role *in vivo* and is it involved in the regulation of vascular tone only?

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**Several investigations performed *in vitro* have shown that vascular endothelia can release diffusible compounds capable of inducing hyperpolarization of the smooth muscle fibers. Experiments *in vitro* have shown that these compounds can cause coronary vasodilation and alter cardiac performance. Experiments *in vivo* only showed the occurrence of vasodilation. While it has been shown that the release of these endothelium-derived hyperpolarizing factors (EDHFs) is not impaired by the inhibition of nitric oxide synthase and cyclooxygenase, the precise nature of the compound(s) has not yet been identified. It is possible that they vary depending on the organ and animal species. However, a common feature of the activity of EDHFs is the activation of calcium-dependent potassium channels, inhibitable by charybdotoxin and apamin. Furthermore in the coronary circulation of many species EDHF seems to be a cytochrome P450-dependent non-prostanoid metabolite of arachidonic acid activated by a number of chemical and physical stimuli similar to those which are known to activate endothelial nitric oxide synthase. Using compounds which inhibit cytochrome P450 and blockers of the calcium-dependent potassium channels, researchers can study the physiological and pathophysiological relevance of EDHF *in vivo* thus disclosing the potential therapeutic applications of the basic knowledge in this field.**

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In addition to neurovegetative innervation, several factors participate in the regulation of the vasomotor tone. Local metabolic demand, neurohormones[1], myogenic responses to mean and pulse pressure[2], as well as endothelium-mediated chemical and mechanical stimuli[3-7] are involved in the control of vascular resistance. For a number of years nitric oxide (NO) has been considered a prominent modulator of the endothelium-mediated signaling leading to vasodilation[4-10]. However, growing evidence supports the hypothesis of an additional pathway resistant to NO synthase (NOS) and cyclooxygenase (COX) inhibition, which results in vascular smooth muscle relaxation mediated by a possible endothelium-derived hyperpolarizing factor (EDHF)[11,12]. Although the precise nature of EDHF remains somewhat controversial[13-16], a common footprint is its role in the activation of calcium-dependent potassium ($K^+_{Ca}$) channels which can be inhibited by charybdotoxin, iberiotoxin and apamin[16-21]. *In vitro* studies have identified an NOS-COX-independent hyperpolarization in endothelial cells stimulated by either acetylcholine or bradykinin. The hyperpolarization is fully blocked by the combination of charybdotoxin and apamin[16,17], and partially or fully blocked by the combination of iberiotoxin and apamin[18,19]. Whether the latter combination reduces or suppresses the hyperpolarization depends on the balance between the doses of the agonists (i.e. acetylcholine and bradykinin) and of the two antagonists. Furthermore, to explain this apparent discrepancy it must be considered that apamin selectively blocks the small conductance and charybdotoxin blocks both intermediate and large conductance $K^+_{Ca}$ channels, whereas iberiotoxin more selectively inhibits the latter[20,21]. Recently, Feletou and Vanhoutte[22] have reported that EDHF-mediated responses are sensitive to the combination of two toxins, charybdotoxin plus apamin, but do not involve ATP-sensitive or large conductance $K^+_{Ca}$ channels .

Since *in vivo* hyperpolarization cannot be directly measured, only indirect evidence of an involvement of a hyperpolarizing factor can be obtained using the above report-

ed channel antagonists, in particular apamin and charybdotoxin. In some species and vascular beds, where the nature of the EDHF synthase seems identifiable[13], specific inhibitors of this synthase can be used. For the moment, since the precise nature of EDHF is still unknown in many species and vascular beds, the vast majority of data supporting the existence of an EDHF is based on *in vitro* preparations in which vascular hyperpolarization can be directly measured.

### *In vitro* studies

**Vasodilation.** A smooth muscle resistant to NOS-COX inhibition hyperpolarization has been obtained *in vitro* using several agents[11-17,22-27] including acetylcholine, bradykinin, histamine, substance P, adenosine diphosphate, vasoactive intestinal peptide and endothelins, as well as by mechanical stimuli such as increased shear stress[28] or pulsatile stretch[29] (Fig. 1). Exercise training, in which pulse pressure increases, has also been shown to enhance endothelium-mediated vasorelaxation by both NO and hyperpolarization-dependent signaling[30], further suggesting a potential role for $K^+_{Ca}$ channel activation in response to enhanced pulsatile stretch. It is noteworthy that the hyperpolarizing response induced by chemical and mechanical stimuli can be prevented by inhibitors of cytochrome P450 (CYP) oxidases[13,31,32]. In fact, in isolated coronary vasculature of many species, EDHF has been suggested to be a CYP-dependent non-prostanoid metabolite of arachidonic acid[13,31,32], while a CYP metabolite-mediated hyperpolarization and coronary vasodilation have also been demonstrated in anesthetized animals[19,33] (see below). Recently, studies by the Busse group have shown that CYP-2C fulfills the properties of an EDHF synthase in porcine coronary arteries[13].

In spite of the findings in favor of the role of endothelial CYP oxidases, other investigations seem to implicate that $K^+$ itself is an EDHF[14] or that NO, which is usually not fully inhibited by NOS inhibitors, fully accounts for EDHF[15]. In fact, $K^+_{Ca}$ channels may also be activated by NO and cyclic guanosin-mono-phosphate (cGMP)[34,35], suggesting a response of $K^+_{Ca}$ channel conductance to NO[36] and shear stress signaling as well[9]. Several studies performed in large conductance vessels have shown a direct correlation between residual responses to acetylcholine and the persistence of NO release despite NOS inhibition[15,37]. Residual NO release could induce $K^+_{Ca}$ activation, as both NO and cGMP can activate these channels[34,35]. Yet, the amount of NO required for the activation of these channels is reported to be much higher than the amount usually released in physiological conditions and controversy exists as to whether or not physiologically stimulated (e.g. by increased shear stress and pulse pressure) release of NO is able to induce hyperpolarization of the vascular smooth muscle cells[15,38-41].

In contrast to the opinion that inhibition-resistant residual NO can be responsible for vascular smooth muscle hyperpolarization, are the results which show that NO inhibitors are able to fully prevent *in vivo* acetylcholine-mediated dilation in conductance arterioles (>100 μ)[27] but not in smaller vessels. These results are consistent with the fact that as the vessel size decreases (< 100 μ), the relative amount of EDHF released by the endothelium of the microvessels increases progressively while NO release decreases (Fig. 1). As a consequence also a progressive increase in the relative importance of EDHF occurs in the small vessels[16,29,42]. These results seem to limit the importance of NO as a hyperpolarizing factor.

**Myocardial depression.** Given the great access of the coronary microvessels to the mass of myocardial cells, and considering the fact that capillary endothelia are not in juxtaposition with smooth muscle cells, but are in close apposition to cardiac myocytes, it is likely that the

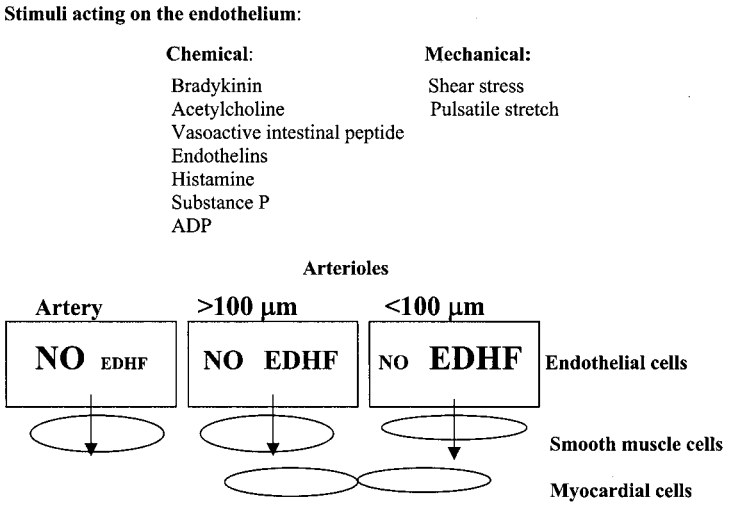


**Figure 1.** *Chemical and mechanical stimuli which induce the release of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). A schematic representation of the balance between NO and EDHF release in artery and arterioles is reported. ADP = adenosine diphosphate.*

latter can be exposed to the effect of the compounds released by the vascular endothelium, i.e. of substances which might therefore be expected to influence myocardial function. This effect has been observed in the case of NO, which increases myocardial contractility at low concentrations and reduces it when produced in large quantities[43].

In 1993 Fort and Lewis[44] demonstrated that a factor released by the coronary endothelium stimulated by bradykinin causes a transient clearly evident reduction of myocardial contractility. Since the inhibition of NO release attenuated but did not suppress the response, they suggested that a factor different from NO might have been responsible for the bradykinin-induced cardiodepression. Their opinion was that the mode of action of this compound should concern the opening of the ATP-sensitive $K^+$ channels. Among the endothelial factors mediating the effect of bradykinin on myocardial performance they suggested a possible role of prostacyclin, calcitonin gene-related peptide and a hyperpolarizing factor not yet well defined.

Although its nature has not yet been unequivocally identified, in the coronary vasculature of many species EDHF, as said above, has been suggested to be a CYP-dependent non-prostanoid metabolite of arachidonic acid[13,32]. Studying the effects on cardiac function exerted by some of the metabolite of CYP, i.e. the epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15 EETs), Moffat et al.[45] concluded that, when infused, EETs have no effects on myocardial contractility of normal hearts; EETs, however, may be responsible for cardiodepression and vasodilation, when they are released from the endothelium in response to ischemia-reperfusion. Subsequently, Van Rollins et al.[46] and Weintraub et al.[47] suggested that infused EETs cannot reach the smooth muscle fibers of the coronary vasculature, especially if the doses are relatively low, because they are avidly incorporated into the phospholipids of the endothelial cells. However bradykinin, which activated endothelial phospholipase, can induce the release of the incorporated EETs from the lipids of endothelial cells, thus allowing coronary vasodilation.

Starting from these considerations our group investigated whether the effect of bradykinin on contractility is mediated by endothelial release of CYP metabolites[48,49]. In Wistar rat hearts isolated and perfused at constant flow we found that the blockade of CYP prevents bradykinin from causing negative inotropic effects, both in the presence and in the absence of NOS-COX inhibition. The suppression of the bradykinin-induced cardiodepression was also obtained by the preliminary removal of the endothelium by triton X-100. All these results suggest that the endothelial CYP plays a pivotal role in mediating the negative inotropic effect of bradykinin. Since a high dose of 14-15 EET (50 ng/l for 20 min), but not of other tested isomers (8-9 and 11-12 EETs), is able to reproduce the negative inotropic effect of bradykinin, it is likely that the reduction in contractility by bradykinin is the result of the release of 14-15 EET from the endothelium. Furthermore, a potentiation of bradykinin-dependent cardiodepression after 14-15 EET has been observed. In addition to the studies of Moffat et al.[45] with ischemia-reperfusion, our results demonstrated for the first time the relevance of EETs/EDHF in isolated hearts perfused at a normal flow rate.

### *In vivo* studies

Despite growing experimental *in vitro* and *ex vivo* evidence supporting the role of EDHF-$K^+_{Ca}$ channel-dependent signaling in vascular smooth muscle relaxation and myocardial contractility, *in vivo* data remain scant, and have mainly been focused on agonist-stimulated vasodilation. To our knowledge in the literature there are no *in vivo* studies which aim at ascertaining a possible role of EDHF in myocardial contractility.

Experiments performed in conscious and anesthetized dogs[50-52] showed that the inhibition of NO release by intracoronary administration of L-arginine analogues, with[52] and without[50,51] COX inhibition, reduces but does not suppress the vasodilation obtained by injection of acetylcholine into a large coronary artery. Since in isolated aortic rings of the rat, acetylcholine was seen to produce vasoconstriction instead of vasodilation after removal of the endothelium[53], it may be argued that after NO inhibition the endothelium is still capable of responding to acetylcholine with a mechanism independent of the NO-cGMP pathway. For the above described reasons this mechanism should be based on a $K^+_{Ca}$ channel-mediated smooth muscle hyperpolarization carried out by residual NO or by another hyperpolarizing factor.

An *in vivo* role for EDHF-$K^+_{Ca}$ channels in NOS-COX resistant acetylcholine-induced dilation has recently been reported by Nishikawa et al.[19]. In this study, residual dilation after NOS-COX inhibition in < 100 μ diameter coronary canine arterioles was prevented by suffusion with $K^+$ buffer or by $K^+_{Ca}$ blockade obtained with iberiotoxin and apamin. However, NOS-COX inhibition alone was effective in blocking acetylcholine dilation in less distal arterioles, supporting the hypothesis of a more prominent role of NO signaling in such vessels[54].

Another group studied the involvement of $K^+_{Ca}$ channels *in vivo*, particularly targeting bradykinin-dependent signaling. In one study, Node et al.[18] reported that charybdotoxin or iberiotoxin combined with L-nitro-arginine-methylester (L-NAME) prevented bradykinin-dependent dilation, and found that this pathway is important in post-ischemic dilation. Later on, the same group reported that also ischemic protection by 17β-estradiol, largely activated by NO and bradykinin, was prevented by iberiotoxin + L-NAME[55]. This suggests that $K^+_{Ca}$ channel activation, like $K^+_{ATP}$-channel activation[56], plays an important role in modifying the impact of coro-

nary supply/demand imbalance. So far, only one study[57] demonstrated the relevance of this pathway in mechanically stimulated vasodilation *in vivo*. In this study, using apamin plus charybdotoxin, the authors showed the involvement of $K^+_{Ca}$ channels in pulse-perfusion signaling. The contribution of $K^+_{Ca}$ channels to *in vivo* regulation of coronary flow by perfusion pulsatility was tested in anesthetized dogs. The blockade of NOS and of $K^+_{Ca}$ channels respectively reduced the flow increases obtained with enhanced pulsatility by 50%, while the combination of both blockades nearly prevented it. Thus, it was argued that physiological responses to pulse-vascular signaling *in vivo* involves a hyperpolarizing pathway which may become particularly important when NO-dependent signaling is impaired. The combined role of NO and $K^+_{Ca}$ channels could explain the beneficial effects of dynamic exercise (in which pulsatility increases) on coronary flow reserve and ischemic dysfunction[18,30]. The residual dilation to acetylcholine that remains after NOS-COX inhibition has completely been blocked by miconazole or metyrapone[19] and by clotrimazole or 17-octadecynoicacid[33] in studies performed in a canine model *in vivo*. All these substances are inhibitors of CYP enzymes, thus confirming that this pathway is involved in acetylcholine-induced coronary vasodilation.

In brief, from the above reported findings it can be concluded that an EDHF can still cause vasodilation after NOS-COX inhibition, acting on small and intermediate $K^+_{Ca}$ channels. The release of this factor is induced by similar stimuli which cause the release of NO. A CYP non-prostanoid metabolite of arachidonic acid, i.e. an EET acid, is likely to be a candidate as EDHF in the coronary circulation of many species. One of these acids is able to induce cardiodepression, thus explaining the negative inotropic effect of bradykinin. Also *in vivo* studies confirm that a hyperpolarizing mechanism is involved in vasodilation induced both by chemical and mechanical stimuli.

The present challenge of the research in this field is to understand the nature of EDHF in every individual species and organ as well as the relevance of the above reported findings to the physiology and pathophysiology of the cardiovascular system. Several human diseases are characterized by reduced NO synthesis and release, a condition in which the EDHF role is enhanced, as it occurs in atherosclerotic coronary heart disease and other cardiovascular diseases[16,18,30,38,55]. The hope is that the research in this field can shed some light on the detailed mechanisms of these pathological conditions.

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